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Oral glucose stimulates insulin secretion much stronger than intravenous glucose making up for the incretin effect mediated by the gut peptides glucose-dependent insulin secreting polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). GIP (from the intestinal k-cells) and GLP-1 (L-cells) are released in response to oral nutrients. While GIP has much less activity in type 2 diabetics, GLP-1 is still effective. GLP-1 receptors were detected in endocrine pancreas, brain, lung, heart, blood vessels, stomach, and intestine suggesting a broad array of biological functions of GLP-1. Transgenic studies in rodents and pigs proved the importance of the incretins for glucose homeostasis. At the endocrine pancreas GLP-1 restores the first-phase insulin response to glucose, stimulates insulin secretion and suppresses glucagon release. GLP-1 does not induce hypoglycemia since the stimulation of insulin secretion is strictly glucose-dependent. GLP-1 regulates gastric emptying thereby reducing postprandial glucose levels. Furthermore, GLP-1 increases satiety, reduces food intake and decreases body weight. Based upon these properties GLP-1 appears as an ideal tool for the treatment of type 2 diabetes. However, upon release GLP-1 is rapidly degraded mainly by the dipeptidyl-peptidase 4 (DPP 4). Thus, native human GLP-1 cannot be orally or subcutaneously supplied and, therefore, can not be utilized for the treatment of diabetics. However, several stable GLP-1 receptor agonists (GLP-1RA) are now available, either exendin-derived peptides or analogs of natural GLP-1. GLP-1 receptor agonists are able to restore a normal glucose tolerance in a significant proportion of type 2 diabetics complementing conventional antidiabetic drugs. After bariatric surgery there is evidence that incretins support the normalization of glucose homeostasis in adipositas. GLP-1 RAs exert multiple extrapancreatic actions, among them cardio- and neuroprotective protective effects. Conclusion: Restoring physiological conditions, incretins such as GLP-1 are interesting drug candidates, so far mainly established in the therapy of diabetes mellitus.
PL-05
Genetics of human cardiomyopathy
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Application of genomic technologies has enriched the discovery of inherited gene mutations that cause primary disorders of heart muscle. The two most common primary heart muscle diseases are hypertrophic (HCM) and dilated (DCM) cardiomyopathy. HCM is characterized by left ventricular hypertrophy, diastolic dysfunction with normal or enhanced systolic performance and a unique histopathology: myocyte hypertrophy, disarray and fibrosis. Dilated cardiomyopathy (DCM) exhibits enlarged ventricular volumes with depressed systolic performance and nonspecific histopathology. Both HCM and DCM increased risk for arrhythmias, sudden death, and heart failure. Human molecular genetic studies have demonstrated that these unique pathologies can both result from dominant mutations in genes that encode protein components of the sarcomere, the contractile unit in striated muscles. This presentation will review the prevalence and spectrum of sarcomere protein gene mutations in HCM and DCM patients and the prevalence and clinical impact of rare sarcomere gene variants that are found in the general population. The cell and molecular mechanisms by which gene mutations cause HCM and DCM will be explored by biophysical and biochemical analyses of mutant proteins and assessments of cell and animal models that carry human HCM or DCM mutations. The application of transcriptional analyses to define key molecules that drive the histopathologic features of cardiomyopathy will be discussed, with a focus on potential therapeutic targets that may be appropriate for translation into the clinical setting. Finally, emerging strategies to silence the expression of mutant alleles will be considered, to address their relevance as therapeutic options and to explore the mechanisms of disease.

PL-06
Dynamics of the auditory cortex and the perception of sounds
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Circuits of the neocortex are believed to mediate conscious perception of the world. Strong evidence arises from the seminal observations by the neurosurgeon Wilder Penfield that microstimulation of sensory cortices can evoke hallucinations in awake patients. Given the fine and complex anatomy of neocortical circuits it still remains unclear how such relatively crude stimulation can lead to meaningful neuronal activity.

We have applied in vivo two-photon calcium imaging to analyze sound-evoked activity patterns in local neuronal ensembles of the mouse auditory cortex. We found that activity patterns are highly constrained into few discrete response modes, which is surprising given how many patterns could theoretically generated by the combination of already a few neurons. Using on-line synthesis of sounds to tailor stimuli that allow precise probing of a given neuronal population, we observe highly non-linear dynamics indicating an antagonistic ‘winner-takes-all’-like competition between response patterns. Our observations suggest that local non-linear dynamics shape the cortical representation of sounds into a basis set of spontaneous categories that are available for behavioral decisions. Furthermore, such dynamics may serve as a potential explanation how non-specific activity elicited by artificial stimulation may converge on activity patterns that are typically observed by natural sensory stimuli.

In a next step, we have established procedures for optogenetic stimulation of the mouse auditory cortex in awake mice trained to discriminate two ‘target’ sounds in a go/no-go paradigm. The behavioral responses to a third stimulus presented in interspersed catch trials allows measuring the perceived similarity to either one of the two ‘target sounds’. In this paradigm we assessed the generalization behavior across sound cues and optogenetically evoked activity. This strategy offers an entry point to a detailed analysis how artificially evoked patterns of neuronal are perceived in an experimentally accessible model organism.
Properties of the paracellular pathway in the kidney have been viewed for a long time as a static element in renal function. The proximal tubule comprising leaky junctional strands, the TAL expressing a steady cation selective paracellular pathway and the collecting duct showing a large paracellular tightness. Recently, substantial knowledge has been gained on the properties, regulation and composition of tight junctions. The major junction protein family, the claudins, have been characterized in more detail and a first structural model could be resolved.

It is appealing to review and integrate this knowledge, keeping in mind some major questions on the understanding of renal tubular transport mechanisms: How does solvent drag in the proximal tubule work through a network of claudins with a distinct selectivity and permeability for different solutes and water? How do regulatory pathways influence the tight junction properties to contribute to the control of renal tubular salt transport? Are paracellular proteins or their regulators involved in the pathomechanism of kidney disease? Does water absorption occur in the distal convoluted tubule given the claudin composition of the junction? Does Chloride use the paracellular route in the collecting duct? Although there are more questions than answers, insights from knockout models for a variety of renal tubular claudins will be discussed with respect to physiology and disease.

The adrenal gland hormone aldosterone is thought to play a key role in K⁺ homeostasis. High K⁺ intake stimulates the release of aldosterone. In the kidney aldosterone exerts its effects primarily in the aldosterone-sensitive distal nephron (ASDN), where it binds to the mineralocorticoid receptor (MR) to increase the expression and/or activity of transporters and channels that favour electrogenic Na⁺ reabsorption and Ca²⁺ homeostasis. The major junction protein family, the claudins, have been characterized in more detail and a first structural model could be resolved.

It is appealing to review and integrate this knowledge, keeping in mind some major questions on the understanding of renal tubular transport mechanisms: How does solvent drag in the proximal tubule work through a network of claudins with a distinct selectivity and permeability for different solutes and water? How do regulatory pathways influence the tight junction properties to contribute to the control of renal tubular salt transport? Are paracellular proteins or their regulators involved in the pathomechanism of kidney disease? Does water absorption occur in the distal convoluted tubule given the claudin composition of the junction? Does Chloride use the paracellular route in the collecting duct? Although there are more questions than answers, insights from knockout models for a variety of renal tubular claudins will be discussed with respect to physiology and disease.
Phosphate is an essential component of bones, cell membranes, DNA, signaling cascades, or cellular energy metabolism. Phosphate deficiency affects most of these systems causing rickets, energy depletion or insulin resistance. However, in industrialized countries, dietary intake with modern Western diet exceeds phosphate requirements several-fold. Phosphate is added to many foods as preservative, anti-oxidant or a natural component. High dietary intake of phosphate as well as high-normal serum phosphate levels are associated in large epidemiological studies with increased risk for cardiovascular and renal disease and overall mortality in the healthy population. This risk is even increased in patients with preexisting cardiovascular or renal disease.

We aimed to examine how dietary phosphate intake could affect cardiovascular functions. Mice were given low (0.1%) or high (1.2%) phosphate (Pi) diets for 1–5 days and showed higher systolic blood pressure (approx. + 10 mm Hg) after three days which persisted thereafter. Moreover, markers of cardiac hypertrophy were increased by high phosphate diet for 5 days. High dietary phosphate intake caused elevated aldosterone levels and renin mRNA abundance within 24 hours. Also, PTH and FGF23 increased within minutes and hours, respectively, whereas vitamin D3 was first high- and later decreased in the high phosphate group. Renal expression of salt transporting proteins including NHE3, NKCC2, the three subunits of the epithelial sodium channel (ENaC) as well as in NKCC1, the K+ channel leading to cell membrane depolarization and increased cytosolic Ca2+ concentration. Mutations of other genes found in APA (Na+/K+-ATPase, plasma membrane Ca2+-ATPase, L-type voltage activated Ca2+ channel) also interfere with the ion homeostasis, but the pathophysiological mechanisms of those mutations are still under investigation.

Tyrosine kinase (TK) inhibitors that block vascular endothelial growth factor receptors (VEGFR) are applied in carcinoma treatment. Their use may be limited by a rapidly developing serious arterial hypertension. We tested if the VEGFR antagonistic TK inhibitor sunitinib induces alterations in renal vascular function and sodium handling which may contribute to the early pathogenesis of VEGFR inhibitor-induced hypertension.

In vitro, sunitinib did neither enhance agonist-induced vasoconstriction nor blunt endothelium-dependent vasodilation in human and rat renal resistance arteries. In rats, four days of sunitinib treatment caused arterial pressure and RVR to rise by 25-30 mmHg and 5 mmHg*ml⁻¹*min⁻¹*kg⁻¹ kidney weight, respectively. In this early phase of sunitinib-induced hypertension, endothelium-dependent renal vasodilation and its NO-dependent component as well as α₁-adrenoceptor-dependent renal vasoconstriction were not significantly altered while angiotensin II-induced renal vasoconstriction was blunted in sunitinib-treated rats. Sunitinib significantly reduced fractional Na+ excretion while fractional Li+ excretion remained unaffected suggesting that sunitinib-induced hypertension is at least in part due to interference with an inhibitory action of VEGF on Na+ reabsorption in the distal tubule or collecting duct. To test this, murine cortical collecting duct (M1) cells were treated with VEGF. VEGF caused a significant reduction in α₁-, β₁- and γ-subunit mRNA abundances of the epithelial sodium channel (ENaC) as well as in α-ENaC protein abundance in M1 cells. These effects were antagonized by sunitinib. Within four days, amiloride reduced arterial pressure in sunitinib-treated rats by 10 mmHg (p < 0.05) but not in control rats while the acute natriuretic responses to amiloride were similar in both groups. Our data indicate that early sunitinib-induced hypertension is associated with modest alterations in renal vascular function but markedly increased renal sodium reabsorption. Our data further indicate that VEGF negatively regulates renal collecting duct ENaC. Blockade of an inhibitory action of endogenous VEGF on renal epithelial Na+ reabsorption may be a pathogenetic mechanism that contributes to VEGFR inhibitor-induced hypertension.
The kidneys participate in numerous metabolic processes and have a high requirement for oxygen and energy supply. This demand is mostly met, however may be interrupted by transient ischemia/reperfusion events, where tissue-protective mechanism are required. The Y-box binding protein-1 (YB-1) is the prototypic member of the cold shock family of RNA/DNA binding proteins that is involved in pleiotropic cytoprotective functions. Recent findings indicate YB-1 secretion via a non-classical pathway and profound extracellular effects mediated by Notch-3 receptors. In vitro studies have also identified YB-1 as a key regulator of inflammatory mediators, such as RANTES/CCL5, MCP1/CCL2, GM-CSF, and IL-2 in monocytic cells. Given that the conventional Ybx1 knockout is embryonic lethal, we have generated a conditional Ybx1 knockout mouse, which we have crossed onto LysM-Cre mice to analyze the contribution of YB-1 to monocytic cell function.

Initial analyses indicate that the Ybx1(fl/fl)LysM-Cre strain develops normally and shows no overt aberrations. Since we previously observed a reduction in infiltrating inflammatory cells in Notch3-deficient animals as well as a mutual autoregulatory loop between YB-1 and Notch3, we hypothesized a relevance of the YB-1/receptor Notch-3 axis for the chemotactic response. Furthermore, YB-1 expression in monocytic cells directs cell-specific CCL5 expression and is linked with cell differentiation.

Therefore we utilized the murine unilateral ureteral obstruction (UUO) model, which mirrors hallmarks of tubulointerstitial injury and damage. Our data demonstrate an increase in tissue resident ‘sentinel’ cells (F4/80+/GR-1-). Following disease induction, we observe an enhanced recruitment of CD45+ and CD3+ infiltrating cells within the kidney, increased CCL5 expression as well as a mild increase in tissue damage and fibrosis. In a separate model of antibody-induced nephrotoxic serum nephritis, the loss of monocytic YB-1 resulted in a dramatic enhancement in disease severity. Similarly, knockdown of YB-1 was accompanied by severe ischemia/reperfusion damage of the kidneys.

Thus, our findings point to a critical role of monocytic YB-1 in the resolution of inflammatory processes, which may largely be due to a disturbed monocytic cell differentiation and perturbation of CCL5 chemokine expression. A temporal upregulation of YB-1 expression may therefore be beneficial for inflammatory kidney disease outcome and relevant for ischemia/reperfusion damage.
**S1-01**
The ion channels of the inner mitochondrial membrane

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A brief overview of Mitochondrial Inner Membrane (MIM) ion channels will be presented. Their biophysical properties will be mentioned, and attention will be drawn to still incompletely explored aspects, such as biogenesis, relationship to plasma membrane channels, selective pharmacology and in some cases molecular identity, regulation, interactions with other mitochondrial proteins, physiological and pathological roles. The focus will be on those channels not dealt with in other talks of the symposium: matrix [Ca\(^{2+}\)] (potentially) modulates - besides the “Big” Ca\(^{2+}\)-activated K\(^+\) channel, mtBK(Ca), and the Mitochondrial Permeability Transition Pore (MPTP) - also intermediate- and small-conductance K\(^+\) channels, mtIK(Ca) and mtSK(Ca). The mitochondrial ATP-sensitive K\(^+\) channel, mtK(ATP) is, after the MPTP, the most extensively studied MIM channel, yet our understanding of it is still limited; the identification of ROMK2 as its pore-forming component, three years ago, so far has not been adequately followed up. The yeast MIM protein Sym1 forms a cation-selective channel; its mammalian homolog MPV17 is under study because mutations are associated with mitochondrial DNA depletion syndrome (MDDS); an in-depth characterization would clearly be welcome. TASK-3 is a two-pore “background” K\(^+\) channel (K2P) thought to contribute to the vitality of malignant cells, present in the MIM of at least some normal and cancerous cells; its downregulation has a strong impact on mitochondrial parameters and function and promotes apoptosis, pointing to a possible functional analogy with MIM Kv-family channels. The long-sought “Ca\(^{2+}\)” uniporter MCU is not the only divalent cation-permeable channel of the MIM: Mrs2 transports Mg\(^{2+}\), a physiologically important role. Anion-selective channels deserve as much attention as cation-selective ones, although they are receiving less. The Inner Membrane Anion Channel (IMAC) can be equated with the “107 pS” pore that was the first MIM channel to be identified and studied by patch-clamp. A higher conductance weakly anionic channel has also been described. The literature contains several scattered reports of mitochondrial pore activities, both anion- and cation-selective, which still await an in-depth characterization, e.g. a pH-sensitive cation-selective conductance recently observed in planar bilayer work. The study of mitochondrial channels may also be defined to include activities – studied in vitro - by proteins involved in the transport of peptides/proteins (e.g. TIM22, TIM23) or small molecules (mitochondrial carriers), although whether these transport systems contribute significantly to the ion permeability of MIM under physiological conditions remains dubious. In summary: a fertile research field with still much to contribute to molecular physiology and medicine.

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**S1-02**
Regulation and physiology of the mitochondrial calcium uniporter MCU

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The recently identified Mitochondrial Calcium Uniporter (MCU) is a 40 kDa protein of the inner mitochondrial mem- brane responsible for Ca\(^{2+}\) uptake into the matrix, which plays a role in the control of aerobic metabolism and cellu- lar signaling under physiological and pathological condi- tions. Recombinant or in vitro-expressed MCU alone is able to form calcium-permeable ion channels with a very low conductance (6 pS in 100 mM Ca\(^{2+}\)). However, by now it is becoming clear that some characteristic features of the mito- chondrial Ca\(^{2+}\) uptake machinery (e.g. the observation that mitochondrial Ca\(^{2+}\) uptake varies greatly among different cells and tissues and that the channel displays low activity at resting state but an increased activity after cellular stimula- tion) are due to the important contribution of several modu- lators of the channel-forming protein. Indeed, mitochondrial calcium accumulation was recently shown to depend on a complex composed of an inner-membrane channel (MCU and MCUb, a dominant-negative subunit) and regulatory subunits (MICU1, MICU2, MCUr1, and EMRE). In particular, both MICU1 and MICU2 are regulated by calcium through their EF-hand domains, thus accounting for the sigmoidal response of MCU to [Ca\(^{2+}\)] in situ and allowing tight physio- logical control. At low [Ca\(^{2+}\)], the dominant effect of MICU2 largely shuts down MCU activity; at higher [Ca\(^{2+}\)], the stimu- latory effect of MICU1 allows the prompt response of mi- tochondria to Ca\(^{2+}\) signals generated in the cytoplasm. The role of the recently discovered MICU3 in regulating channel activity and the mitochondrial calcium uptake will also be discussed.

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**S1-03**
Uncoupling proteins – by name or function?

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The original uncoupling protein, UCP1, is truly uncoupling, bioenergetically as well as physiologically. The other two members of the phylogenic uncoupling protein family, UCP2 and UCP3, are probably not uncoupling – and even if they were, the amounts of these proteins found in the mitochondria are so small – 2-3 orders lower than that found of UCP1 in brown-fat mitochondria – that an uncoupling function would not be physiologically discernable. Even the protoUCP1, found in fishes and even in marsupials, is probably not uncoupling: uncoupling is a property acquired during the evolution of the true mammals. – Other proteins given UCP names – UCP4 and UCP5 – do not even belong phylogenically to the uncoupling protein (sub)family. The uncoupling proteins are members of the large family of mitochondrial inner membrane transporters, all having a tri- partite structure and a molecular weight around 33000. A
feature that is common for several members of this family, particularly the adenine nucleotide transported (ANT), is that they – in the absence of cognate substrate for transport – can transport fatty acids over the mitochondrial membrane. As certain fatty acids can diffuse (flip-flop) over the membrane in the hydrogenated form, a proton-conducting circuit can be formed, a circuit that short-circuits the mitochondrial membrane potential and thus is uncoupling. Likely even UCP1 as well as UCP2/UCP3 can do this, but there is no reason to think that this process has physiological relevance. Importantly, also fatty acids that cannot flip-flop can induce uncoupling through UCP1, demonstrating that this is a unique process not related to what can be observed in any other mitochondrial transporter.

In the innate situation, in the cell, UCP1 activity is blocked by cytosolic purine nucleotides: ATP, ADP, GTP, GDP. Experimentally with isolated mitochondria, GDP is commonly used. Fatty acids have the ability to functionally compete with the inhibition caused by GDP, showing apparently competitive kinetics, but it does not seem as if GDP and fatty acids compete for the same binding site physically. How uncoupling molecularly occurs through UCP1 activity is surprisingly unsettled, even more than 3 decades after its identification. Importantly, it is unlikely that UCP1 actually transports protons over the mitochondrial membrane. UCP1 activity is associated with a high permeability for anions, but the actually transported species is still not established. The functional roles of UCP2 and UCP3 also remain unsettled; the possibility that they can become activated by ROS and thus counteract further ROS production has not been unequivocally supported by recent experiments. Only the function of UCP1 as the molecular mediator of nonshivering thermogenesis is firmly established.

**S1-04**

**New answer to an old question: regulation of mitochondrial function by cytosolic calcium, pathophysiological consequences**

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Cytosolic calcium (Ca2+cyt) coordinates cellular work load and ATP regeneration. The paradigmatic view is that Ca2+cyt after its uptake via the Ca2+ uniporter activates matrix dehydrogenases for regulation of mitochondrial OXPHOS. We can show that the extent of this mechanism is low and that its importance was largely over-interpreted in the past. Instead, we have shown at isolated brain mitochondria (BM) that Ca2+cyt regulates the glutamate/malate- as well as the glycerol-3-phosphate (G3P)-dependent rates of OXPHOS via reversible activation of aralar the mitochondrial glutamate/ aspartate carrier and the mitochondrial G3PDH5,6,15. In contrast, pyruvate/malate dependent OXPHOS is only slightly influenced by Ca2+cyt. The pyruvate formation in connection with the malate aspartate shuttle (MAS) and the G3P shuttle (G3PS) is tightly controlled by Ca2+cyt. Via NADH/NAD+, the pyruvate formation is tightly linked to both shuttles4,8,9. Therefore a rise of Ca2+cyt causes large enhancements of OXPHOS due to increased pyruvate supply. Changes of state 3 respiration are accompanied by respective changes of mitochondrial Δψ. Ca2+cyt also controls the rate of mitochondrial ROS-formation. Both the MAS and G3PS, together with the pyruvate generating enzymes (LDH, GAPDH) act as a metabolic pyruvate supply unit which we called “Mitochondrial Gas Pedal”3,8,10. These results were obtained with reconstituted systems consisting of isolated mitochondria and soluble enzymes of the MAS or G3PS.

We now demonstrate at synaptosomes and intact fibroblasts both fed with glucose that the addition of BAPTA decreases the cytosolic Ca2+ from 76±11 nM to 15±11 nM. At this low Ca2+cyt the MAS is not active anymore. As a consequence pyruvate formation stops and the pyruvate concentration starts to decrease. After 15 minutes, the pyruvate concentration is decreased by 40 % causing a decreased pyruvate supply to mitochondria. Therefore the respiration decreased by 45 %. An pyruvate addition, however, restores the original rate of respiration within seconds but Ca2+cyt remains on its low level. Similar results were obtained at cultivated human fibroblasts. These data clearly show that activation of mitochondrial function is regulated via the pyruvate supply to mitochondria and not by changes in Ca concentration. Moreover this regulation does not need the uptake of Ca2+cyt via the mitochondrial Ca uniporter.

For generalization of our hypothesis we investigated mitochondria from different tissues. Comparing mitochondria isolated from white (m. rectus femoris) and red (m. soleus) muscle we found that the rate of pyruvate oxidation can be stimulated by Ca2+cyt only to a small extent whereas the glutamate oxidation was largely activated. White muscle mitochondria also oxidize G3P but red muscle mitochondria do not. This difference was confirmed by different mRNA pattern, too.

We conclude that the MPG probably acts in all tissues which oxidize pyruvate in mitochondria but we detected different combinations of these shuttles. Heart and red muscle contain the MAS only. Astrocytes and brown adipose tissue contain only the G3PS but in neurons and white muscle both shuttles are present. At sufficiently low Ca2+cyt (<<100 nM) mitochondria are in a substrate-limited state with decreased Δψ limiting OXPHOS but also avoiding ROS-formation. We assume that healthy neurons adjust such a condition at physiological rest (e.g. sleep). Pathological conditions with permanently but modestly increased Ca2+cyt (to only 100-300 nM) should have the disadvantage of elevated ROS formation.

**S1-05**

Regulation of the mitochondrial BKCa-channel by the respiratory chain

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In recent years, a number of potassium channels present in the inner membrane of mitochondria have been discovered. Their physiological roles are still unclear. However, it has been observed that potassium channels affect mitochondrial matrix swelling, regulate the concentration of reactive oxygen species, change the mitochondrial membrane potential, and transport calcium into mitochondria. Additionally, it has been shown that activation of mitochondrial potassium channels protects against necrotic and apoptotic cell death during myocardial infarction or cerebral hypoxia. These findings stimulated an intensive study of the pharmacology of mitochondrial ion channels and contributed to the development of many hypotheses concerning the role of mitochondrial ion channels in cell death.

In our study, we described pharmacological and electrophysiological properties of the large-conductance Ca2+-regulated potassium channel (mitoBKCa channel) using patch-clamping mitoplasts isolated from the astrocytoma U-87 MG cell line. Western blot analysis, immuno-gold electron microscopy, high-resolution immunofluorescence assays and polymerase chain reaction demonstrated the presence of the BKCa channel with b4 regulatory subunit in the inner mitochondrial membrane of the human astrocytoma cells. Also, we showed that substrates of the respiratory chain (e.g. succinate) decrease the activity of the channel at positive voltages. This effect was abolished by inhibitors of the respiratory chain (e.g. antimycin). Our findings indicate possible structural and functional coupling of the mitoBKCa channel with the mitochondrial respiratory chain in human astrocytoma U-87 MG cells.


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**S1-06**

The mitochondrial permeability transition pore: channel formation by F-ATP synthases

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The “permeability transition” (PT) denotes a Ca2+-dependent permeability increase of the inner membrane of mammalian mitochondria to solutes with molecular masses up to about 1,500 Da. Recognized since the early days of mitochondrial research, its nature remained a mystery for 60 years [1]. Permeability increases have also been described in yeast and Drosophila mitochondria, but whether the PT has been evolutionarily conserved has been the matter of debate (reviewed in [2]). We recently discovered that mammalian F-ATP synthase forms Ca2+- and oxidant-activated channels that possess all the electrophysiological features of the PT pore [3]. I will discuss recent data on channel formation by F-ATP synthases of yeast [4] and Drosophila [5] indicating that transition of F-ATP dimers to form PT pores is a conserved evolutionary feature with distinct species-specific differences.

References


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**S1-07**

*Pseudomonas aeruginosa* pyocyanin induced neutrophil death is mediated by direct mitochondrial effects controlled by acid sphingomyelinase and Cftr


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Pulmonary infections with *Pseudomonas aeruginosa* are the major clinical problem of patients with cystic fibrosis, but they are also critical in immunocompromised or ventilated patients. Cystic fibrosis is caused by mutations of the cystic fibrosis transmembrane conductance regulator CFTR and constitutes the most common autosomal recessive disorder in western countries. Many *P. aeruginosa* strains are resistant to antibiotics limiting therapeutic options. It has been previously shown that neutrophils are central in the defense against *P. aeruginosa*. Thus, we investigated interactions of *P. aeruginosa* and, in particular its soluble toxin pyocyanin, with neutrophils. The neutrophil response is altered in cystic fibrosis lungs, in which neutrophils accumulate and contribute to chronic inflammation, a hallmark of cystic fibrosis lungs. Our studies show that high concentrations of pyocyanin trigger apoptosis of wildtype neutrophils via direct interaction of pyocyanin with the mitochondrial respiratory chain resulting in release of reactive oxygen species (ROS), activation of mitochondrial acid sphingomyelinase, formation of mitochondrial ceramide and finally release cytochrome c from mitochondria. Lower concentrations of pyocyanin induce ROS by inhibition of the mitochondrial Kv1.3 channel also resulting in acid sphingomyelinase activation and ceramide release. Genetic deficiency of the acid sphingomyelinase or the cystic fibrosis transmembrane conductance regulator (Cftr) prevents activation of this pathway and pyocyanin-induced neutrophil death. The reduced death on the other hand correlates with an increased release of Interleukin 8 from pyocyanin-activated acid sphingomyelinase and cystic fibrosis neutrophils compared to wildtype cells. The studies indicate a novel mechanism of pyocyanin-induced death of neutrophils. This process is altered in cystic fibrosis neutrophils, which may contribute to the chronic inflammation in cystic fibrosis lungs.

**S2**

Pathomechanisms of Familial Hypertrophic Cardiomyopathy, a cardiac disease of the sarcomere

**S2-01**

*Bringing genetics to medicine: hypertrophic cardiomyopathy - present and future*

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Hypertrophic cardiomyopathy (HCM) was the first inherited cardiomyopathy to be characterized at the molecular level. Approximately 25 years ago, landmark studies demonstrated that sarcomere mutations cause disease, ushering in the field of cardiovascular genetics. Initially, genetic testing could only be performed in highly specialized research laboratories able to perform meticulous linkage analysis and candidate gene sequencing in large, well-characterized families. However, DNA sequencing methodology has evolved tremendously over the past decade, allowing genetic testing to now be commercially available. Disease-causing (pathogenic) sarcomere mutations are routinely identified in HCM patients in clinical practice. Cascade family testing also allows precise identification of at-risk relatives in the preclinical stage—prior to diagnosis. These individuals have inherited the mutation that causes HCM in their family and are therefore at risk for developing disease, but currently have normal left ventricular wall thickness and no diagnostic clinical manifestations (G+/LVH-). In addition to guiding care of the individual and the family, studying this intriguing population provides a valuable opportunity to characterize early phenotypes of sarcomere mutations. Moreover, preclinical mutation carriers may be a key group to target for new treatment approaches designed to modify the natural history of disease. By being able to initiate treatment in an at-risk population before irreversible changes to the heart’s structure and function have occurred, disease-modifying therapy may be more successful. Ongoing and future basic science and clinical translational collaborations will continue to dissect the precise pathways driving how these sarcomere mutations lead to the complex phenotype of HCM, and identify rational targets for novel therapeutic strategies designed to interrupt the emergence of disease.
S2-02  
Myocardial dysfunction in hypertrophic cardiomyopathy (HCM): primary effects of sarcomeric protein mutations versus secondary E-C coupling remodeling  
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We have recently shown (Coppini et al, Circulation 2013) that in cardiac muscle from HCM patients primary changes in myofilament function, related to the presence of mutant sarcomeric proteins responsible for the disease, are always associated with secondary abnormalities due to adverse remodeling of cardiomyocyte E-C coupling. The latter are likely major contributors of the mechanical dysfunction and arrhythmogenesis of HCM human hearts. Here we characterize the changes in sarcomere function and E-C coupling that occur in two HCM mouse models carrying different mutations in cTnT (R92Q and E163R). Echocardiography showed LV hypertrophy, enhanced contractility, diastolic dysfunction and enlarged left atria in both HCM models; the phenotype was more pronounced in the R92Q mice. In E163R ventricular myofibrils, in spite of a significant increase in the rate of the initial isometric slow phase of relaxation, overall relaxation from maximal activation was impaired and prolonged vs WT and R92Q myofibrils that exhibited similar relaxation kinetics. Resting tension was higher in the E163Q compared to WT and R92Q myofibrils. Isometric ATPase both at rest and at maximal Ca2+-activation and the energy cost of tension generation were increased in E163R vs WT and R92Q skinned trabeculae. Myofilament Ca2+-sensitivity was increased in both mutant lines compared to WT; the change was larger in the R92Q preparations. R92Q intact cardiomyocytes and trabeculae compared to WT and E163R preparations showed blunted response to inotropic interventions, reduced amplitude and slower decay of Ca2+-transients with reduced SERCA function. Twitch kinetics were prolonged in both HCM mouse models, despite Ca2+-transient kinetics was faster and SERCA function unchanged in the E163R mice. Intact preparations of both HCM mouse models showed increased probability of arrhythmogenic behavior that increased in response to isoproterenol. The results suggest that similar HCM phenotypes can be generated through different pathogenic pathways. We employed the R92Q mouse model to assess whether long-term oral treatment with ranolazine, a late Na+ current blocker, is capable to prevent the HCM phenotype and the associated myocardial remodeling. Echocardiographic measurements showed that 1 year-old R92Q mice treated since birth with the drug lacked the left ventricular hypertrophy, hypercontractility and diastolic dysfunction found in the R92Q-untreated mice. Gadolinium-contrast magnetic resonance showed that the intramyocardial fibrosis of the R92Q-untreated hearts was largely reduced in the treated mice. Mechanical experiments in intact ventricular trabeculae showed that the alterations observed in the R92Q-untreated mice were mostly reversed in the R92Q-treated mice. Both amelioration of cardiomyocyte function and reduction of extracellular fibrosis may contribute to the positive effect of the long-term treatment with ranolazine.

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S2-03  
Energy deficiency as target of treatment in hypertrophic cardiomyopathy  
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Hypertrophic cardiomyopathy (HCM) is a genetically inherited cardiac disease characterized by asymmetric hypertrophy of the left ventricle in the absence of other cardiac or systemic diseases. The exact disease mechanism leading from the genetic defect to manifest HCM are largely unknown. Moreover, disease onset varies even in family members carrying the same mutation. Sarcomere mutations might induce energy depletion for which until now there was no direct evidence at sarcomere level in human HCM. We performed in vivo and in vitro studies to investigate if mutations in genes encoding myosin-binding protein C (MYBP3) and myosin heavy chain (MYH7) underlie changes in the energetic cost of contraction in the development of human HCM. Energetic cost of contraction was studied in vitro by measurements of force development and ATPase activity in cardiac muscle strips from manifest HCM patients with and without sarcomere mutations. In addition, in vivo, the ratio between external work (EW) and myocardial oxygen consumption (MVO2) to obtain myocardial external efficiency (MEE) was determined in pre-hypertrophic mutation carriers, manifest HCM patients and healthy controls using [11C]-acetate positron emission tomography and cardiovascular magnetic resonance imaging. Tension cost (TC), i.e. ATPase activity during force development, was higher in HCM patients carrying sarcomere mutations compared with sarcomere mutation-negative HCM patients. In addition, in vivo, the ratio between external work (EW) and myocardial oxygen consumption (MVO2) to obtain myocardial external efficiency (MEE) was determined in pre-hypertrophic mutation carriers, manifest HCM patients and healthy controls using [11C]-acetate positron emission tomography and cardiovascular magnetic resonance imaging. Tension cost (TC), i.e. ATPase activity during force development, was higher in HCM patients carrying sarcomere mutations compared with sarcomere mutation-negative HCM patients. TC was highest in HCM patients carrying MYH7 mutations at maximal and submaximal, more physiological, [Ca2+]. EW was significantly lower in mutation carriers, while MVO2 did not differ. MEE was significantly lower in mutation carriers compared with controls, showing the lowest efficiency in MYH7 mutation carriers. MEE was even more depressed in patients with manifest HCM. Here, we provide direct evidence that sarcomere mutations perturb the energetic cost of cardiac contraction. Gene-specific severity of cardiac abnormalities may underlie differences in disease onset and suggests that early initiation of metabolic treatment may be beneficial, in particular, in MYH7 mutation carriers.
Familial Hypertrophic Cardiomyopathy (FHC) is characterized morphologically by left ventricular hypertrophy, myocyte and myofibrillar disarray, and interstitial fibrosis. Among genotyped patients about 1/3 are heterozygous for missense mutations in β-myosin heavy chain (β-MyHC) and in myosin binding protein C (cMyBPC), respectively. It is still unclear how altogether several hundred different mutations in at least 20 different sarcomeric and also some non-sarcomeric proteins result in the characteristic features of FHC. Generally, it is assumed that the FHC phenotype is triggered by the respective mutation, while a specific direct effect common to all FHC-mutations has not been identified. Previously we found for different β-MyHC missense mutations in slow skeletal muscle fibers from FHC-patients a large functional variation in force-pCa-relations ranging from normal (control-like) to significantly shifted curves compared to controls. Evidence suggested that the heterogeneity of the force-pCa-relations presumably results from fiber-to-fiber variation in abundance of mutated β-MyHC.

Based on this, we hypothesized that unequal expression of the mutated protein from cardiomyocyte to cardiomyocyte could trigger the development of FHC-typical features, at least in the case of β-MyHC-mutations. If the mutated myosin is not equally expressed in all myocytes, increased or reduced force generation caused by the respective mutation would lead to imbalanced force generation among neighboring cells.

To test this hypothesis we recently extended our functional studies and mRNA-quantification to single cardiomyocytes isolated from myocardium of FHC-patients. Functional analysis of cardiomyocytes showed a large variation of calcium-sensitivity from cell-to-cell, which may well be due to a variation in abundance of mutated β-MyHC. At the mRNA-level, we found a large variation in mutant vs. wildtype β-MyHC-mRNA expression in individual cardiomyocytes of the same tissue samples, ranging from almost pure mutant to almost pure wildtype mRNA.

These data support our hypothesis that cell-to-cell allelic imbalance occurs in FHC-patients myocardium and is associated with functional imbalances from cardiomyocyte to cardiomyocyte. Over the years, this could induce distortions within the cellular network of FHC-affected myocardium, resulting in myocyte disarray. Such structural distortions may gradually activate stretch-sensitive cell signaling in cardiomyocytes and non-myocyte cells, leading to interstitial fibrosis and hypertrophy.
Optogenetic dissection of amygdala intercalated cells in fear circuits
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Increasing evidence suggests that parallel plastic processes in the amygdala involve inhibitory elements to control fear and extinction memory. GABAergic medial paracapsular intercalated cells (mpITCs) are thought to relay activity from basolateral nucleus (BLA) and prefrontal cortex to inhibit central amygdala output during suppression of fear. Recently, projection diversity and differential behavioral activation of mpITCs in distinct fear states suggest additional functions. Here, we use a combination of electrophysiological, anatomical and optogenetic approaches to delineate functional properties of novel circuitry of mpITCs. We demonstrate that mpITCs receive convergent sensory thalamic and cortical inputs that undergo fear learning-related changes and are dynamically modulated via presynaptic GABAB receptors recruited by GABA released from the mpITC network. Among mpITCs, we identify cells that inhibit but are also mutually activated by BLA principal neurons. Thus, mpITCs take part in fear learning-modulated feed-forward and feedback inhibitory circuits to simultaneously control amygdala input and output nuclei. Our findings place mpITCs in a unique position to gate acquired amygdala-dependent behaviors via their direct sensory inputs.

Endocannabinoids in circuits of the extended amygdala: impact for fear sustainment
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The vast majority of experimental studies assess fear as a rapidly beginning and dissipating state of apprehension in response to a threat (“phasic fear”), while anxiety and anxiety disorders are based on a more long-lasting state of apprehension that is typically elicited by less predictable threats (“sustained fear”). One accepted rat model (Davis et al., 2010) puts neuronal connections within the “extended amygdala”, involving the central amygdala and the bed nucleus of the stria terminalis (BNST), in a key position for mediation of sustained fear, although this model awaits explanation on a synaptic and mechanistic level. Along an independent line of evidence, endocannabinoids have been shown to regulate synaptic interactions in the amygdala associated with habituation and extinction. Therefore we hypothesized that the endocannabinoid system is critical also for controlling sustained states of fear in the extended amygdala.

We developed a fear training paradigm involving non-anticipated conditioned stimuli allowing to distinguish phasic and sustained fear states in mice, used genetic mouse lines with cell-type specific deficiency or rescue of cannabinoid type 1 (CB1) receptors, and locally expressed channelrhodopsins through adenoviral transfection in specific amygdaloid nuclei. Through combined optogenetic and electrophysiological approaches we studied CB1-mediated synaptic interactions in specific amygdala to BNST pathways, and through local pharmacological intervention in behaving animals we assessed their functional relevance for conditioned fear. Overall our data indicate that stimulation of CB1 receptors on central and basal amygdala inputs to non-GABAergic neurons in the dorsal BNST are both necessary and sufficient for controlling the transition between phasic and sustained components of fear.


New players in angiogenesis and vascular homeostasis

Roles of miRNAs for cardiovascular development and remodelling
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miRNAs have multiple roles in many physiological processes. The miRNAs miR-1 and miR-133a belong to the most abundant miRNAs of the heart and are transcribed from two genomic clusters. To investigate the function of these miRNAs in cardiac development and physiology we have generated loss of function mutants for both genomic clusters. We discovered a function of the miR-1/133a clusters in the specification of the early cardiomyocytes by supporting the transition from an immature embryonic condition, characterized by smooth muscle gene expression, to a more mature fetal stage. This transition is fundamental for development and function of the embryonic heart; however re-expression of smooth muscle marker genes is also a hallmark of remodeling processes in the adult heart. At embryonic stages these miRNAs function primarily by repression of the direct miR-1 target gene myocardin and overexpression of myocardin in
the embryonic heart recapitulates the miR-1/133a loss of function phenotype. We show that myocardin also regulates the miR-1/133a clusters in a negative feedback-loop, thus these miRNAs together with other identified molecular targets form a regulon that has defined functions in cardiomyocyte development.

S4-02
Non-coding RNAs in cardiovascular aging
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Aging is the major risk factor for developing cardiovascular complications like endothelial dysfunction, atherosclerosis, formation, acute myocardial infarction and heart failure. The mechanisms involved in cardiovascular aging are poorly understood, but non-coding RNAs, including microRNAs, have emerged as key biological regulators. We have identified that age-induced miR-29 has a crucial role in atheroma by regulating the expression of extracellular matrix proteins in the aorta. Furthermore, we recently described the crucial role for miR-34a in cardiac aging, which regulates cardiomyocyte apoptosis and telomere length. Current studies in the laboratory focus on determining the role of long non-coding RNAs (lncRNAs) in aging of the endothelium and how these affect organ homeostasis and cellular metabolism. We first showed that many lncRNAs (>200 nt) are expressed in endothelium and that the lncRNA MALAT1 is required for endothelial proliferation in vitro and in vivo. Several lncRNAs are also regulated during endothelial aging, most notably Meg3 and H19, which are up- and downregulated during aging, respectively. Inhibition of Meg3 and H19 in vitro affects endothelial function and senescence and ongoing mechanistic studies will elucidate the mechanism by which these lncRNAs affect endothelial aging.

S4-03
The F-BAR protein NOSTRIN and its function in the cardiovascular system
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Endothelial cells are surprisingly diverse in appearance and remarkably multifaceted in respect to the physiological functions they execute. This is most evident when one compares the highly mobile endothelial tip cell with a quiescent endothelial cell lining the inner vessel wall. Tip cells are highly polarised and characterised by numerous filopodial protrusions, through which they sense guidance cues and lead the growing vessel to establish the stereotypic vascular pattern during development. In contrast, the quiescent endothelial cell in a mature vessel is flattened out, stationary and characterised by tight cell/cell contacts. The distinct endothelial cell functions are associated with specific requirements in respect to membrane shape and cytoskeleton dynamics. Recently, membrane-bending proteins of the BAR family have been found to play an important role in the control of membrane shape and in coupling membrane remodeling with cytoskeleton dynamics in a number of cell types. The property to sculpt the membrane is mediated by their common N-terminal BAR domain, which binds phospholipids through defined positively charged amino acids and displays intrinsic curvature. In addition to the BAR domain BAR proteins contain a number of protein/protein interaction domains to link to cytoskeletal components and regulators. They are involved in processes such as endocytosis, membrane protrusion formation, migration or cytokinesis. We have investigated the function of the F-BAR protein NOSTRIN in endothelial cells in adult vasculature as well as during vascular development. NOSTRIN modulates the subcellular trafficking of endothelial nitric oxide synthase (eNOS) by mediating the interaction between eNOS and the endocytic machinery components dynamin and N-WASP; hence the name nitric oxide synthase traffic inducer. Here we discuss the consequences of the loss of NOSTRIN for developmental angiogenesis and cardiovascular function upon antisense morpholino nucleotide-mediated knockdown in zebrafish embryos or in NOSTRIN knockout mice.

S4-05
Semaphorin-3C signals through Neuropilin-1 and PlexinD1 receptors to inhibit pathological angiogenesis
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Preterm babies are at risk of developing retinopathy of prematurity which causes visual impairment due to destructive neangiogenesis after degeneration of the retinal microvascularity. This study was aimed at studying how the neuronal guidance molecule Semaphorin-3C (Sema3C) acts on endothelial cells and if it can be used to interfere with pathological angiogenesis in the eye. Sema3C exerted potent inhibiting effects in cellular models of angiogenesis. In an endothelial cell xenotransplantation assay Sema3C acted primarily on immature microvessels by inducing endothelial cell apoptosis. Intravitreal administration of recombinant Sema3C disrupted endothelial tip cell formation and cell-cell contacts, which led to decreased vascular bed expansion and vessel branching in the growing retinal vasculature of newborn mice, while not affecting mature vessels in the adult retina. Sema3C administration strongly inhibited the formation of pathological pre-retinal vascular tufts during oxygen-induced retinopathy. Mechanistically, Sema3C signaled through the receptors Neuropilin-1 and PlexinD1, induced VE-cadherin internalization and abrogated VEGF-induced activation of the kinases AKT, FAK and p38MAPK. This led to disruption of endothelial cell junctions, focal adhesions and cytoskeleton assembly resulting decreased cell migration and cell survival. Thus this study identified Sema3C as a potent inhibitor of pathological retinal angiogenesis.
S5

Imaging subcellular structures and molecular organization to understand brain function

S5-01

In vivo STED microscopy of the visual cortex of adult mice


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Two-photon microscopy and less extensive also confocal microscopy are widespread techniques to visualize fluorescently labeled cells in living cells, tissue or living animals. However, the limit of this technique is the diffraction limited resolution of about half of the wavelength of light (~ 200–350 nm) which had been overcome by a whole family of superresolution microscopy or nanoscopy concepts such as STED, RESOLFT, PALM, STORM etc. STED microscopy, which was implemented first, stands out for its imaging capabilities in tissue: It is live-cell compatible, especially when using standard fluorescent proteins such as EGFP or EYFP, it is able to record 3D images from inside transparent tissue, and the imaging speed is fast compared to other superresolution methods. Recently, we have developed an upright scanning STED microscope to image the dynamics of dendritic spines in the molecular layer of the visual cortex in a living, anesthetized mouse. With Lifeact, an actin binding peptide, fused to the yellow fluorescent protein (EYFP) we labeled actin. We recorded actin in dendritic arborisation and spines with a resolution of 50–70 nm at a depth down to 40 µm [1]. These results show that STED microscopy becomes a valuable tool to study morphological changes in the living mouse brain.


S5-02

Transcriptome network analysis identifies a developmental switch gene that limits regenerative ability in the adult CNS

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Traumatic central nervous system (CNS) injuries often result in permanent disabilities due to axon regeneration failure. Mechanistically, both non-permissive environment and reduced intrinsic growth ability have been proposed to account for the regenerative failure in the adult. While progress has been made in characterizing extracellular growth inhibitors expressed in the adult CNS, our current understanding of the molecular signature that leads to neurons losing their ability to regenerate is still fragmentary. Here we used a systematic and unbiased approach to identify genes whose expression correlated both positively with loss of axon elongation during later stages of embryonic development, and negatively with conditions necessary to gain growth competence in the adult. RNA sequencing (RNA-Seq) identified a developmental switch that limits axon growth and regeneration. In vitro and in vivo silencing or pharmacological blockade following administration of an important therapeutic class of drugs promoted axon growth and CNS regeneration. Given that our pharmacological approach is already used clinically to manage a wide range of neurological disorders, our results could significantly impact on the development of therapies aimed at promoting structural plasticity and regeneration following a variety of CNS trauma.

S5-03

Astrocyte signalling in spike-timing dependent depression

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Spike-timing dependent plasticity is a plausible mechanism for synaptic plasticity in development, circuit refinement and learning and memory formation. We have been investigating the cellular mechanisms that lead to long-term depression of synaptic strength that is induced by an activity pattern, in which the synaptic activity follows the postsynaptic generation of an action potential. The signaling cascade for the induction of this type of depression requires retrograde endocannabinoid signaling and activation of presynaptic NMDA receptors. We found that the postsynaptic synthesis of endocannabinoids results in the activation of astrocytes that surround the synapse. Activation of astrocytic cannabinoid receptors results in calcium signaling in the astrocyte processes and the release of glutamate onto presynaptic NMDA receptors. Thus astrocyte calcium signaling represents an important step in the modification of synaptic strength.

S5-04

Second generation optical control of metabotropic glutamate receptors

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Metabotropic glutamate receptors are G-protein coupled receptors and perform important tasks in synaptic communication. They have recently been optically controlled by means of a bioconjugation strategy employing cysteine-maleimide chemistry. Although neurons were amenable to optical control, thiol-maleimide labeling has its drawbacks,
such as the introduction of a free cysteine by mutagenesis and the instability of the maleimide in aqueous environments (both before and after labeling) and its possible cross-reactivity. We therefore present a more generalized approach to achieve optical control over the metabotropic glutamate receptor 2 (mGlur2) by using the SNAP-tag technology. With this second generation photochromic tethered system a highly modular photosensitive ligand was synthesized and is labeled to a SNAP_mGlur2 chimera expressed in HEK293T cells. The tethered ligand stays inactive in the dark until illuminated. The resulting photoreceptor can then be used to trigger activation and deactivation of mGlur2 signaling repeatedly when exposed to UV and green light, respectively, followed by GIRK1/2 channel electrophysiology. Generally, this method can be expanded to all SNAP-tagged biomolecules due to its synthetic modularity and the vast number of SNAP-tagged proteins already available.

In our study we could observe that a co-culture of normal human keratinocytes (NHK) and melanocytes induce a re-distribution of MVBs of NHK close to the area of interaction of both cell types. Characterization of EVs revealed an endosomal origin and the labelling allows the observation of the interaction with melanocytes. We have isolated and characterized EVs, of NHK from different donors (Caucasian, Caucasian after UVB irradiation and Black) and incubated them with melanocytes in monoculture or in reconstructed epidermis. Exosomes secreted by Black NHK or by Caucasian NHK stimulated by UVB and applied to Caucasian melanocytes or Caucasian reconstructed epidermis increased the pigmentation genes expression and the tyrosinase activity, resulting in enhanced melanin synthesis. The data also pinpoints components, among which miRNAs, that control pigmentation.

Our studies reveal an unexpected physiological role for exosomes in human pigmentation and open a new avenue to regulate pigmentation in normal and diseased states.

S6-02
The vesicle behind the fusion behind the transdifferentiation —functional RNA transfer from blood to brain by extracellular vesicles in inflammation

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Mechanisms how the immune system signals to the brain in response to systemic inflammation are not fully understood. Transgenic mice expressing Cre recombinase specifically in the hematopoietic lineage (vav1-iCre mice) in a Cre reporter background display recombination and marker gene expression in Purkinje neurons. Here we show that reporter gene expression in neurons is caused by intercellular transfer of functional Cre recombinase messenger RNA contained in secreted extracellular vesicles (EVs), particularly in exosomes. Specifically, exosomes as well as microvesicles from the blood of vav1Cre mice contain Cre mRNA but not protein. Intracerebral injection is sufficient to induce recombination in neurons, formally demonstrating transfer of functional RNA. Physiologically, while Cre mediated recombination events in the brain occur very rarely in healthy animals, their number increases considerably in different injury models, particularly under inflammatory conditions, and extend beyond Purkinje neurons to other neuronal populations in cortex, hippocampus and substantia nigra. Recombined Purkinje neurons differ in their miRNA profile from their non-recombined counterparts, indicating physiological significance. Together, we present evidence that EVs can communicate RNA-based signals directly from the hematopoietic system to the brain in response to peripheral inflammation. In general, using the Cre-lox system allows tracing of EV-mediated RNA transfer in vivo without potentially confounding experimental manipulations.
S6-03
Delivery on demand: exosomes as ‘care packages’ from glia cells for stressed neurons
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Oligodendrocytes are the myelin forming glial cells of the CNS. Moreover, oligodendrocytes support neurons to maintain long term axonal integrity by as yet not fully understood mechanisms. Our recent work revealed that secreted extracellular vesicles, termed exosomes, are involved in bidirectional neuron-glia interaction. Exosomes are released by oligodendrocytes in response to neurotransmitter signalling by activation of glial ionotropic glutamate receptors. These exosomes are internalized by neurons via endocytosis and the exosome cargo is functionally recovered by the recipient neurons. Neurons appear to benefit from exosome internalization by increased resistance to stress conditions such as oxidative stress, starvation or ischemia. Oligodendroglial exosomes have the ability to impact neurons in several ways by modulating action potential firing, axonal transport, signal transduction, and neuronal gene expression. To determine the specific role of exosomes in glial support, we studied PLP- and CNP-deficient mice characterized by secondary axonal degeneration. Intriguingly, exosomes released from PLP- and CNP-deficient oligodendrocytes exhibit quantitative, qualitative, and functional abnormalities. In summary, we propose that oligodendroglial exosomes function as vehicles for the transfer of biomolecules from oligodendrocytes to neurons and are implicated in neuroprotection and glial maintenance of axonal integrity.

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Relevant Publications:


S6-04
Cancer exosomes dynamics in vitro and in vivo
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Exosomes are involved in many intercellular communication processes but also cancer progression. Deciphering exosome-release mechanics and dynamics is essential to understand the physiology of exosomes. However, tools to study this process directly have been lacking. We describe a novel imaging approach that can visualize multivesicular body (MVB) fusion with the plasma membrane (PM) directly, an essential step for exosome release. We used our technique to follow MVB-PM fusion dynamics in living tumor cell lines and provide evidence that SNARE molecules and G-protein-coupled receptor signaling control the functional release of exosomes, affecting invasive behavior. Apart from these findings our approach will be imperative for monitoring the dynamics of exosome release and their post-fusion fate, but will also allow researchers to uncover further new insights into the endosomal-exosomal pathway, including their biogenesis, maturation and function.

S6-05
Therapeutic potentials of mesenchymal stem cell-derived extracellular vesicles
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More than 400 NIH registered clinical trials applied MSCs to treat a variety of different diseases such as myocardial infarction, stroke and graft-versus-host disease (GvHD). Initially, MSCs were thought to replace lost cells in damaged tissues. Despite contrary reports regarding the outcome of MSC treatments, MSCs seem to exert their beneficial effects by the secretion of immunosuppressive factors and/or small extracellular vesicles (EVs, 80-160 nm), such as exosomes and microvesicles, rather than by interacting into affected tissues.

After setting up techniques for the characterization and large scale preparation of EVs, we have treated a steroid-refractory GvHD patient with EVs/exosomes enriched from MSC...
supernatants (MSC-EVs) (Kordelas et al., 2014). In addition we investigate the MSC-EVs’ therapeutic potential to exert neuroprotective functions in animal models for stroke and preterm brain injury. So fare, we observed beneficial effects in all settings. At the functional level MSC-EVs were shown to exert immunosuppressive functions in vivo and in vitro. In addition, they seem to promote pro-regenerative processes. Due to the contrary reports regarding the outcome of MSC treatments and the fact that MSCs are a very heterogeneous, ill-defined class of fibroblastic cells with adipogenic, cartilagenic and osteogenic differentiation potentials, we consider that not all human MSCs release therapeutic effective EVs. To this end, we harvested EVs from cell-culture supernatants of different donor-derived MSCs and analyzed their immunomodulatory properties on human T cells. Indeed, huge differences were observed in the MSC-EVs’ capabilities to suppress T cell functions. Currently, we are improving the platform to produces MSC-EVs for the clinical setting and search for surrogates to discriminate therapeutic effective and no-effective MSC-EV samples.

S8
Integration of sensory information and memory formation in the hippocampus

S8-01
Multimodal signal integration in thalamo-hippocampal memory networks
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The anterior thalamus provides highly-processed information to the hippocampal formation. The head-direction signal and theta rhythm are believed to be the main signals that propagate within the thalamo-hippocampal circuitry. Thalamic head-direction cells are tightly coupled with the hippocampal place cells. The place cells are also functionally entrained by limbic theta oscillations and their spiking depends on the amplitude and frequency of theta rhythm. The head directional representation integrates external sensory (landmark) information as well as internal idiothetic signals. The idiothetic signals integrate vestibular, proprioceptive and motor efference information. Concurrently, theta oscillations mediate the processing of idiothetic and neuromodulatory signals. The idiothetic signals reflect self-motion information, while the cholinergic neuromodulatory inputs from medial septum regulate the degree of information processing within hippocampo-diencephalic network. Medial septum does not merely generate theta rhythm, but actively integrates sensorimotor stimuli that reflect sniffing rate. Recent findings suggest that theta rhythm generated across the septo-hippocampal axis integrates sensorimotor signals. Such integration may provide temporal oscillatory synchronization of septum-innervated limbic structures with the olfactory information. This temporal coupling incorporates the integration of intrinsic theta and extrinsic sensorimotor signals on each theta cycle. Here, I will address the functional significance of this integration for the episodic memory formation. The thalamo-hippocampal pathways mediating head-direction and theta rhythm signals share similar connectivity. The anatomical substrates of these parallel pathways involve separate diencephalic nuclei. However, the two pathways interact at multiple levels within hippocampo-diencephalic circuitry and I summarize here the main findings that demonstrate this interaction. Such additive effect indicates that the head direction and theta systems alone have only partial effect on the hippocampal spatial representation. It is likely that these parallel signals require functional integration to support hippocampal spatial learning. The firing patterns of the neurons that encode the head-direction signal as well as those that encode theta rhythmicity derive from two main factors: afferent inputs and biophysical cellular properties. The first factor depends on the synaptic inputs, and defines what information the cell is conveying, while the second factor is based on the properties of membrane channels, and determines how the cell will propagate this information. Here, I will review the nature of the head-direction signal together with the biophysical properties of anterodorsal thalamic neurons.

S8-02
All sensory modalities activate different subregions of the hippocampus to activate a number of memory based processes
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In rodents all sensory modalities send information via the anterior thalamus, prefrontal cortex, parietal cortex, retrosplenial cortex, and perihinal cortex to the entorhinal cortex. The entorhinal cortex send this information to different subregions of the hippocampus. For example, layer 2 of the entorhinal cortex projects to the dentate gyrus and CA3 and layer 3 of the entorhinal cortex projects to CA1. The dorsal dentate gyrus (dDG) subregion of the hippocampus processes all sensory (e.g. objects) information including idiothetic inputs to create a spatial representation of the environment via a conjunctive encoding process and in addition engages a memory based spatial pattern separation process. The ventral dentate gyrus (vDG) subregion processes odor information and a memory based odor pattern separation. The dorsal CA3 received inputs from the DG, but also receives information from all sensory modalities via the entorhinal cortex in order to generate associative processes including arbitrary associations and also pattern completion which is important for retrieval of familiar information. The
CA1 subregion receives information from CA3, but also from entorhinal cortex in order to generate memory based temporal processing of information including temporal pattern separation, temporal pattern completion and trace conditioning. Data will be presented to support the above mentioned roles for the dDG and vDG, CA3 and CA1 subregions of the hippocampus.

S8-03
Subregional distinctions in the role of synaptic plasticity for hippocampal information storage and memory
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The neural mechanisms for spatial memory formation are believed to comprise an integration of processes mediated by hippocampal synaptic plasticity in the form of long-term potentiation (LTP) and long-term depression (LTD) (Morris, 2006; Kemp and Manahan-Vaughan, 2007). Novel space consists of several types of information, however, that may evoke differential synaptic responses in individual hippocampal subregions. We have observed that in all hippocampal subregions (CA1, CA3, dentate gyrus), and at multiple types of hippocampal synapses (perforant path-dentate gyrus, mossy fiber-CA3, Schaffer collateral-CA1) exploration of a novel empty environment facilitates the expression of robust LTP (>24h) in freely behaving rats (Kemp and Manahan-Vaughan, 2008; Hagena and Manahan-Vaughan, 2011). In contrast, LTD facilitation is subregion- and synapse-specific and dependent on the nature of the cues. For example, In the CA1 region, partially concealed, small, contextual cues have a facilitatory effect on LTD. On the other hand, LTD in the dentate gyrus is facilitated by large directional cues. Thus, although LTP is facilitated uniformly (but synapse-specifically) in both areas by the same novel environment, LTD is facilitated in a region-specific manner, and at multiple types of hippocampal synapses, (perforant path-dentate gyrus, mossy fiber-CA3, Schaffer collateral-CA1) exploration of a novel empty environment facilitates the expression of robust LTP (>24h) in freely behaving rats (Kemp and Manahan-Vaughan, 2008; Hagena and Manahan-Vaughan, 2011). In contrast, LTD facilitation is subregion- and synapse-specific and dependent on the nature of the cues. For example, In the CA1 region, partially concealed, small, contextual cues have a facilitatory effect on LTD. On the other hand, LTD in the dentate gyrus is facilitated by large directional cues. Thus, although LTP is facilitated uniformly (but synapse-specifically) in both areas by the same novel environment, LTD is facilitated in a region-specific manner, and was based on type of novel spatial cue presented (Kemp and Manahan-Vaughan, 2008). Strikingly, active exploration is not required: facilitation of LTD by spatial contextual information also occurs when the information is presented on a computer screen whilst the animal is stationary (Kemp and Manahan-Vaughan, 2011). Patterned afferent stimulation is not a prerequisite for the appearance of synaptic plasticity in association with declarative learning-plasticity also occurs when merely test-pulse stimulation is given (Manahan-Vaughan & Braunewell, 1999). Furthermore, effects are also not species-specific, as mice also respond to spatial learning events by expressing synaptic plasticity. Taken together, these data suggest that LTP and LTD contribute to the encoding and storage of different components of a spatial representation and that LTD may be specifically involved in storing information about spatial context.

References


S8-04
Dopaminergic modulation of inhibition in the CA1 region of the ventral hippocampus
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Although the actions of dopamine in the CA1 region of the hippocampus are thought to be important for novelty detection and transfer of information into long-term memory, the specific actions of dopamine in this brain area are not fully characterized. Hyperactivity of the dopaminergic system has been linked to the pathophysiology of schizophrenia and repeated exposure to drugs of abuse can result in behavioral sensitization that may contribute to the state of addiction. In the current studies, GABA_A currents were initially measured in acutely dissociated CA1 neurons from rat ventral hippocampal tissue and the effect of activating D_3 dopamine receptors with PD128907 (300 nM) was assessed. GABA_A currents were inhibited in this preparation, consistent with a postsynaptic localization of these D_3 receptors. Mono-synaptic IPSCs were recorded in CA1 neurons from ventral hippocampal slices and the interaction of PD128907 with either mu opioid receptor-expressing or cannabinoid receptor-expressing GABAergic inputs was determined by prior administration of either DAMGO, or WIN55212. DAMGO (1 microM) occluded any further inhibition by PD128907 (1 microM), whereas WIN55212 (1 microM) did not, suggesting that D_3 receptor activation selectively inhibited the MOR-expressing GABAergic input. Either neurogliaform or parvalbumin containing basket cells are interneuron types known to express mu opioid receptors and are therefore potential sources of the GABAergic input targeted by this post-synaptic D_3 receptor-mediated activity. Such a mechanism is an example of how dopamine actions in this region of the ventral hippocampus may contribute to the pathophysiology of schizophrenia and/or drug addiction via disinhibition of CA1 pyramidal neurons.
In psychosis, sensory information processing is disrupted. All three levels are affected: the generation of the percept, the creation of a representation, and the triggering of a behavioural response. Whereas the primary sensory and prefrontal cortices are intrinsically involved in the generation of the percept, the hippocampus is crucial for the creation of spatial and associative representations, both of which critically depend on appropriate processing of sensory information. In animal models of psychosis, hippocampal function is profoundly impaired, and this can be detected at the level of hippocampus-dependent learning and memory, hippocampal synaptic plasticity, and hippocampus-prefrontal cortex information relay and modulation. Disruptions of glutamatergic signalling may underlie the pathophysiology of psychosis and schizophrenia. Antagonism of the N-methyl-D-aspartate receptor (NMDAR) leads to similar molecular, cellular, cognitive and behavioural changes in rodents and/or humans to those that have been identified to occur in psychosis. One of the main loci of change occurs in the hippocampus, raising the question as to whether changes in hippocampal glutamatergic transmission may drive changes in GABAergic and dopaminergic-mediated signaling in psychosis. NMDAR antagonists such as MK801, PCP and ketamine all elicit similar psychosis-related effects, with MK801 inducing the most potent psychotomimetic reactions. We have observed a profound and sustained loss of the hippocampus to express long-term potentiation (LTP) and to support long-term spatial memory following MK801 treatment of rodents to emulate first-episode psychosis. In parallel, the complement and degree of expression of glutamatergic and GABAergic receptors changes within the hippocampus and prefrontal cortex. Furthermore, hippocampal neuronal excitability is persistently increased. These findings indicate that in psychosis, deficits in hippocampus-dependent memory are caused by a loss of hippocampal LTP that arises through enhanced hippocampal neuronal excitability, altered glutamate and GABA receptor expression and an uncoupling of the hippocampus-prefrontal cortex circuitry. These alterations can be expected to result in the disruption of sensory information processing related to the creation of cognitive representations that are characteristic of psychosis.
Molecular insights into TRPA1-mediated nociception
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The perception of external and internal stimuli is critical for survival. In vertebrates, chemical, mechanical and thermal stimuli are detected by specialized sensory neurons which transfer these signals via the spinal cord to the brain. An important subset of these neurons, so-called nociceptors, senses noxious stimuli using specific ion channels as primary signal detectors. Among those are TRPA1 channels which are critically involved in different types of pain in mice and humans. Despite intensive study of TRPA1 channels, little is known about their regulation, especially during different pain states.

We have recently shown that TRPA1-mediated nociceptive behavior can be sensitized in vivo by activating TRPA1 with its ligand mustard oil and by activation of inflammatory signaling pathways. Interestingly, these stimuli increased TRPA1 plasma membrane levels and therefore overall TRPA1 function suggesting that TRPA1 translocation to the membrane represents one of the mechanisms controlling TRPA1-mediated nociception.

In order to get insights into the molecular mechanisms modulating TRPA1-mediated nociceptive signaling we established a mass spectrometry-based proteomics approach to identify proteins associated with endogenous TRPA1 channels in sensory neurons of mice. These efforts led to the identification of Annexin A2 (Anxa2), which is coexpressed with TRPA1 in sensory neurons. Functional studies suggest that Anxa2 limits the availability of TRPA1 channels and consequently regulates TRPA1-dependent nociception in mice. Our findings underscore the notion that protein-protein interactions critically shape nociceptive signaling in sensory neurons which has far reaching implications for the development of new pain therapies.

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Functional interaction of signal transducer gp130 and TRPA1 for mechanonociception and neuropathic pain
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Proinflammatory cytokines augment nociceptor sensitivity by fast modification of transducer ion channels such as TRPV1 or TRPA1 via phosphorylation. Moreover, long-lasting changes in ion channel machinery of nociceptive primary afferents results from the activity of cytokines of the IL-6 family. Mice with a null mutation of the IL-6 signal transducer gp130 in primary nociceptive afferents (SNS-gp130−/−) are largely protected from hypersensitivity to mechanical stimuli in mouse models of pathological pain. Therefore, we set out to investigate how neuronal gp130 regulates mechanonociception.

Proinflammatory cytokines augment nociceptor sensitivity by fast modification of transducer ion channels such as TRPV1 or TRPA1 via phosphorylation. Moreover, long-lasting changes in ion channel machinery of nociceptive primary afferents results from the activity of cytokines of the IL-6 family. Mice with a null mutation of the IL-6 signal transducer gp130 in primary nociceptive afferents (SNS-gp130−/−) are largely protected from hypersensitivity to mechanical stimuli in mouse models of pathological pain. Therefore, we set out to investigate how neuronal gp130 regulates mechanonociception.

SNS-gp130−/− mice revealed reduced mechanonociception in vivo and this was associated with a reduced mechanosensitivity of nociceptive primary afferents in vitro. In line with these findings, mRNA expression of the mechanotransducer ion channel Transient Receptor Potential Ankyrin 1 (TRPA1) was significantly decreased in dorsal root ganglia (DRG). This was reflected by a reduced number of neurons responding with Ca2+ transients to TRPA1 agonists in primary DRG neuron cultures. In the spared nerve injury (SNI) model for neuropathic pain, SNS-gp130−/− mice exhibited a significantly reduced and only transient mechanical hypersensitivity. Although, total TRPA1 mRNA levels were unaltered in DRG explants after SNI, a functional overrepresentation of the mechanotransducer was observed in intact but not injured neurons of control mice. The elevated functional expression was associated with the presence of gp130 as it was not found in SNS-gp130−/− mice.
Our results closely link pro-inflammatory cytokines of the IL-6/gp130 family to the mechanotransducing channel TRPA1, and suggest that gp130 has an essential role in mechanonociception and in the regulation of TRPA1 expression.

**S11-03**  
**New tricks for TRPA1**  
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TRP channels play a key role in sensing environmental and endogenous stimuli. TRPA1 is expressed in primary afferent neurons and senses a multiplicity of exogenous and endogenous chemicals. Agonists can be separated in non-electrophilic and electrophilic agents; the latter act by covalent cysteine modification. So far TRPA1 has been assigned a role in the pathogenesis of inflammatory disease as asthma, colitis and sepsis, cardiovascular control and in glucose homeostasis. Three new developments are discussed.

First, the interplay between TRPV1 and TRPA1, which are coexpressed in sensory neurons. A TRPV1::TRPA1 concatamer expressed in HEK293 cells was responsive to TRPV1 stimuli capsaicin, acidic pH or ethanol, but not to TRPA1 stimuli as allylisothiocyanate, carvacrol or hydrogen peroxide. Atomic force microscopy of solubilised concatamer particles showed a size and volume distribution of TRPV1::TRPA1 similar to the wildtype channels, suggesting the formation of channels consisting of 4 subunits with a 2:2 channel stoichiometry. New constructs suggest that only the first subunit of the dimer is functional, and attempts to functionalize the second subunit are presented.

Second, TRPA1 is involved in transducing photosensitivity and phototoxicity. The wavelength causing an activation of TRPA1 reaches further into the visible blue spectrum than previously reported. The mechanism involves the formation of reactive oxygen species and a covalent modification of TRPA1. This photoactivation could be substantially amplified by the use of aminolaevulinic acid, the entry substrate of the heme pathway and protoporphrin IX. Both agents are used in photodynamic therapy, in which target cells are ablated by phototoxic stress. This treatment is painful and TRPA1 is likely to mediate the respective pain.

Third, acidosis is a hallmark of inflammation and involved in many pathophysiologic states mainly derived from a mismatch of metabolism and perfusion. The main sensors for acidosis, sensitizing and activating primary afferents, are acid sensing ion channels TRPV1 and tandem-pore potassium channels. We demonstrated that the human TRPA1 channel is also gated by acidosis. There are substantial species differences for TRPA1; neither rodent TRPA1, but not even rhesus monkey TRPA1 is sensitive to acidic pH. Especially weak acids like lactic acid, occurring in tissue acidosis activate TRPA1. The importance of the different molecular receptors will be discussed.

**S11-04**  
**Cold sensing receptors: targeting TRPA1 and TRPM8 for pain relief**  
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Investigations into the mechanisms of neuropathic and inflammatory pain have revealed that TRPA1 and TRPM8 could become potential and also interesting targets for controlling persistent pain. At present, different classes of compounds have been identified as TRMP8 and TRPA1 antagonists but, so far, only a small number of these molecules have been investigated in preclinical in vivo studies. For the use as an analgesic drug the pharmacodynamic abilities, like oral availability and the penetration of the blood-brain barrier are of essential additional interest. The major concern regarding preclinical experiments with i.e. TRPA1 antagonists is inherent in different animal species. Therefore, it is not possible to infer that the TRPA1 antagonists tested in rodents would necessarily act in the same way in humans. Several studies have shown effects of TRPA1 modulating agents on cold- and mechanical allodynia in neuropathic and inflammatory pain. In addition to the studies on neuropathic and inflammatory pain, there is a growing list of preclinical evidence implicating TRPA1 in the pathophysiology of headache. TRPA1 immunoreactivity and functional TRPA1-like currents have been found on identified dural afferents in rodents. Distribution of the channel has been reported in both unmyelinated and thinly myelinated axons with terminations in superficial lamina of the trigeminal nucleus caudalis providing an anatomical basis for TRPA1-dependent orofacial nociception. Application of the TRPA1 agonists can increase blood flow in the dura, an effect that is blocked by dural application of either a CGRP receptor or a TRPA1 antagonist. These findings suggest that TRPA1 may contribute to environmental irritant-induced neuronal activation, and the mechanism may be via access of irritants to the meninges through the nasal route and subsequent activation of TRPA1 on meningeal afferents. Summarizing, there is good evidence for the use of cold-receptor modulating compounds as analgesic drugs, although key studies on the most effective mechanism of action and pharmacodynamics are missing.
MicroRNAs (miRNA) are small RNAs that posttranscriptionally regulate gene expression. miRNAs are expressed as long primary miRNA (pri-miRNA) transcripts that are processed in the nucleus to precursor miRNA hairpins (pre-miRNA) by the Microprocessor complex. The pre-miRNAs are exported to the cytoplasm by Exportin 5 and are further processed into mature miRNAs by Dicer and incorporated into the RNA-induced silencing complex (RISC) where they exert their predominant functions in regulating translation and the stability of target genes. miRNAs recognize their targets by base-pair complementarity. The nucleotides 2–7 of the mature miRNA, termed the seed, have been identified as the most essential region for target recognition. Each miRNA is believed to regulate several hundred targets, making up extensive gene expression regulatory networks.

miRNAs are emerging as biomarkers for many diseases, and therapeutically miRNAs can be targeted by antisense inhibitors. Still, the transcription and biogenesis pathways and their influence on disease is less well understood. I will present the first study of in vivo transcriptome-wide pri-miRNA processing using next-generation sequencing of chromatin-associated pri-miRNAs. In this work, we have identified a distinctive Microprocessor signature in the transcriptome profile, from which efficiency of the endogenous processing event can be accurately quantified. This analysis reveals differential susceptibility to Microprocessor cleavage as a key regulatory step in miRNA biogenesis. Processing is highly variable among pri-miRNAs and a better predictor of miRNA abundance than primary transcription itself. Processing is also largely stable across three cell lines, suggesting a major contribution of sequence determinants. Based on differential processing efficiencies we define functionality for short sequence features adjacent to the pre-miRNA hairpin. In conclusion, we identify Microprocessor as the main hub for diversified miRNA output and suggest a role for uncoupling miRNA biogenesis from host gene expression, which could be important for furthering our understanding of how differential miRNA expression is mediated and its regulation in disease.

Mammalian genomes encode numerous long noncoding RNAs (lncRNAs), which play a major, but still largely unexplored role in the regulation of gene expression. It is becoming increasingly evident that lncRNAs are directly involved in regulating gene expression, serving as signals, decoys, guides, and scaffolds. Regarding the guiding function of lncRNA, I will present data showing that lncRNA can directly interact with homopurine-stretches in double-stranded DNA, forming a triple-stranded DNA:RNA structure. Such DNA:RNA triplexes may target chromatin modifying enzymes to specific genomic sites, modulating gene expression by altering the chromatin structure and epigenetic signature. To prove that formation of DNA:RNA triplexes is a general mechanism that guides chromatin modifying enzymes to regulatory gene sequences, we study lncRNA-dependent regulation of the proto-oncogene SPHK1. We found that Khps1, an antisense RNA covering the SPHK1 promoter, regulates SPHK1 transcription by direct binding to a purine-rich sequence at the SPHK1 promoter. Tethering Khps1 to complementary DNA sequences is required to establish a transcription-permissive chromatin structure at the SPHK1 promoter that enables binding of E2F1 and activates transcription. Our results reveal a novel function of lncRNAs, serving as a potent address label to guide chromatin modifying proteins to specific gene sequences and regulate gene expression.

Genomic imprinting results in gene expression from only one defined parental allele. Imprinted genes are mainly organized in clusters and often regulated by imprinting control regions (ICRs), that are differentially methylated on the parental chromosomes. Appropriate regulation of imprinted genes is important for normal development and imprinting defects are associated with several complex diseases like the Beckwith-Wiedemann syndrome (BWS) or the Silver-Russell syndrome (SRS). These two growth defects are caused by different (epi-)mutations affecting two clusters of imprinted genes on chromosome 11p15.5, enabling functional insights in imprinting regulation. One cluster (IC1) contains the genes encoding the fetal growth factor IGF2 and the long noncoding RNA (lncRNA) H19. IC1 serves as a model region for studies on transcriptional regulation of imprinted genes and their effects on normal and aberrant growth and also tumor development. The presentation will highlight previous and recent data on the ICR1 based regulation of the imprinted H19 lncRNA and how this gene modifies the expression of
other imprinted genes relevant for somatic growth within an Imprinted Gene Network (IGN).

**S13-04**

**DNA-methylation in differentiation and cancer**

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Epigenetics refers to the establishment and inheritance of potentially reversible cellular phenotypes which are not brought about by changes in the primary DNA sequence. This pragmatic definition includes a broad range of biological phenomena like position effect variegation, imprinting, non-equivalence of parental chromosomes, X chromosome inactivation in female cells, cell type-specific gene expression, differential reproductive and social behavior in state building insects and functional deletion of tumor suppressor genes in cancer cells.

Among the still growing list of molecules and mechanisms involved in these complex regulatory mechanisms methylation of the DNA base cytosine at carbon atom number 5 in the sequence context of a CG dinucleotide ("CpG methylation") is the longest known and best studied. However, the development of comprehensive high-resolution DNA methylation profiling techniques and the (re)discovery of 5’ hydroxymethylcytosine as well as non-CpG cytosine methylation in recent years had a tremendous impact on the field.

In my talk I will discuss how DNA methylation is involved in cell type-specific gene expression and differentiation processes, taking the hematopoietic system as an example. New findings about X-chromosone inactivation will illustrate the impact of newly developed comprehensive profiling approaches on decade old textbook knowledge.

In the field of cancer biology the functional deletion of tumor suppressor genes due to aberrant hypermethylation of regulatory sequences is recognized as a hallmark of cancer cells with numerous examples involving all cellular functions and signaling pathways. I will focus on the function and importance of 5’ hydroxymethylcytosine and the possible classification of human tumors according to their DNA methylation pattern which is now firmly established for several human malignancies.

Finally, I will discuss how the quantitative analysis of DNA methylation with single CpG resolution has already reached the clinics. Aberrant hypermethylation of the DNA repair gene MGMT, for example, is a predictive and prognostic factor in glioblastoma, a malignant brain tumor, which is assessed in the routine workup of patient biopsies.

**S14**

**Atherosclerosis: new players live on stage**

**S14-01**

**The role of neutrophils in atherosclerosis**

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Due to their rare presence in atherosclerotic lesions, the involvement of neutrophils in the pathophysiology of atherosclerosis has largely been neglected. However, over the past couple of years, studies have provided convincing evidence for the presence of neutrophils and their secretory products in atherosclerotic plaques and further revealed the causal contribution of neutrophils during various stages of atherosclerosis. Here I will summarize mechanisms underlying hyperlipidemia-mediated neutrophilia, how neutrophils may enter atherosclerotic lesions, and mechanisms of neutrophil-driven atherosclerosis.

**S14-02**

**Lost in transdifferentiation: smooth muscle cells, macrophages & atherosclerosis**

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Atherosclerosis is a widespread and devastating disease, but the origins of cells within atherosclerotic plaques are not well defined. Here, we used genetic inducible fate mapping in apolipoprotein E-deficient mice to investigate the role of vascular smooth muscle cells (SMCs) in atherosclerotic plaque formation. Mature SMCs in the arterial media were genetically pulse-labeled prior to disease development and then their fate was monitored during disease progression. We found that medial SMCs can undergo clonal expansion and convert to macrophage-like cells that have lost classic SMC marker expression and make up a major component of advanced atherosclerotic lesions. Smooth muscle-to-macrophage transdifferentiation supports an important role of SMC plasticity in atherogenesis and might be a novel target for the treatment of atherosclerosis as well as other diseases with a smooth muscle component.
S14-03
Signaling through S1P in atherosclerosis
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Sphingosine-1-phosphate (S1P) is a signaling lipid generated by the sphingosine kinases-1 and -2 (SPHK1/2). S1P is generated by different vascular cells such as endothelial and smooth muscle cells. It is also synthesized and stored in large quantities in platelets. S1P plays a key role in maintaining the vascular homeostasis, during remodeling of the vessel wall upon vascular injury and in modulating inflammatory responses during atherosclerotic plaque development. Recent data from several groups including ours suggest that S1P represents a potent mediator between blood coagulation and inflammation. Activation of blood coagulation and the impact of activated coagulation factors such as the activated factor-X (FXa) and thrombin may regulate the local S1P availability at vascular lesion sites. In particular, we recently demonstrated that FXa enhances transcription of SPHK1 and induces S1P synthesis and release in human vascular smooth muscle cells (SMC). The functional consequences involve increased SMC mitogenesis and migration. FXa induced a time- (3–6 h) and concentration-dependent (3–30 nM) increase of SPHK1 mRNA and protein expression in human aortic SMC, resulting in an increased synthesis of S1P. FXa-stimulated transcription of SPHK1 was mediated by the protease-activated receptor-1 (PAR-1) and PAR-2. In human carotid artery plaques, expression of SPHK1 was observed at SMC-rich sites and was co-localized with intraplaque FX/Fxa content. FXa-induced SPHK1 transcription was attenuated by inhibitors of Rho kinase (Y27632) and by protein kinase C (PKC) isoforms (GF109203X). In addition, FXa rapidly induced the activation of the small GTPase Rho A. Inhibition of signaling pathways which regulate SPHK1 expression, a pharmacological inhibition of SPHK activation or a siRNA-mediated SPHK1 knockdown attenuated the mitogenic and chemotactic response of human SMC to FXa.

From these data, it is concluded that FXa induces SPHK1 expression and increases S1P formation in human SMC independent of thrombin actions. The underlying mechanism involves the activation of Rho A and PKC signaling. In addition to its key function in coagulation, this direct effect of FXa on human SMC may increase cell proliferation and migration at sites of vessel injury and may thereby contribute to the progression of vascular lesions.

S14-04
Purinergic receptors in atherosclerosis
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**Background:** A solid body of evidence supports a role of extracellular nucleotides such as ATP, ADP, UTP, or UDP and their receptors in innate and adaptive immunity. The binding of extracellular nucleotides to purinergic receptors promotes inflammation as a danger signal in various chronic inflammatory diseases. The ATP-binding P2Y<sub>2</sub>- and the UDP-binding P2Y<sub>6</sub>-receptor are involved in inflammatory cell recruitment. Since atherosclerosis is a chronic inflammatory disease, we hypothesize contribution of extracellular ATP and P2Y<sub>2</sub> in vascular inflammation and atherosclerosis.

**Methods and Results:** P2Y<sub>2</sub> and P2Y<sub>6</sub>, both G-protein coupled purinergic receptors, are overexpressed in murine atherosclerotic lesions. Stimulation with ATP or UDP induces leukocyte rolling and adhesion via P2Y<sub>2</sub> and P2Y<sub>6</sub> respectively assessed by intravital microscopy. To investigate the role of P2Y<sub>2</sub> and P2Y<sub>6</sub> in atherosclerosis both strains were crossed with LDLR<sup>−/−</sup> mice and fed a high cholesterol diet for 16 weeks. P2Y<sub>2</sub>- deficiency and P2Y<sub>6</sub>-deficiency reduces atherosclerosis compared to P2Y<sub>2</sub>/P2Y<sub>6</sub>-competent LDLR<sup>−/−</sup> mice, whereas stimulation with extracellular ATP increases atherosclerosis. Atherosclerotic lesions from P2Y<sub>2</sub> and P2Y<sub>6</sub>-deficient mice are less inflamed due to reduced content of macrophages.

**Conclusion:** We could show for the first time that extracellular ATP induces vascular inflammation and atherosclerosis via P2Y<sub>2</sub>. P2Y<sub>6</sub>-deficiency is athero-protective. Thus, purinergic receptors P2Y<sub>2</sub> and P2Y<sub>6</sub> are potential targets for an anti-atherogenic therapy.
S15
Tumor pathophysiology from molecular mechanisms to clinical implications

S15-01
Ion channels in tumor stroma cell activation
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Cancer and host cells within the tumor stroma strongly influence each other and create a tumor microenvironment with distinct physical and chemical properties that are permissive for tumor progression. This is particularly valid in pancreatic ductal adenocarcinoma (PDAC). PDAC is characterized by a pronounced fibrosis (desmoplasia) resulting from the mutual growth factor-dependent stimulation of pancreatic stellate cells (PSCs) and cancer cells. Hence, typical features of the PDAC microenvironment include local hypoxia and acidosis, as well as high interstitial pressure and an abundance of growth factors. There is growing evidence that ion channels like transient receptor potential (TRP) channels, STIM/ORAI proteins and other transport proteins play important roles in modifying and sensing the tumor microenvironment. Moreover, they are essential cellular components of the transduction and effector cascades underlying cellular responses to the tumor microenvironment.

One of the effects caused by a hypoxic tumor microenvironment is to stimulate tumor and stroma cell migration. The most prominent stromal cell type in PDAC is the pancreatic stellate cell which is responsible for the excessive matrix production in PDAC. Here we focused on the TRPC family of which several members are expressed in PSCs. We could show that TRPC1, 3 and 6 channels play distinct roles in PSC migration in response to different stimuli such as PDGF or hypoxia. Notably, they are either upregulated in the PDAC stroma or by hypoxia in an in vitro setting. Mechanistically, TRPC channels affect PSC migration by cooperating with other Ca^{2+}-sensitive ion channels (e.g. K_{Ca}-3.1) or other intracellular Ca^{2+}-sensitive effector proteins such as calpain. In summary, our results indicate that TRPC channels are important mediators in the adaptation of PSCs to the PDAC microenvironment since they are part of the signal transduction pathways.

S15-02
Focal adhesion signaling hubs: promoters of tumor cell survival and therapy resistance
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Focal adhesions are composed of transmembrane receptors, cytoplasmic adapter proteins and extracellular matrix (ECM) for integrating cells into tissues. This integration is conducted through structure and signal transduction. In recent years we were able to identify various focal adhesion proteins with fundamental roles in cancer cell therapy resistance, phenomena termed cell adhesion mediated radioresistance (CAM-RR) and drug resistance (CAM-DR). Overcoming therapy resistance remains one of the major unsolved challenges. Integrins, cell adhesion molecules and critical components of focal adhesion, emerged as potential cancer targets easily druggable due to their membranous expression. Among the 18 alpha and 8 beta integrin subunits, targeting beta1 integrins has become most apparent for radiochemosensitization in a variety of tumor models in our hands. The lecture will summarize the effects of beta1 integrin inhibition in head and neck cancers and glioma including mechanistic explanations. Further, owing to the fact that receptor tyrosine kinases are essential parts of focal adhesions, I will present data on simultaneous targeting of beta1 integrins and epidermal growth factor receptor in head and neck cancers. Mutual and cooperative interactions between these two receptors promote cancer cell survival, proliferation and treatment resistance. By bioinformatics analysis for the reactome, we were able to delineate resistance-mediating pathways and the alterations achieved by beta1 integrin/EGFR targeting for radiosensitization. A distinction between a responder and a non-responder treatment group allows further differentiation relevant for bench-to-bedside translation and personalization of this therapeutic approach in the clinic.
S15-03
Post-transcriptional control of tumor cell fate by miRNAs and RNA-binding proteins
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The oncogenic potential of tumor cells is controlled at various layers including the post-transcriptional regulation of mRNA fate by RNA-binding proteins (RBPs) and microRNAs (miRNAs).

One key regulator of cytoplasmic mRNA fate in tumor cells is the oncofetal IGF2 mRNA binding protein 1 (IGF2BP1). Next to its close homolog IGF2BP3, IGF2BP1 is de novo synthesized in various aggressive malignancies. In accord with its upregulation in human cancer, in vitro studies revealed that IGF2BP1 promotes the survival, migration and invasiveness of tumor-derived cells. These oncogenic roles of IGF2BP1 rely on its association with a plethora of mRNAs including oncogene-encoding transcripts. The role of IGF2BP1 in promoting tumor cell proliferation is partly facilitated via its association with the MYC mRNA. This prevents MYC mRNA degradation resulting in an enhanced expression of the oncogene. More recently, we observed that IGF2BP1 also prevents the decay of the ZEB1 and LEF1 mRNAs, presumably involving miRNA-dependent feed-back regulation. Consistently, IGF2BP1 promotes a mesenchymal-like tumor cell phenotype by enhancing the expression of “EMT-driving” transcriptional regulators like ZEB1, LEF1 or SLUG. In addition to the control of pro-mesenchymal transcription, IGF2BP1 enhances tumor cell migration by modulating dynamics of the actin cytoskeleton. IGF2BP1 controls ACTB mRNA translation and regulates the availability of monomeric actin by the indirect inhibition of HSP27 phosphorylation. In concert these effects result in an IGF2BP1-dependent enhancement of tumor cell migration, invasiveness as well as proliferation. The interconnected mechanisms underlying IGF2BP1-directed control of tumor cell fate will be discussed.

S15-04
Changes in tumour physiology induced by vascular targeting agents: relevance for their clinical application
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A functional vascular supply, formed from the host normal vasculature by the process of angiogenesis, is essential for the growth and development of solid tumours. This importance makes the tumour vasculature a promising therapeutic target and two major groups of vascular targeting agents (VDAs) have been developed. These include inhibitors of the angiogenesis process (angiogenesis inhibitors; AIs) or agents that destroy the already established vessels (vascular disrupting agents; VDAs) and examples of both types are under clinical evaluation. The tumour vascular supply plays a significant role in determining the physiological conditions within the tumour microenvironment, as it governs both oxygen and nutrient supply as well as waste removal (pH homeostasis). Any treatment that affects the vascular supply has the potential to change the physiological parameters. Indeed, VTAs have been shown to modify blood perfusion, vessel permeability, and oxygenation status of the tissues. Physiological changes induced by VTAs has also been shown to have critical impact on the tumour response to conventional therapies. As radiation therapy is hugely dependent on oxygenation status of the tissues, an increase or decrease in this parameter can markedly influence the outcome of such a treatment. Vessel permeability and tissue perfusion are key parameters for successful treatment with chemotherapy, and these can be massively altered using VTAs. Since the clinical potential of VTAs will not be fully realised when used alone, but rather in combination with more conventional treatments, the vascular changes induced by either AIs or VDAs has the potential to significantly impact the outcome of the conventional treatments with which they will be combined. Furthermore, VTA-induced changes in the tumour physiology may also modify malignant progression, as additional studies have shown that tumour physiology influences the metastatic potential of tumours. This presentation will review the physiologic changes induced by AIs and VDAs and highlight the significance of these changes with regards to their clinical application.

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S15-05
Nano drug delivery systems for passive tumor targeting – hope or hype?
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Drug therapy in cancer is very often limited by rapid drug metabolism and elimination, low drug concentrations in the desired tissue and serious side effects in healthy tissue and organs. Therefore a more specific and effective drug transport to the tumor cells, combined with a site specific controlled release, would result in less frequent drug applications, more effective and selective killing of the tumor cells and a better quality of life (less side effects). A commonly used principle to increase the delivery efficiency to tumors is to take advantage of the higher permeability of tumor vasculature. This effect has been discovered by H. Maeda and it is known as the “Enhanced Permeability Retention” Effect (EPR effect, passive targeting).[1] Many nanoscaled drug delivery systems (NDDS) have been published and patented which were claimed to be superior carriers for tumor therapy. Examples include liposomes, polymers and nanoparticles, polymer micelles, carbon nanotubes, metal or metal oxide nanoparticles. These NDDS differ in the used materials
(inorganic, polymers, lipids), sizes, aggregation states (solid, liquid), shapes, charges, flexibility, biodegradability, heterogeneity and other parameters. The question arises, which carriers are the most selective ones and what are the key parameters? Comparing the reported values for optimal drug carriers from the literature, conflicting values are seen. The contradictions might arise from different tumor models, but also from different (and often insufficient) characterization of the nanomaterials. Therefore, we try to understand and to compare different, but well characterized NDSS in several tumor models. The results show, that even small changes of the particle size might have an important impact on tumor accumulation and liver uptake. Despite the “stealthy” PEG-shell of the biodegradable nanoparticles, a quite high liver uptake was observed. The best results have been obtained with flexible HPMA polymers [3] and hydroxyethylstarch (HES). [4] The most effective results have been achieved with long circulating, star-shaped flexible HPMA polymers with a pH-sensitive cleavage of the covalently bound drug. [5] The in vivo results on tumor bearing mice also demonstrated that passive targeting needs long circulation times, as the highest accumulation was seen after 1–2 days. Therefore, an effective prevention of the RES-mediated uptake is a necessity. Surprisingly, in our attempts to improve tumor delivery, we also observed unintended ovarian [6] and also adrenal (current studies) accumulation with non-covalently and covalently linked marker molecules. In summary, NDSS mediated passive targeting is a clinically proven way of better efficiency and less side effects (e.g. Doxyl). Translation from the preclinical into the clinical state requires appropriate characterization techniques and the feasibility of pharmaceutical development, which favors less complex structures.


S17
Molecular and cellular mechanisms of homeostasis in the central nervous system

S17-01
Homeostatic coupling of exo- and endocytosis by the synaptic adhesion molecule N-cadherin
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Under physiological conditions in the intact brain, synapses are transmitting information encoded in electrical signals that regularly occur at frequencies around 100 Hz. To achieve this high-frequency transmission, glutamatergic synapses in cortical neurons have to release vesicles at high release rates. This raises the need for homeostatic control of the size of the presynapse, because ongoing vesicle fusion would rapidly and dramatically expand the presynaptic membrane area. Since standard clathrin-dependent endocytosis is a rather slow process with time constants of several seconds, facilitation of compensatory endocytosis is required to enable efficient synapse homeostasis. However, the homeostatic coupling of exo- and endocytosis is not well understood both in terms of physiological processes and molecular mechanisms.

Transsynaptic cell adhesion molecules are thought to be important for long-term synapse stabilization, but the molecular and physiological mechanisms involved are largely unknown. The homophilic synaptic adhesion molecule N-cadherin has been suggested in loss-of-function studies to affect both exo- and endocytosis of synaptic vesicles. In addition, it has been shown by our group to be required for synaptic targeting of another adhesion system, the transsynaptically interacting proteins Neuroligin1 and Neurexins. Because postsynaptic Neuroligin1 regulates vesicle release at mature synapses, this cooperation might explain the effects of N-cadherin on exocytosis. Recent data obtained with simultaneous imaging of both exo- and endocytosis is presented, demonstrating that N-cadherin is also of crucial importance for controlling compensatory endocytosis. In summary, our recent data strongly suggest that the N-cadherin transsynaptic adhesion system has a regulatory influence on both vesicle exocytosis and compensatory endocytosis. This places N-cadherin in a center stage position within a novel molecular mechanism for efficiently coupling exo- and endocytosis during high network activity.
Activin A is a member of the transforming growth factor-β (TGF-β) family. In the nervous system, it was originally identified as a neurotrophic and neuroprotective factor. More recent evidence from our and other laboratories implicated activin receptor signaling also in the regulation of both excitatory and inhibitory synaptic transmission under physiological conditions. Activin signaling is responsive to both physiological and pathological stimuli. For example, activin signaling is significantly enhanced by novel environmental stimuli as well as by brief trains of action potentials which induce long-term potentiation at glutamatergic synapses. A more pronounced, large-scale version of this physiological responsiveness is observed after epileptic activity or acute injury. Canonical activin signaling involves activin binding to heteromeric receptor complexes consisting of type I and type II serine/threonine kinase receptors, which then phosphorylate the recruited receptor SMADs 2 and 3. The latter multimerize with SMAD4 in the cytosol, translocate to the nucleus and modulate transcription of activin target genes. In addition to this canonical pathway, activin receptors might also act on other signaling systems, in particular mitogen-activated protein kinase (MAPK) signaling.

At the excitatory Schaffer collateral-CA1 pyramidal cell synapse, activin strengthens NMDA receptor currents and promotes long-term potentiation (LTP). Importantly, the optimized performance of glutamatergic synapses by activin signaling does not come at the expense of enhanced vulnerability to excitotoxic injury. On the contrary, activin signaling affords neuroprotection in various brain lesion models, including kainic acid injection.

Activin also regulates the function of GABAergic synapses of the hippocampus in several aspects: (i) spontaneous GABA release is reduced, (ii) paired-pulse depression is enhanced, (iii) tonic inhibition by extrasynaptic GABA(A) receptors is reduced, and (iv) GABA(B) receptor-activated K⁺ currents (GIRK) are decreased. Furthermore, activin controls the responsiveness of GABA(A) receptors to allosteric modulation in a highly site-specific fashion: Whereas the action of diazepam is enhanced, the augmenting effect of alcohol is strongly dampened by activin. In summary, activin signaling is intimately involved in the daily operations of synaptic networks that are linked to cognitive functions and affective behavior.
from visually identified GFP-labeled GABAergic interneurons in GFP-GAD67 mice within the same area disclosed adaptive alterations in the excitability of non fast-spiking (Non Fs) and fast-spiking (Fs) interneurons in the lesion-treated animals: Non Fs interneurons displayed a depolarized membrane potential as well as a higher frequency of spontaneous excitatory postsynaptic currents (sEPSCs). In contrast, Fs interneurons showed a reduced sEPSCs amplitude. This suggests a synaptic downscaling of excitatory inputs targeting Fs interneurons, which may prevent the recruitment of this specific population of interneurons to the hyperexcitable network. As a consequence, this mechanism may seriously affect neuronal network function and exacerbate hyperexcitability. However, it may also be important to protect this particular vulnerable population of GABAergic neurons from excitotoxicity. For the complete understanding of the overall adaptive changes in neocortical function following focal brain injuries future studies should address additional long-lasting survival times as well as the potential changes of GABAergic inhibition and hyperexcitability in the homotopic region of the contralateral hemisphere.

S17-05
Stable neural function from sloppy underlying components
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A fundamental question in neuroscience is how neurons and nervous systems develop, control, and maintain their electrical signaling properties in spite of ongoing protein turnover and activity perturbations. In this talk I will summarize efforts to address this question both experimentally and using theory and computational modeling. I will show how a simple yet robust and flexible model of regulation can be derived from generic assumptions about the molecular biology underlying channel expression, and how this model ties recent experimental data with long-standing questions about the inherent variability of neuronal properties. I will also demonstrate that “homeostatic” regulation critically depends on the complement of ion channels expressed in cells: in some cases loss of specific ion channels can be completely compensated, in others the homeostatic mechanisms itself can cause pathological loss of function. Finally, I will offer ideas of how neurons and nervous systems can reliably cope with and respond to global perturbations such as temperature fluctuations and neuromodulation.
**W1**

**W1-01**

**pHoenix – an optogenetic tool to modulate synaptic vesicle acidification**

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At chemical synapses information is transmitted by release of neurotransmitters from synaptic vesicles causing the activation of specific postsynaptic receptors. Following neurotransmitter release, vesicles are recycled from the presynaptic membrane, acidified and refilled with neurotransmitters via specialized transporter proteins. Here, we present pHoenix, a light-activated proton pump that specifically targets to synaptic vesicles and allows for simultaneous imaging of intravesicular pH. We apply pHoenix to monitor light-induced vesicular acidification and the corresponding recovery of postsynaptic currents (EPSCs) in bafilomycin-treated cells. Thus, pHoenix is used to study the energetics of neurotransmitter uptake and to analyze fill-state dependent release probability of synaptic vesicles.

**W1-02**

**Imaging with the patch pipette: basics and applications of Scanning Ion Conductance Microscopy**

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Recent advances in fluorescence microscopy allow the investigation of living cells beyond the diffraction limit. However, mapping the three-dimensional surface structure of the cell at this resolution has been limited to denaturated samples due to the required sample preparation for most scanning probe microscopy techniques. Scanning Ion Conductance Microscopy (SICM) utilizes the current through the opening of a glass micro- or nanopipette as conventionally used in patch-clamp experiments to determine the topography of insulating surfaces such as cellular membranes. In contrast to other scanning probe techniques, SICM neither requires denaturing sample preparation nor physical contact between sample and probe tip, hence allowing the unperturbed observation of the cell membrane topography. In recent years, SICM has been applied to obtain nanometre resolution images and time-lapse recordings of living cells or to obtain information about local volume changes during cellular migration. Furthermore, SICM has been combined with various other techniques such as fluorescence microscopy-revealing spatial information about the distribution of proteins-or patch-clamp recordings-enabling researchers to map the ion channel distribution in defined membrane structures. Here, I will detail the operating principle of SICM, discuss advantages and pitfalls and review recent applications of SICM and SICM in combination with other techniques to explore subcellular membrane movements and polarized protein distribution.

**W1-03**

**A technical guide to two-photon uncaging**

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The primary function of neurones as fundamental functional units of the nervous system is to receive and process incoming signals to determine an output. Neurones receive thousands of inputs throughout their complex dendritic structures, where the inputs signals are integrated and processed before reaching the soma and an output is determined. Two-photon uncaging allows experimenters to examine how neurones integrate synaptic inputs with single spines resolution. These individual synaptic inputs can be stimulated in precisely defined patterns using two-photon photolysis of caged-glutamate (e.g. MNI-glutamate). Two-photon glutamate uncaging has shown that dendrites process excitatory inputs in a linear, supra-linear or sub-linear manner, and has revealed the spatio-temporal input patterns required for these different integration modes.

In this talk, I will present a how-to guide to two-photon uncaging, discussing typical experiments, common pitfalls and useful tips. Finally, I will discuss the advantages and drawbacks of the technique and propose future directions.

**W1-04**

**Combined patch-clamp recording and dendritic calcium imaging in vivo**

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The vast majority of excitatory synaptic inputs to neurones of the mammalian central nervous system are located in the dendrites. Neurones process these inputs and generate an output signal, a single action potential or a burst of several action potentials, which is then transmitted to downstream neurons. It is known from numerous studies performed in brain slice preparations that dendrites play an important and active role in this process and are, thus, more than passive information conductors. However, the principles of dendritic input integration in vivo remain still largely unknown.

To study the process of input-output-transformation on the level of single neurones in vivo we perform combined somatic patch-clamp recordings and high-speed two-photon dendritic calcium imaging in anesthetized mice. This experimental strategy allows at the same time recording of the inputs that a given neurone receives as well as recording of the subsequently generated neuronal output. Here, we will discuss the design and practical implementation of these combined
patch-clamp and imaging experiments. We will also present example applications of this approach, including an in vivo study performed in the CA1 region of the hippocampus. This work identifies a novel type of NMDA receptor-dependent non-linear process in the dendrites of CA1 pyramidal neurons, which is essential for burst firing in these neurons in vivo.

In conclusion, the combination of two-photon imaging of dendritic calcium signals and electrophysiological recordings from single neurons has provided many essential insights on how information is processes on the level of individual neurons.

References:

W1-05
Studying arrhythmogenic events in mouse cardiomyocytes

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During the development of chronic heart disease the heart undergoes an extensive remodeling process including structural and morphological alterations but also changes in the expression of ion channels and proteins involved in intracellular calcium homeostasis together with regulating proteins in single cardiomyocytes. This functional and electrical remodeling is most often associated with an enhanced susceptibility to lethal arrhythmia. Up to 50% of patients suffering from heart failure die due to the sudden cardiac arrest as a consequence of such arrhythmia. Since pharmacological treatments to prevent remodeling or handle arrhythmia are still of limited success especially with regard to long-term mortality reduction a deeper understanding of the mechanisms leading to the susceptibility to arrhythmia and its maintenance is the key to new treatments. Whether the mouse is a suitable model to study arrhythmia is a matter of debate because translation to man might be limited due to differences in cardiac electrophysiology. However, mouse models are easy to generate, increasingly used for studies and might help to elucidate potential arrhythmogenic mechanisms on the cellular level. Here, we give a short introduction to cardiac arrhythmogenic remodeling, an overview what is regarded as arrhythmogenic events and as main part how these can be detected on the single cellular level by the example of adult mouse cardiomyocytes. We will summarize and discuss methodical procedures including the detection of spontaneous calcium releases like calcium sparks, calcium waves and spontaneous calcium transients using the calcium indicator dyes Indo1-AM and Fluo4-AM with wide-field and confocal laser scanning microscopy. Furthermore, the registration of early afterdepolarizations and spontaneous action potentials using the patch clamp technique will be illustrated. We will present different electrical stimulation protocols and pharmacological approaches suitable to provoke respective events and illustrate their effects with concrete examples. We will show options how the collected data may be analyzed and what conclusions could be drawn regarding underlying mechanisms. Finally, we will illustrate how these events on the single cellular level are reflected in in-vivo ECG-recordings in living mice.

W1-06
In vivo application of optogenetics in the auditory system

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Why consider optogenetics for stimulation of the auditory system? Electrical cochlear implants are by far the most successful neuroprostheses implanted in over 300,000 people worldwide and enable open speech comprehension in a majority of users. However, the method suffers from low frequency resolution due to wide current spread from stimulation contacts, which limits the number of independently usable channels and compromises speech recognition in noise, music appreciation or prosody comprehension. Our goal is to overcome these drawbacks by pursuing an optogenetic approach: Optical cochlear implants activate spiral ganglion neurons genetically modified to spike upon light stimulation (Cochlear Optogenetics). Optical stimulation can be spatially confined and thus promises lower spread of excitation in the cochlea. Accordingly, an increased number of independent stimulation channels is expected to enhance frequency resolution and intensity coding. We have investigated cochlear optogenetics employing various transgenic rodent models as well as virus-mediated expression of channelrhodopsin variants in spiral ganglion neurons. Viral transduction via transuterine and postnatal injection was established. Blue light stimulation of the spiral
ganglion via fiber-coupled lasers activated the auditory pathway, as demonstrated by recordings of neuronal population responses along the auditory pathway. For estimation of the spatial confinement of optical stimulation, it is mandatory to develop and establish multichannel optical implants in collaboration with semiconductor experts. Recently, we have implanted rodent cochleae with flexible µLED arrays accommodating approximately 100 µLEDs per 1 cm. Positioning of cochlear probes was assessed in 3D models derived from x-ray tomography. We will discuss the use of fast opsins as well as strategies for virus-mediated transduction and implantable optical stimulators.

**W1-07**

**Imaging of whole mouse brains with the ultramicroscope**

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It would be very helpful for the analysis of neuronal networks of the brain, if one could visualize these networks in 3 dimensions. Up to now this was only possible with limited resolution by sequential slicing and reconstruction of the brain. This time consuming attempt is easily hampered by artifacts as shrinkage and distortion induced by standard histological procedures. To overcome these problems we used a microscopy based on extreme darkfield illumination with a light sheet, so-called ultramicroscopy. This microscopy allows optical sectioning of whole mouse brains and was combined with an approach to clear fixed neuronal tissue: Mouse brains were made completely transparent by immersion in clearing solutions of the same refractive index as protein. By illuminating the brains with blue light neurons labeled with GFP were visualized by fluorescence. This way we could detect single neurons in the neocortex and hippocampus inside whole brains. Also the dendritic trees and axons of these neurons could be visualized in the brains. New developments in clearing technology will be discussed. Emphasis will be given on the description of new optics for the generation of super thin light sheets and correction devices for objectives for the use in clearing solutions. Many proteins can be labelled in transgenic mice with genetically encoded fluorescent markers. Using these markers our approach will represent a high-throughput screening method for protein expression in 3 D. This expression can be monitored with µm resolution and should allow the elucidation of complex neuronal networks in the brain and spinal cord. We show that ultramicroscopy allows also optical sectioning and detailed 3D reconstruction of the circulatory system of whole mouse embryos by imaging autofluorescent structures. Also other applications like e.g. visualization of nerve bundles in whole embryos and visualization of plaques in brains of mice with Alzheimers disease will be shown. In general the method is well suited for high-throughput phenotype screening of transgenic mice and thus will benefit the investigation of disease models.

**References**


**W1-08**

**Structure function relationships revealed by juxtacellular recordings in freely moving rats**

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Place, head-direction, grid and border cells in the hippocampal formation constitute a brain “GPS” for spatial cognition. Despite such advances, a mechanistic understanding of how individual neurons, or specific cell types contribute to certain spatial representations is still lacking. To address this issue, we developed a novel head-mountable, pipette-positioning device, which allowed us to record and juxtacellularly label single neurons in trained rats engaged in exploratory behavior (Tang et al., 2014a). With our novel method we revealed the existence of highly-selective microcircuits between functionally-identified neurons in medial entorhinal cortex of the rat. We provide evidence that grid cells might be preferentially recruited from the pyramidal cell population formed in an anatomical grid in layer 2 of Medial Entorhinal Cortex (MEC). Border cells on the other hand were almost exclusively observed in stellate neurons. These data point towards the existence of strong structure-function relationships in layer 2 of MEC (Ray et al., 2014; Tang et al., 2014b). We further applied the technique in other hippocampal structures and provided novel insights to the input-output structures of spatial modulated cells. Altogether, these results indicate that our new method can be used for resolving the cellular identity of spatially modulated neurons, which form the basis of the animal’s internal representation of space, but this approach can easily be extended to other unrestrained behaviors.
Anoctamins (TMEM16), a family of transmembrane proteins, are known to function as both Ca²⁺-dependent ion channels and scramblases. Whereas anoctamin 1 and 2 (ANO1 and ANO2) have been proved to be Ca²⁺-dependent Cl⁻ channels, the ion channel function of other anoctamins remains less well understood. The purpose of the study is to clarify the channel function of heterologously expressed and in the retinal pigment epithelium (RPE) endogenously expressed anoctamin 4 (ANO4).

By whole-cell-configuration of the patch-clamp technique, currents of heterologously expressed ANO4 in HEK293 were recorded. ANO4 sequence was modified by site-directed mutagenesis. Ano4 expression in ARPE-19 cells and mouse RPE was investigated by immunohistochemistry and ANO4 siRNA knock-down in ARPE-19.

ANO4 expression in HEK cells elicited Ca²⁺-dependent non-selective cation currents which were sensitive to niflumic acid. The currents showed a weak Eisenmann I-VI selectivity for monovalent cations. Using sequence homology analysis, we identified in the assumed pore region of anoctamin a negatively charged amino acid in ANO4. In the same region ANO1 and 2 contain only positively charged conserved amino acid side chains. In order to demonstrate that the membrane currents in transfected cells result from ion channel pore activity of ANO4, we generated two mutants of ANO4: exchanging the negative amino acid into a non-charged one (E-ANO4-G) and a positively charged one (E-ANO4-K). The E-ANO4-G mutation failed to induce increased membrane conductance whereas the E-ANO4-K variant led to Ca²⁺-dependent Cl⁻ channel currents indicating that the conserved negatively charged amino acid is the major determinant for ion selectivity. We detected endogenously expressed ANO4 in situ in the mouse RPE of sagittal sections of the retina as well as in the human RPE cell line ARPE-19. siRNA mediated knockdown of ANO4 led to a reduction of the endogenous ANO4 expression as well as ENaC expression and activity.

The regulation of the amiloride-sensitive epithelial Na⁺ channel (ENaC) is mainly mediated by the mineralocorticoid hormone aldosterone which stimulates the expression and membrane insertion of ENaC. Further, ENaC membrane abundance depends on the rate of retrieval from the apical membrane. The E3 ubiquitin ligase ‘neural precursor cell expressed developmentally down-regulated protein’ (Nedd4-2). Nedd4-2 enables the ubiquitination of ENaC, followed by endocytosis of the ubiquitinated channels and proteasomal degradation of the protein. Dysfunction of this regulatory mechanism leads to an increased channel surface density and ENaC overactivity. Recently, ENaC was further identified as being an important regulator of the nanomechanical properties (i.e. stiffness) of endothelial cells: the more endothelial ENaC (ENaC) in the plasma membrane, the stiffer the cell. Endothelial stiffness in turn seem to be related to the development of endothelial dysfunction and thus
cardiovascular pathologies. Although the molecular structures of ENaC and EnNaC seem to be similar, their regulation is different. Amiloride, a specific inhibitor of ENaC function, was shown to decrease EnNaC surface abundance and cortical stiffness of the cell within five minutes. This novel observation discloses an apparent discrepancy to the classic amiloride response in renal epithelium.

To study the underlying mechanism of the amiloride-induced disappearance of EnNaC, cortical stiffness of endothelial cells was monitored over 30 minutes after acute application of amiloride (1 µM) or benzamil, another specific ENaC blocker, (0.1 µM). Therefore, paired nanoindentation measurements using Atomic Force Microscopy (AFM) were performed in a time dependent manner. In addition the time course of EnNaC disappearance was observed by using Quantum Dot-mediated immunostaining of EnNaC at the surface of endothelial cells.

Analysis of the paired time course experiments revealed that amiloride leads to an immediate significant decrease in cortical stiffness within the first 2 minutes from 0.99±0.19 pN/nm to 0.81±0.14 pN/nm (n=7). Low doses of benzamil (0.01 µM), another specific blocker of ENaC function, also led to an immediate softening of the endothelial cortex 0.97±0.22 pN/nm to 0.87±0.09 pN/nm (n=6). After the first decline in stiffness no further change of the cortical stiffness within 30 minutes could be detected. From these data it is hypothesized that inhibition of Na+ influx via EnNaC initiates a rapid rearrangement of the cortical actin-myosin web, leading to a softening of the cell cortex.

**OS1-04**

Enhanced activation of an amino-terminally truncated isoform of voltage-gated proton channel HVCN1 enriched in malignant B cells


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HVCN1 is the only mammalian voltage-gated proton channel. In human B lymphocytes, HVCN1 associates with the B Cell Receptor (BCR) and is required for optimal BCR signaling and redox control. HVCN1 is expressed in malignant B cells that rely on BCR signaling, such as Chronic Lymphocytic Leukemia (CLL) cells. Compared with normal B lymphocytes, HVCN1 expression is higher in B-cell lines and in B cells from patients with Chronic Lymphocytic Leukemia. We found that HVCN1 was expressed in B cells as two protein isoforms. The shorter isoform (HVCN1S) was enriched in B cells from a cohort of 76 CLL patients. When overexpressed in a B-cell lymphoma line, HVCN1S responded more profoundly to PKC-dependent phosphorylation. This more potent enhanced gating response was mediated by increased phosphorylation of the same residue responsible for enhanced gating in HVCN1L, Thr39. Although B cells from CLL patients expressed both isoforms, their PMA response was comparable with that of cells heterologously expressing HVCN1S alone, indicating that this isoform dominates. Furthermore, the association of HVCN1S with the BCR was weaker, which resulted in its diminished internalization upon BCR stimulation. Finally, HVCN1S conferred a proliferative and migratory advantage, as well as enhanced BCR-dependent signaling. Overall, our data show for the first time the existence of a shorter isoform of HVCN1 with enhanced gating that is specifically enriched in malignant B cells. HVCN1S properties suggest it may contribute to the pathogenesis of BCR dependent B-cell malignancies.

**OS1-05**

The role of ORAI channels and their interplay with NADPH oxidase 2 in bacterial peptide-induced innate immune responses

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**Question and state of the art:** One of the major events during phagocytosis is recognition of bacterial peptides by formyl peptide receptors (FPRs); an event followed by Ca2+ mobilization and production of reactive oxygen species (ROS) via the NADPH-oxidase (NOX) enzymes. We have previously shown that oxidation reduces store operated Ca2+ entry (SOCE) by inhibiting ORAI1 and 2 but not ORAI3 channels and identified upregulation of ORAI3 expression as an adaptive mechanism against oxidative stress. However, the roles of ORAI channels and their activators STIM1 and STIM2 and the molecular mechanisms by which they might regulate NOX activity, and vice versa, are still not understood.
**Results:** Here, we show that besides ORAI1 and STIM1, primary human monocytes express high levels of ORAI3 and STIM2. The ORAI1/ORAI3 expression ratio correlates with the redox sensitivity of SOCE as well as with the viability of the human monocytes under oxidative stress. Electron paramagnetic resonance (EPR) and fluorescent ROS measurements indicate that primary human monocytes generate superoxide (O2•−) upon activation of SOCE either by FPR activation by bacterial peptides or by passive store depletion. This SOCE-induced O2•− production was significantly reduced by the NOX inhibitor DPI, under Ca2+-free conditions. Therefore, the most cancer cells. Regulators of SOCE include the serum response factor (SRF) and the SRF sensitive transcription factor NFκB (p65). As shown recently, constitutively active SRF-VP16 induces hepatocellular carcinoma (HCC) formation in mice (Ohrnberger et al., 2014). Store operated Ca2+ entry (SOCE) is the predominant Ca2+ entry mechanism in murine hepatocellular carcinoma (HepG2) cells.

**Introduction:** Serum response factor (SRF) is a ubiquitously expressed transcription factor involved in cellular proliferation, differentiation and resistance to apoptosis. Expression and activity of SRF is deregulated under pathophysiological conditions. As shown recently, constitutively active SRF-VP16 induces hepatocellular carcinoma (HCC) formation in mice (Ohrnberger et al., 2014). Store operated Ca2+ entry (SOCE) is the predominant Ca2+ entry mechanism in most cancer cells. Regulators of SOCE include the serum and glucocorticoid inducible kinase SGK1. Therefore, the present study explored whether expression of constitutively active SRF (SRF-VP16) affects the protein expression of SGK1, the SGK1 sensitive transcription factor NFκB subunit p65, ERK5 (extracellular signal regulated kinase 5) and the SGK1/NFκB sensitive pore forming Ca2+ channel protein Orai1.

**Methods:** Protein expression was determined by Western blotting, mRNA level by quantitative RT-PCR, cytosolic Ca2+ activity by Fura-2 fluorescence and proliferation by MTT assay. Experiments were performed in SRF-VP16 transfected mice expressing constitutively active SRF-VP16 in hepatocytes and HepG2 cells.

**Results:** The protein abundance of SGK1, NFκB-p65, ERK5 and Orai1 was increased in HCC tumor tissue from mice expressing SRF-VP16, as compared to non-transformed liver tissue. Similarly, SGK1, ERK5 and Orai1 protein abundance and transcript level of c-Fos was markedly increased upon SRF-VP16 transfection in HepG2 cells. SRF-VP16 expression further increased SOCE, an effect almost abolished by Orai1 inhibitor 2-APB (2-Aminoethoxysydiphenyborate, 50 µM), SGK1 inhibitor EMD638683 (50 µM) and NFκB inhibitor wogonin (100 nM). HepG2 cells transfected with SRF-VP16 demonstrated increased cell proliferation relative to control. **Conclusion:** Dysregulated SRF stimulates SOCE via triggering a signaling cascade involving SGK1, NFκB, ERK5 and Orai1. Thus, Ca2+ signaling contributes to SRF mediated cellular proliferation and survival.


**OS1-06**

**Serum response factor mediates Orai1 expression and store operated Ca2+ entry in murine hepatocellular carcinoma**

*OS1-06* Serum response factor mediates Orai1 expression and store operated Ca2+ entry in murine hepatocellular carcinoma

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**Introduction:** Serum response factor (SRF) is a ubiquitously expressed transcription factor involved in cellular proliferation, differentiation and resistance to apoptosis. Expression and activity of SRF is deregulated under pathophysiological conditions. As shown recently, constitutively active SRF-VP16 induces hepatocellular carcinoma (HCC) formation in mice (Ohrnberger et al., 2014). Store operated Ca2+ entry (SOCE) is the predominant Ca2+ entry mechanism in most cancer cells. Regulators of SOCE include the serum and glucocorticoid inducible kinase SGK1. Therefore, the present study explored whether expression of constitutively active SRF (SRF-VP16) affects the protein expression of SGK1, the SGK1 sensitive transcription factor NFκB subunit p65, ERK5 (extracellular signal regulated kinase 5) and the SGK1/NFκB sensitive pore forming Ca2+ channel protein Orai1.

**Methods:** Protein expression was determined by Western blotting, mRNA level by quantitative RT-PCR, cytosolic Ca2+ activity by Fura-2 fluorescence and proliferation by MTT assay. Experiments were performed in SRF-VP16 transfected mice expressing constitutively active SRF-VP16 in hepatocytes and HepG2 cells.

**Results:** The protein abundance of SGK1, NFκB-p65, ERK5 and Orai1 was increased in HCC tumor tissue from mice expressing SRF-VP16, as compared to non-transformed liver tissue. Similarly, SGK1, ERK5 and Orai1 protein abundance and transcript level of c-Fos was markedly increased upon SRF-VP16 transfection in HepG2 cells. SRF-VP16 expression further increased SOCE, an effect almost abolished by Orai1 inhibitor 2-APB (2-Aminoethoxysydiphenyborate, 50 µM), SGK1 inhibitor EMD638683 (50 µM) and NFκB inhibitor wogonin (100 nM). HepG2 cells transfected with SRF-VP16 demonstrated increased cell proliferation relative to control. **Conclusion:** Dysregulated SRF stimulates SOCE via triggering a signaling cascade involving SGK1, NFκB, ERK5 and Orai1. Thus, Ca2+ signaling contributes to SRF mediated cellular proliferation and survival.


**OS1-07**

**The bile acid-sensitive ion channel (BASIC) is activated by alterations of its membrane environment**

*OS1-07* The bile acid-sensitive ion channel (BASIC) is activated by alterations of its membrane environment

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Along with the acid-sensing ion channels (ASICs) and the epithelial Na+ channel (ENaC), the bile acid-sensitive ion channel (BASIC) forms the DEG/ENaC ion channel family in mammals. In rat and mouse BASIC is expressed in brain, liver and intestine, in humans it is restricted to the intestinal tract. In rat and mouse liver BASIC is mainly found in bile duct epithelial cells. When expressed in Xenopus oocytes rat BASIC is activated by bile acids and other steroid-derivatives but not cholesterol. However, the physiological function and the mechanism of activation by bile acids are still unclear.

Due to their amphiphilic nature bile acids are detergents and thus membrane active molecules. Therefore we tested whether bile acids increase rBASIC activity by directly binding to the channel or indirectly by interfering with the properties of the membrane environment. Membrane-active substances other than bile acids affect the bile acid induced rBASIC activity when co-applied and also increase the activity of rBASIC when applied alone, suggesting that BASIC is sensitive for changes in its membrane environment. In addition, we show by patch-clamp fluorometry that the kinetic of bile acid binding to the membrane and activation of rBASIC strongly correlate, further supporting that membrane alterations affect rBASIC activity.

To investigate the structural basis for the membrane sensitivity of BASIC we generated chimeras between rBASIC and the bile acid-insensitive rASIC1a. Data obtained from these chimerase show that the extracellular and the transmembrane domains are important for membrane sensitivity. Collectively, our results suggest that rBASIC is sensitive to changes of the plasma membrane and that the entire structure of the channel is crucial for this sensitivity.
Bile acids activate the human epithelial sodium channel probably by interacting with its degenerin site

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Question: The epithelial sodium channel (ENaC) is probably a heterotrimeric channel with three well-characterized subunits (αβγ). In humans, but not in mice and rats, an additional δ-subunit exists which is expressed in various epithelial and non-epithelial tissues including the brain. Recently, it has been reported that rat δβγ ENaC can be activated by bile acids. The stimulation of ENaC by bile acids may be physiologically and pathophysiologically relevant in humans. The aim of this study was to investigate whether the human bile acids chenodeoxycholic (CDCA), cholic (CA) and deoxycholic (DCA) modulate human ENaC in its αβγ and δβγ-configuration. Furthermore, we studied the biophysical mechanism/s by which bile acids activate ENaC.

Methods: Human δβγ- and δβγENaC were heterologously expressed in Xenopus laevis oocytes. Channel activity was investigated by measuring the amiloride-sensitive currents using the two-electrode voltage-clamp method and the outside-out configuration of the patch-clamp technique. A molecular docking approach and site-directed mutagenesis were used to identify putative bile acid binding site.

Result: We demonstrated that bile acids activate δβγENaC more potently than αβγENaC. Moreover, αβγENaC is activated preferentially by tauro-conjugated bile acids whereas δβγENaC is activated by both, tauro-conjugated and unconjugated bile acids. Bile acids stimulate ENaC-mediated currents by increasing the open probability of active channels without recruiting additional “near-silent” channels known to be activated by proteases. Stimulation of ENaC activity by bile acids was accompanied by a ~8% reduction of the single-channel current amplitude which indicates an interaction of the bile acids with a region close to the channel pore. Analysis of the known ASIC1 (acid-sensing ion channel) crystal structure and a molecular docking approach suggested that bile acids may bind to the pore region at the degenerin site of ENaC. This prediction was tested by site-directed mutagenesis. Substitution of a single amino acid residue within the degenerin site of βENaC (N521C or N521A) significantly reduced the stimulatory effect of bile acids on ENaC.

Conclusion: The finding that mutating the degenerin site reduces the stimulatory effect of bile acids on ENaC suggests that this site is critical for the functional interaction of bile acids with the channel. To our knowledge, this is the first study demonstrating a possible functional role of the degenerin site in ligand-mediated ENaC activation.
OS2-02
Dynamic regulation of intracellular chloride concentration by EAAT glutamate transporters
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Cerebellar Bergmann glia cells are located in the Purkinje cell layer between the Purkinje neurons and contribute to the clearance of glutamate and GABA from the synaptic cleft. These cells express two different excitatory amino acid transporters, EAAT1/GLAST and EAAT2/GLT-1 that function not only as secondary-active glutamate transporters but also as anion channels. To study the role of these EAAT anion channels in determining intracellular [Cl-] we used fluorescent lifetime imaging microscopy (FLIM) with the chloride-sensitive fluorescent dye MQAE (Kaneko et al. J Neurosci: 24, 7931-7938 (2004)) in acute cerebellar slices of wild type and GLAST-/- knockout mice. MQAE is collisionally quenched by chloride ions, and we used calibration with different chloride concentration after permealization with nigericin and tributyltin for exact determination of [Cl-]int in these cells. In WT mice, intracellular chloride concentrations are significantly lower compared to GLAST-/- animals. In WT animals, we determined a mean internal chloride concentration of 33.8 ± 0.6 mM (mean ± SEM, n = 64 slices from 23 animals). In GLAST/-/ - animals intracellular [Cl-]int was significantly higher 41 ± 0.9 mM (n = 27 slices from 9 animals). Similar intracellular anion concentrations as in GLAST/-/- animals were obtained in WT glial cells after isoform-specific pharmacological block of GLAST by UCHP-101 (Abrahamsen et al. J Neurosci: 33, 1068-1087 (2013)). EAAT anion channels are activated by glutamate, and we used local electrical stimulations as well as the application of high glutamate concentrations (500 µM) to study the potential role of these channels in the dynamic regulation of glial anion concentrations. Both maneuvers decreased the intracellular chloride concentration significantly. Around 60 min after the application of 500 µM glutamate we obtained mean [Cl-]int of Bergmann glia cells of wild type mice of 29.8 ± 0.6 mM (n = 10 slices from 3 animals). Local electrical stimulation (50 Hz, 150 µs pulse duration, 2 mA) reduced [Cl-]int within 80 secs to 28.3 ± 0.8 mM (n = 3 slices from 1 animal). EAAT1 anion channels thus decrease intracellular chloride concentration in Bergmann glia in response to excitatory synaptic transmission. Since GABA uptake is stoichiometrically coupled to the chloride gradient, this mechanism allows stimulation of GABA uptake by EAAT glutamate transporters and permits a crosstalk between excitatory and inhibitory synaptic transmission in the cerebellum.

Figure 1

OS2-03
Coexistence of different forms of timing-dependent LTP at hippocampal CA3-CA1 synapses
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Long-term potentiation (LTP) and long-term depression (LTD) are commonly used to study learning and memory processes in vitro and can be induced with various stimulation patterns. A reliable tool to mimic experience-dependent synaptic plasticity is spike timing-dependent plasticity (STDP), which consists of repetitive single presynaptic stimulation paired with 1-4 postsynaptically triggered action potentials (AP). Interestingly, subtle changes in the number of postsynaptic APs used for pairing and in the number of overall repetition of pairings, respectively, led to t-LTP forms which markedly differ in the recruited intracellular signaling cascades and t-LTP expression.

Applying different STDP paradigms at hippocampal CA3-CA1 synapses, we can induce robust timing-dependent (t-) LTP using whole cell patch clamp techniques in acute slices of rats and mice. We use either 1EPSP/1AP (1:1) or 1EPSP/4AP (1:4) pairings (1 presynaptically induced excitatory postsynaptic potential paired with one or four postsynaptic AP, respectively).

We show that the 1:1 pairing leads to presynaptically expressed t-LTP, which is induced in a dopamine dependent manner, while a 1:4 paradigm leads to postsynaptically expressed and BDNF dependent t-LTP. We further show that this 1:4 pairing induced t-LTP is mediated via BDNF action at postsynaptic TrkB receptors. Postsynaptic elevation of cAMP combined with repeated 4AP burst firing leads to a BDNF dependent increase of evoked EPSPs, mimicking and occluding the 1:4 pairing induced t-LTP. This proves the specificity of the postsynaptic stimulation pattern for BDNF signaling and also suggests postsynaptic BDNF secretion as underlying mechanism for the 1:4 t-LTP. Independence of signaling and expression mechanisms between the 1:1 and the 1:4 paradigm induced t-LTP is shown by the absence of t-LTP occlusion between these 2 protocols. Additionally
to these differences regarding t-LTP at the stimulated synapse, hetero-synaptic effects as well as adaptive intrinsic changes are evident from our study. In experiments in which a non-potentiated second synaptic input is analyzed as control pathway, our BDNF-dependent 1:4 protocol leads to a hetero-synaptic spread of BDNF and a subsequent transient increase of evoked EPSPs in the independent control (for 1:1: no change). Parallel to synaptic plasticity, we observe an additional type of plasticity, which is indicated by AP frequency changes in potentiated neurons.

Taken together our data show, that similarly effective STDPE can be induced with various patterns of STDPE protocols at CA3-CA1 synapses. However, these t-LTP forms are mediated by activation of distinct signaling cascades and induced by different patterns of stimulation. Hence, our data indicate a multitude of potential forms of memory formation, which are expressed at a single type of synapse in the hippocampus.

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OS2-05
Modulation of endothelial cell apoptosis, differentiation, and proliferation by extracellular matrix rigidity

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Introduction: Own earlier studies showed, that upon shear stress stimulation, endothelial cells (EC) actively release the growth factor FGF-2 (J Vasc Res. 48; J Biol Chem. 277). This release of FGF-2 was initiated by protease activation, a process that was further controlled by specific cell matrix adhesion. Proteolytic digestion of the extracellular matrix change its stiffness and might have profound effects on cell matrix interactions. However, up to date it is studied insufficiently how such matrix modifications are established and whether they influence EC phenotype. In this study, we tried to address that question and draw the hypothesis that, similar to stem cells, the phenotype of EC might be dependent on the elastic properties of the extracellular matrix (e.g. the vascular wall). We paid special interest on cellular phenotypes and signaling responses induced by changes in the matrix stiffness. Since during vascular remodeling processes, matrix proteases are often activated and seem to be critically involved in generation and maintenance of signaling cascades in adaptive vascular remodeling, we proposed that due to those proteolytic activities the matrix stiffness is altered and influences EC phenotype.

Methods and results: To investigate acute signaling effects induced by proteolytic matrix degradation, EC were grown on plastic dishes coated with fibronectin and subsequently exposed to elastase (0.3 to 0.5 U/ml). As soon as 2-5 min after elastase treatment EC showed substantial re-organization of focal adhesion sites. In order to verify that matrix stiffness was the effective signal, EC were seeded on matrix-coated polyacrylamide gels exhibiting increasing stiffness (Young’s modulus ranged from approx. 4 to 140 kPa). Parallel to matrix flexibility, EC showed changes in morphology from a flat and spread phenotype to a more rounded shape. Furthermore, EC grown on intermediate flexible matrices are intending to form new capillary-like structures, which were absent on gels with high rigidity. Here EC showed to highest proliferative index.

Conclusion: These results indicate that, proteolysis of the matrix induces distinct signaling cascades. Thus, our hypothesis that fragmentation of matrix proteins changes the microarchitecture and show modulator properties for adhesion dependent signaling was verified. Moreover, EC grown on a soft matrix tend to go into apoptosis. Cells grown on intermediate flexible matrices are intending to form new vessels, and, finally, cells on stiff matrices are highly proliferative. This in vitro culture regime might serve as a model system to study not only development and progression of vascular aneurysms but also of arteriosclerosis and high blood pressure.
**OS2-06**

**Actin depolymerisation and crosslinking join forces with myosin II to drive secretion via compression of fused secretory vesicles**

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In recent years it has become evident that actin-based forces are required for secretion of poorly soluble vesicle contents. It is now well established that actin and myosin are specifically recruited to the surface of exocytic vesicles following fusion with the plasma membrane and promote active extrusion of cargo. Yet, little is known about the molecular mechanisms that regulate actin coat formation and drive coat contraction on fused granules. We have recently demonstrated that actomyosin dependent compression of fused lamellar bodies (LBs) is essential to drive secretion of pulmonary surfactant.

Here we provide a detailed kinetic analysis of the molecules regulating actin coat contraction on fused LBs. We demonstrate that Rock1 and myosin light chain kinase (MLCK) translocate to fused LBs and activate myosin II on actin coat. Yet, myosin II activity is not sufficient for efficient actin coat contraction. In addition, Rock1 regulates cofilin-1 activity. Regulated actin depolymerisation by cofilin-1 and actin crosslinking via α-actinin is essential for full contraction of the actin coat. In summary, our data support a model in which actin depolymerisation and crosslinking join forces with myosin II to contract actin coats around fused secretory vesicles to drive secretion.

**OS2-07**

**The clathrin-inhibitor Pitstop-2 disrupts the nucleocyttoplasmic barrier: implications for non-viral gene therapy**

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Several thousands of nuclear pore complexes (NPCs) perforate the nuclear envelope of each eukaryotic cell. These elaborate proteinaceous assemblies mediate all nucleocytoplasmic transport highly selectively through a central channel residing within a rigid and well-structured NPC scaffold. The selectivity of the NPCs is the major obstacle for non-viral gene therapy due to the prevention of exogenously applied therapeutic macromolecules from nuclear entry. Selectivity is attributed to highly dynamic and disordered Phenylalanine-Glycine rich proteins (FG-nups) within the NPC central channel. Limited understanding of the FG-nup arrangement hinders development of strategies directed at disrupting the NPC permeability barrier. We explore an alternative approach to enhancing the NPC permeability for exogenous macromolecules by targeting the NPC scaffold. The latter is rich in proteins with striking structural similarities to clathrin coat proteins which implies a common evolution of NPC and clathrin proteins. We hypothesized that disruption of NPC scaffold proteins by a clathrin coat protein disruptors perturbs the NPC barrier. To test this hypothesis we studied the effect of the newly developed clathrin coat inhibitor pitstop-2 on NPC structure and permeability in several cell lines using atomic force and confocal scanning microscopy. Treatment with Pitstop-2 causes fragmentation of the NPC scaffold which is paralleled by a collapse of the NPC permeability barrier. In conclusion, our data provide the first functional indication of the evolutionary relation between clathrin and NPC scaffold proteins. In addition, Pitstop-2-induced breakdown of the NPC permeability barrier may be exploited for gene therapeutic purposes.

**OS2-08**

**Interleukin-1β induced PDZK1 downregulation reduces NHE3 activity in intestinal epithelial cells**

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**Question:** The expression of the NHE3-binding PDZ adapter protein PDZK1 is strongly decreased in the inflamed murine and human intestinal mucosa. We searched whether this inflammation-associated PDZK1 downregulation is a direct consequence of the release of proinflammatory cytokines, by exposing intestinal epithelial Caco-2BBE cells to tumor necrosis factor alpha (TNF-α), interferon-γ (IFN-γ) and interleukin-1β (IL-1β) alone and in combination.

**Methods:** Real-time PCRs and Western blots were done to analyse mRNA and protein expression after cytokine treatments. Transient transfections using different PDZK1 5' flanking regions were performed and promoter activities were measured by luciferase assays. pH-fluorimetry was performed by utilizing BCECF dye, to analyse NHE3 activity. **Results:** Among the cytokines tested, only IL-1β significantly decreased PDZK1 promoter activity, mRNA and protein expression in a time- and dose-dependent manner. Inhibition of PDZK1 expression by IL-1β resulted in decreased acide-activated NHE3 activity, without a change in NHE3 protein expression. Deletion of a distal region in the PDZK1 promoter, from -4689 to -3995bp strongly diminished basal expression of PDZK1 by IL-1β meditated inhibition. This region of the promoter harbours putative binding sites for NF-kB, AP-1, SP-1 and retinoid X receptor alpha (RXR-α) proteins. IL-1β-mediated downregulation of PDZK1 protein expression and promoter activity was not dependent on NF-kB and p38 MAPK pathways. 9-cis retinoic acid, a ligand for RXR-α, attenuated IL-1β-induced decrease in PDZK1 protein expression in Caco-2BBE cells. Moreover, RXR-α mRNA expression was decreased in Caco-2BBE cells treated with IL-1β and also in intestinal mucosa of patients with active ulcerative colitis.
Conclusion: The results suggest that the strong decrease in PDZK1 expression during intestinal inflammation is, at least in part, a direct consequence of reduced PDZK1 promoter activity, possibly via IL-1β-induced reduction of RFXα expression, and therefore will be amenable by pharmacological treatment.

OS3 Stem cells and cardiac function

OS3-01 Cardiomyocyte specific knockout of p38MAPK induces severe cardiac dilation and metabolic alterations after Angiotensin II treatment.

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Angiotensin II (AngII) (1,5mg/kg/d) over 14 days. Echocardiography was performed at baseline and day 2, 7 and 14 of AngII-treatment as well as telemetric blood pressure measurements for 7 days at baseline and during AngII-treatment. Experiments were performed in HL-1 cardiomyocytes and in Ampkα1 deficient and wild-type mice following pressure overload by transverse aortic constriction (TAC) and angiotensin II infusion.

Question: Our data show that p38MAPK protects the heart from pressure overload induced cardiac remodelling and seems to play a newly discovered role in cardiac metabolism.

OS3-02 Ampkα1 isoform induces activation of Ap-1 via Pkcz1 in cardiomyocytes


AMP-activated protein kinase (Ampk) is a crucial regulator of cellular energy metabolism and plays a decisive role in the myocardial response to increased cardiac stress. The current study investigated possible isoform specific effects of Ampkα1 in cardiomyocytes.

Methods: Experiments were performed in HL-1 cardiomyocytes and in Ampkα1 deficient and wild-type mice following pressure overload by transverse aortic constriction (TAC) and angiotensin II infusion.

Results: Overexpression of constitutively active Ampkα1 in HL1 cardiomyocytes significantly increased phosphorylation of protein kinase C ζ (Pkkζ), AP-1 transcriptional activity, as well as mRNA expression of c-Fos, Il6 and Ncx1. The stimulatory effect of constitutively active Ampkα1 on AP-1 activity was blunted by Pkkζ silencing. Silencing of Ampkα1 and Pkkζ, but not Ampkζ2 further blunted the AP-1 activation and c-Fos, Il6 and Ncx1 mRNA expression induced by angiotensin II. Following angiotensin II infusion, an increased...
expression of cardiac Ampkα1 was paralleled by increased Pkcζ abundance as well as mRNA expression of c-Fos, Il6 and Ncx1 in wild-type mice, but not in Ampkα1 deficient mice. Similarly, following TAC, cardiac function was better preserved in Ampkα1 deficient mice as in wild-type mice, paralleled by a blunted increase of Pkcζ abundance and c-Fos, Il6 and Ncx1 mRNA expression.

Conclusions: Ampkα1 has isoform specific effects in cardiac tissue. Ampkα1 promotes activation of AP-1 in a Pkcζ-dependent manner and might therefore participate in myocardial remodelling.

OS3-03
Role of cytosolic renin in ischemic heart conditions
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Introduction: The renin-angiotensin system is known to increase oxidative stress, exert pro-inflammatory effects and interfere with cardiac energy metabolism. Correspondingly, inhibitors of the RAS belong to the most potent drugs in the treatment of hypertension and cardiac failure, markedly increasing life span of patients. In the heart an alternative renin transcript is expressed, which encodes for a non-secretory renin protein that is located in the cytosol or within mitochondria. Cytosolic renin expression is increased after acute myocardial infarction. Aim of the study was to investigate the effect of cytosolic renin overexpression in the ischemic heart in vivo.

Methods: High dose isoproterenol leads to tachycardia and hypotension, resulting in ischemic conditions in the heart. Ten weeks old male control rats (TG-) and CX- (exon2-9)renin transgenic rats (TG+) (n=10) were exposed to two consecutively given doses of 80 mg/kg body weight isoproterenol s. c. in an interval of 24 hours. Five TG- and TG+ rats of the same age received 0.9 % NaCl s. c. as control. Cardiac MRI was performed two weeks before and two days after the injections. As high-dose-isoproterenol leads to apical dysfunction with a systolic “ballooning” of the apex, apical function was measured in the apical short axis slices as regional stroke volume in percent of enddiastolic volume.

Results: Left ventricular mass per body weight was similar in both groups (TG-: 2.08 ± 0.15 mg/g, TG+: 2.18 ± 0.18 mg/g; p=0.07). High dose isoproterenol injection led to death in six out of ten TG- rats, but only in one out of ten TG+ rats. Apical function of the hearts of the surviving TG- rats was decreased, but remained unchanged in TG+ rats. Basal ejection fraction was higher in TG- than in TG+ rats (68.92 ± 5.59 % vs. 63.18 ± 2.84 %; p<0.01, n=15). Two days after isoproterenol injection, ejection fraction was reduced in the surviving TG- rats (from 66.2 ± 1.14 % to 58.05 ± 4.37 %), but even increased in isoproterenol treated TG+ rats, compared to basal levels (from 63.01 ± 0.74 % to 69.41 ± 2.1 %; p<0.01).

Conclusion: In contrast to the negative effects of secretory renin, cytosolic renin is a major protective factor in the ischemic heart disease. However, since angiotensinogen is not present in the cytosol, these effects should be rather angiotensin independent.

OS3-04
Protective outweighs inflammatory role of inducible proteasome subunit Lmp2 in cardiac remodeling
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Introduction: The ubiquitin-proteasome system constitutes the major intracellular pathway for targeted protein degradation of intact as well as damaged proteins. In cardiac tissue, multiple proteasome complexes exist, differing in their assembly of constitutive and inducible subunits. In non-cardiac cells, the latter are associated with improved degradation of damaged proteins as well as optimized antigen processing for MHC surface presentation. To date, the role of inducible proteasome subunits in the heart is unknown, although they seem to be dynamically regulated in early cardiac remodeling as well as inflammatory cardiomyopathy.

Objective: Aim of the study was to identify whether dynamic regulation of inducible subunits, more specifically Lmp2, is limited to the described cardiac conditions, and whether it is driving or restricting the pathogenesis.

Methods & Results: Lmp2 expression in murine hearts of 3-4 month old mice was investigated in response to pressure overload due to 4 weeks transaortic constriction as well as familial hypertrophic cardiomyopathy induced by a homozygous G>A transition on the last nucleotide of exon 6 of the gene encoding cardiac myosin binding protein Mybpc3. Both cardiac conditions were characterized by established hypertrophy and reduced systolic function. Furthermore, both animal models showed an increased expression of Lmp2 between 40-50%. Previously, up to 78% increased expression of Lmp2 was observed in cardiac hypertrophy with preserved systolic function induced by continuous β-adrenergoreceptor stimulation. In this study, Lmp2 incorporation in assembled and functional proteasome complexes was not detectable via activity-based proteasome probes in cardiac tissue under unstimulated conditions, suggesting little participation in baseline proteasomal degradation. Furthermore, Lmp2 KO mice had no distinct phenotype compared to wildtype mice based on visual appearance, body weight, heart weight, echocardiography as well as cardiac proteome. In contrast, Lmp2 KO mice subjected to continuous β-adrenergoreceptor stimulation showed more pronounced cardiac remodeling with exacerbated hypertrophy, the onset of fibrosis visualized by trichrome staining, and reduced systolic function.

In conclusion, induction of Lmp2 is associated with at least three independent cardiac conditions leading to pathologic remodeling and seems to restrict rather than drive the pathogenesis.
EPDC in the adult heart can be reactivated by myocardial infarction (MI) and are considered as endogenous cell source with the potential to mediate cardiac regeneration. EPDC are of mesenchymal origin and express high CD73 which catalyzes the formation of adenosine, an important paracrine factor. We thus explored the extracellular purine metabolism and the impact of the matricellular protein tenasin-C (TNC). We found that cultured rat EPDC rapidly degrade ATP via ADP and AMP to adenosine (HPLC analysis). Surprisingly, NAD is equally well hydrolyzed to adenosine involving ADP-ribose and AMP. Thus, CD73 is the critical bottleneck of both degradation pathways. Quantitative RT-PCR showed that EPDC express a distinct set of both adenosine (A2A > A2B > A3 > A1) and ATP (P2X4 > P2X7 > P2X5; P2Y2 > P2Y4 > P2Y12) receptors. As to the endogenous source of nucleotides, we found quinacrine (an ATP store marker) and Bodipy ATP (a fluorescent ATP analog) localized to vesicular structures suggesting ATP-storing granules. In line with this hypothesis, elevating intracellular Ca++ by ionomycin caused the release of ATP.

CD73 is known to directly bind to proteins of the extracellular matrix, which in turn may modulate its enzymatic activity. We found that TNC is strongly expressed by EPDC in vivo and in vitro as evidenced by quantitative RT-PCR and immunofluorescence. Secretion of TNC is stimulated by TNFα and in vitro as evidenced by quantitative RT-PCR and immunofluorescence. Moreover, TNC strongly promoted migration of EPDC in a haptotaxis assay.

Our data demonstrate that EPDC can release nucleotides and avidly degrade ATP as well as NAD via CD73 to adenosine. EPDC formed in response to MI produce significant TNC which is absent in healthy heart and may facilitate EPDC migration into the injured myocardium.

The angiotensin converting enzyme (ACE) is an ectoenzyme that regulates blood pressure and electrolyte/water balance. The enzyme is expressed in endothelial and vascular smooth muscle cells and has been proposed to be a stem cell marker that influences the mobilization of hematopoietic progenitor cells (HPCs). In addition to its action as peptidase, ACE is also capable of signal transduction by a mechanism initiated by the CK2-mediated phosphorylation of ACE on Ser1270 (human sequence). The aim of this study was to verify the expression of ACE in HPCs and determine the potential role of ACE signaling in HPC mobilization in mice. Although ACE was expressed by a small population of bone marrow (BM) cells it was more strongly expressed in the bone itself (immunohistochemistry and Western blotting). To determine whether ACE played a role in stem cell mobilization, wild-type (WT) and ACE-deficient (ACE.4) mice were treated with vehicle or granulocyte-colony stimulating factor (G-CSF, 250 μg/kg/day, 5 days) before HPC mobilization was assessed by quantifying colony-forming units (CFU) in culture formed by peripheral blood derived mononuclear cells (PB-MNCs). While there was no difference in mobilization between the two vehicle-treated strains, the number of CFUs formed by G-CSF-stimulated ACE.4-derived PB-MNCs was significantly higher than from WT mice. Also, in WT mice the ACE inhibitor Ramipril (10mg/kg/day, 5 days before and during G-CSF administration) increased CFU formation. Transplantation rescue experiments of sublethally irradiated WT animals demonstrated that the loss of ACE in the HPC microenvironment rather than in the HPCs themselves increased HPC mobilization (increase in the number of CFUs in the spleen). To address the mechanism by which ACE deletion affects mobilization, we focused on “ACE signaling” and found that G-CSF elicited the phosphorylation of ACE on Ser1270 in vivo and in vitro, a response sensitive to CK2 inhibition. That ACE phosphorylation may be required for mobilization was assessed using a transgenic mouse expressing a non-phosphorylatable ACE (ACE569) mutant. In these animals, the number of CFUs formed by PB-MNCs after administration of G-CSF was significantly higher than in the WT mice. Moreover, mass spectrometry analysis of the proteins co-precipitated with ACE from WT vs. ACE569 mice revealed that several proteins with putative links to HPC mobilization colocalized with ACE from WT but not ACE569 mice. These data demonstrate an important role for ACE in the HPC microenvironment and revealed that ACE expression/phosphorylation negatively affects the G-CSF-induced HPC mobilization, most likely via interaction with specific proteins.

OS3-07
Stimulation of vasculogenesis and leukopoiesis of embryonic stem cells by extracellular transfer RNA and ribosomal RNA
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Cell injury releases nucleic acids to support inflammation and stem cell activation. Here, the impact of extracellular ribonucleic acid, especially transfer RNA (tRNA), on vasculogenesis and leukopoiesis of mouse embryonic stem (ES) cells was investigated. Extracellular tRNA (ex-tRNA) and whole cell RNA as well as ribosomal RNA (ex-rRNA) but not DNA increased CD31-positive branching points in embryoid bodies. Ex-tRNA and ex-rRNA treatment increased cell numbers of VEGFR2+, CD31+ and VE-cadherin+ vascular cells as well as CD18+, CD45+ and CD68+ cells, indicating leukocyte/macrophage differentiation. This was paralleled
by mRNA and protein expression of hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor-165 (VEGF165) and neuropilin 1 (NRP1) as well as phosphorylation of phosphatidylinositol 3-kinase (PI3K) and VEGF receptor 2 (VEGFR2). Furthermore, ex-tRNA increased protein expression of the pro-angiogenic semaphorin B4 receptorplexin B1 as well as the ephrin-type B receptor 4 (EphB4) and ephrinB2 ligand. Ex-tRNA enhanced cell migration, which was inhibited by the VEGFR2 antagonist SU5614 and the PI3K inhibitor LY294002. This likewise abolished the effects of ex-tRNA on vasculogenesis and leukopoiesis of ES cells. Ex-tRNA increased Nox-1, Nox-2 and Nox-4 mRNA and boosted the generation of reactive oxygen species (ROS) which was inhibited in the presence of radical scavengers and the NADPH oxidase inhibitors apocynin and VAS2870. The latter abolished the effects of ex-tRNA treatment. Our findings indicate that ex-tRNA treatment induces vasculogenesis and leukopoiesis of ES cells via ROS generated by NADPH oxidase and activation of VEGFR2 and PI3K.

**OS3-08**

**Titin phosphorylation is differentially modulated in models of myocardial ischemia and affects cardiac myocyte stiffness**

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**Question:** Titin is a major determinant of myocardial passive stiffness. Titin stiffness is modulated by the relative expression levels of the cardiac isoforms N2BA (3.2–3.7 MDa) and N2B (3.0 MDa) and is further modified by several modifications including phosphorylation of the elastic titin domains PEVK and N2B. Phosphorylation in the PEVK domain by e.g. PKCa or CaM-Kinase II leads to an increase, phosphorylation in the N2B domain by PKA or PKG causes a decrease in titin-based myocardial stiffness. Myocardial ischemia (MI) is known to activate several protein kinases, including PKCa, thereby regulating the onset and extent of cell injury and death. Here, we tested whether myocardial ischemia induces changes in titin phosphorylation and affects its elastic properties.

**Methods:** We analyzed titin parameters in three models of MI: (I) Langendorff-perfused mice hearts, 20’ global ischemia (I)/30’ Reperfusion (R); (II) adult mice hearts with either 3 days permanent LAD ligation or (III) 10 days permanent LAD ligation. Titin isoform composition was tested by agarose-strengthened 2.1% SDS-PAGE. Titin phosphorylation was analyzed by Western blot analysis using phosphospecific antibodies targeting the titin N2B or PEVK domain. Kinase activity was estimated by using phospho-specific antibodies targeting the activated kinases itself or kinase substrates. Passive stiffness was measured using isolated skinned cardiac myocytes.

**Results:** In model I and II titin PEVK phosphorylation was significantly increased. Changes occurred within 20’ after beginning of ischemia (I) and persisted even after 3 days (II) consistent with an increased PKCa activity in both models. After 10 days LAD ligature (III) PEVK phosphorylation and PKCa-activity were unchanged. PKG-dependent N2B phosphorylation was significantly decreased in model I, unchanged in model II but without statistical significance. In model III PKG-dependent N2B phosphorylation was strongly increased probably due to elevated PKG activity. Consistent with increased titin PEVK and reduced N2B phosphorylation passive stiffness was elevated in model I and model II. In contrast, the titin phosphorylation changes 10 days after ischemia suggest a reduction in titin stiffness.

**Conclusion:** Titin-based passive stiffness is rapidly increased early after myocardial ischemia and is adversely modulated after the acute phase. Our data suggest that modification of titin stiffness may play an important role in both the early adaptational and the remodeling phase after myocardial ischemia.

**OS4**

**Vascular functions and circulation**

**OS4-01**

**Deletion of the NADPH oxidase organizing protein Nox01 promotes angiogenesis**

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Reactive oxygen species contribute to angiogenesis and vascular repair. NADPH oxidases of the Nox family are the main source of ROS in the vasculature. Nox01 is a cytosolic protein facilitating assembly on the constitutively active NADPH oxidase of epithelial cells. Although Nox01 is known to be expressed in the vascular system to a low level, its physiological function is unknown. Being constitutively active, we speculate that Nox01 contributes to basal ROS formation in the vascular system and modulates angiogenic responses. This hypothesis was tested in Nox01 knockout mice and cells obtained from these animals. Blood flow recovery after femoral artery occlusion was better in Nox01−/− as compared to WT animals and also capillary density was increased after Nox01 knockout. Similar, ex vivo spheroid outgrowth assays revealed increased tube
formation capacity in lung endothelial cells obtained from NoxO1-/- mice as compared to WT animals. In a spheroid confrontation assay, in which color labeled cells from WT and NoxO1-/- animals are directly studied within the same spheroid, the number of NoxO1-/- cells at the tips was higher than that of wildtype cells. These results suggest that deletion of NoxO1 favors the expression of a tip cell like phenotype.

The NOTCH pathway is one of the main switches for an endothelial cell from a tip cell into a stalk cell phenotype and activation of the NOTCH pathway results in expression of a stalk cell phenotype. Physiologically, NOTCH mediated signalling requires proteases, among them the alpha-secretase ADAM17, to eventually result in the formation of the active NOTCH intracellular signalling domain. Importantly, ADAM17 activity was indeed reduced in NoxO1-/- cells when compared to wildtype as measured by the degradation of an artificial substrate.

We conclude that NoxO1 controls alpha-secretase activity. Deletion of NoxO1 therefore promotes a tip cell phenotype which results in increased angiogenesis.

OS4-02
Hydrostatic pressure reorganizes the endothelial cortical cytoskeleton
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In the vasculature, endothelial cells are exposed to three types of mechanical forces: shear stress due to blood flow, tensile stress due to the compliance of the blood vessel wall and hydrostatic pressure due to vertical transmural pressure. In this project we aimed to separate the effects of hydrostatic pressure from the other mechanical forces and analyzed its impact on cortical acto-myosin organization.

We asked the question whether hydrostatic pressure per se could modulate the organization of the endothelial cortical cytoskeleton and thereby influences nanomechanical properties and endothelial function.

To aim this, we have developed a pressure chamber, based on a hybrid AFM/confocal microscope. Furthermore we established a cell culture compatible pressure chamber for long term cultivation of cells at elevated hydrostatic pressure. Acute (1 h) and chronic (24 h) pressure (e.g. 100 mmHg) was applied, while and acto-myosin organization as well as cortical stiffness were analyzed.

Confocal fluorescence imaging and mechanical nanoindentation measurements of the actin web revealed a time-dependent effect of hydrostatic pressure on cortical stiffness. After 1h of pressure application, cortical stiffness increased by 67 ± 2.4% in comparison to non-pressurized cells, and after 24h by 110 ± 3.1%. While no changes in submembranous actin filaments could be found in the acute experiment (1h), chronically pressurized cells (24h) responded with an increase of actin filaments by 42 ± 1.3%. In contrast to cortical actin, cortical myosin increased by 115 ± 3.2% already after short-term pressure application (1h) and remained elevated over at least 24h. This change is paralleled by its functional activation of 48 ± 4.1%, as confirmed by phospho-myosin light chain fluorescence staining.

We propose a model, describing the response of endothelial cells in terms of acute and chronic hydrostatic pressure. Short-term pressure causes endothelial cortical stiffening due to myosin-induced lateral tension. In contrast, long-term pressure stiffens the endothelial surface by cortical actin web remodeling. Since cortical nanomechanics determines nitric oxide release, hydrostatic pressure per se is possibly an important component in the regulation of blood.

OS4-03
Inhibition of fatty acid amide hydrolase (FAAH) prevents pulmonary hypertension
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Question: Recently, we could show that the endocannabinoid anandamide induces pulmonary vasoconstriction via its degradation to eicosanoids by the enzyme fatty acid amide hydrolase (FAAH). This signaling pathway is also involved in the induction of hypoxic pulmonary vasoconstriction (HPV). Because long lasting HPV can result in chronic pulmonary hypertension (PH), we are now interested in the role of FAAH in the pathogenesis of PH.

Methods: PH was induced by chronic exposure of mice to hypoxia (10% O2) in ventilated chambers for 3 weeks. For analysis of PH, measurements of right ventricular pressure, right ventricular heart weight and vascular wall thickness were performed. We also determined inflammation and/or remodeling by immunostaining and qRT-PCR measurements.

Results: Our data reveal that HPV is diminished in FAAH-/- mice and after pharmacological inhibition of FAAH using the specific inhibitor URB-597. Using LC-MSMS we found that hypoxia increased levels of anandamide and its metabolite arachidonic acid in the lungs of WT animals. Moreover, we could identify pulmonary arterial smooth muscle cells as the source of hypoxia-dependent anandamide production and degradation. Importantly, FAAH-/- mice and WT mice treated with URB-597 were protected against the development of hypoxia-induced PH. This was accompanied by a clear reduction of infiltrating macrophages and neutrophils and decreased expression of vascular remodeling markers in the lung. We also found that URB-597 inhibits cell growth of pulmonary arterial smooth muscle cells, which is critical for the development of PH.

Conclusion: Our data demonstrate that inhibition of FAAH can prevent pulmonary hypertension by blocking inflammation and vascular remodeling.
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Question: The soluble epoxide hydrolase (sEH) metabolizes bioactive polyunsaturated fatty acid (PUFA) epoxides such as the epoxyeicosatrienoic acids (EETs) and epoxydocosapentaenoic acids (EDPs) to their corresponding diols. Given that the docosahexaenoic acid (DHA) diol, 19,20-dihydroxydocosapentaenoic acid (DHDPA) is enriched in postnatal retinas and plays a role in physiological angiogenesis by inhibiting of Notch signaling, the aim of the present study was to investigate the role of sEH and its products in diabetic retinopathy.

Methods: Ins2Akita mice carrying a point mutation on the insulin 2 gene that causes misfolding of the insulin protein were used as animal model of diabetic retinopathy. Animals were treated with vehicle or a sEH inhibitor (sEH-I) from the age of 6 weeks for 6 months. Thereafter, retinopathy was assessed and the number of endothelial cells, pericytes and acellular capillaries were quantified after retinal digestion. Retinal vascular permeability in vivo was estimated by intravenous injection of FITC-BSA. The transendothelial electrical resistance (TEER) was studied in vitro to estimate permeability through endothelial cells treated with different PUFA epoxides and diols.

Results: At 30 weeks, the vehicle-treated Ins2Akita mice demonstrated a pronounced diabetic retinopathy associated with a significant decrease in the numbers of retinal pericytes. At the same time numbers of migrating pericytes and acellular capillaries were increased compared to wild-type littermates, as was the vascular permeability. The latter phenotype was associated with disrupted endothelial cell junctions (as evidenced by non-continuous VE-cadherin staining in retinal vessels) and a marked increase in the expression and enzymatic activity of the sEH in diabetic retinas. This resulted in an increase in 19,20-DHDPA levels. Inhibition of sEH markedly diminished the production of 19,20-DHDPA and significantly attenuated retinal pericyte loss, acellular capillary formation and suppressed the retinal vessel leakage in Ins2Akita mice compared to vehicle treated controls. In in vitro studies, 19,20-DHDPA and the Notch inhibitor DAPT significantly decreased the TEER in primary brain microvascular endothelial cells, and this phenomenon was accompanied with a disorganized VE-cadherin localization along endothelial cell boarders.

Conclusion: These data indicate that the CYP/sEH cascade plays a critical role in the regulation of retinal vessel homeostasis and as the disturbed PUFA metabolism associated with diabetes was associated with increased sEH expression, the inhibition of sEH may serve as a new therapeutic target for diabetic retinopathy.

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Objectives: Mesenteric artery spasms cause non-occlusive mesenteric ischemia (NOMI) that has a high mortality. High plasma catecholamine concentrations and endothelin (ET) system activation facilitate the development of mesenteric artery spasms. RhoA/Rho-kinase (ROCK) activation has been shown to contribute to vasospasms in several vascular beds. The function of human mesenteric arteries and the efficacy of vasodilators currently used in NOMI treatment are not well defined. We characterized the responses to physiological vasoconstrictors, ROCK isoenzyme expression and the ROCK-dependence of ET-1-induced vasoconstriction in small human mesenteric arteries. Further, we tested the efficacy of three vasodilators that are currently administered via the arterial route in NOMI treatment.

Methods: Mesenteric tissue was obtained from patients who underwent elective abdominal surgery and gave informed consent. Mesenteric artery function and ROCK isoenzyme expression were investigated by small vessel myography and Western blot.

Results: ET-1, vasopressin and norepinephrine (NE) elicited concentration-dependent constrictions in small mesenteric arteries. Maximum phenylephrine-induced vasoconstriction was 85 % less than maximum NE-induced vasoconstriction suggesting an important contribution of α2-adrenoceptor-dependent mechanisms. Mesenteric arteries express both ROCK isoenzymes. ROCK inhibition did not significantly affect maximum ET-1-induced vasoconstriction but shifted the concentration response curve to the right (log EC50 -7.67 controls vs. -7.13 mol/l with ROCK inhibition). The cAMP-dependent vasodilators iloprost, prostaglandin E1 (PGE1) and papaverine reduced vascular wall tension to 22-28 % of ET-1-induced (10-7 mol/l) wall tension without statistically significant differences (n = 10 per group). However, logEC50 was significantly less for iloprost (-7.54 ± 0.07 mol/l) than for PGE1 (-6.50 ± 0.10 mol/l) or for papaverine (-6.44 ± 0.14 mol/l) (p < 0.001).

Conclusions: Iloprost, PGE, and papaverine dilate pre-constricted human mesenteric arteries with similar potency and may be suitable for intra-arterial administration in NOMI patients. The high sensitivity of mesenteric arteries to iloprost suggests that lower tissue concentrations may be required than for PGE1 or papaverine to resolve mesenteric artery spasms which may be an advantage under clinical conditions. The modest ROCK-dependence of ET-1-induced vasoconstriction suggests that pharmacological ROCK inhibition is not suitable to resolve acute mesenteric artery spasms.
Introduction: Atherosclerotic lesions are known to occur near arterial bifurcations. Platelets interacting with ultra-large von Willebrand Factor (ULVWF) multimers deposited after CD40 receptor ligation on the endothelial surface may facilitate P-selectin-dependent monocye adhesion at these regions of disturbed flow. A deficiency in ULVWF-cleaving ADAMTS13 protease activity could facilitate monocyte recruitment to the endothelium and subsequent transendothelial migration.

Methods: Human umbilical vein endothelial cells (HUVEC) grown in a parallel plate flow chamber were exposed to arterial shear stress (2.5 or 10 dyn/cm²) and exposed to soluble CD40 ligand (sCD40L) or freshly isolated, thrombin-stimulated human platelets. Isolated quiescent platelets and monocytes from whole blood were labeled with fluorescent dyes and superfused over the HUVEC monolayer. Platelets tethering to ULVWF multimers and adhesion of monocytes to platelets was documented by live cell imaging. ADAMTS13 plasma activity levels and concentrations were measured using a fluorescence resonance energy transfer (FRET) assay. ADAMTS13 expression was monitored by real-time polymerase chain reaction (PCR) and Western blotting. Cell surface-associated ULVWF multimers were visualized by immunofluorescence analysis.

Results: Platelet CD40L as well as sCD40L elicited a prominent deposition of ULVWF multimers on the luminal HUVEC surface to which platelets adhered forming ULVWF multimer-platelet strings. Monocytes were selectively recruited and trapped by the platelets tethered to the ULVWF multimers despite the fast flow. ADAMTS13 abundance was inversely correlated to the differentiation state of the HUVECs. Perfusion with ADAMTS13 at physiological concentrations of 1000 ng/ml completely dissolved the ULVWF multimer-platelet strings formed on the HUVEC surface. In contrast, low concentrations of 500 ng/ml or less, as found in patients with coronary heart disease, allowed the formation of these strings that persisted over minutes.

Conclusion: These findings may contribute to our understanding of how monocytes with the help of platelets tethered to ULVWF multimers deposited on endothelial cells in response to CD40L stimulation overcome the intermediate to high shear stress in conduit arteries, transmigrate into the subendothelial space and differentiate into pro-inflammatory macrophages in the early stages of atherosclerosis. In addition, low ADAMTS13 plasma levels are not sufficient to efficiently cleave emerging ULVWF multimers and may thus contribute to the pro-thrombotic and pro-inflammatory state of the atherosclerotic vessel wall.
**Question:** Chronic exposure of blood vessels to an elevated transmural pressure induces a phenotype switch of vascular smooth muscle cells (VSMCs) to overcome the undue rise in wall stress and eventually recapture normal vascular tone. At the cellular level, overt stretch induces the translocation of the mechanosensitive protein zyxin from focal adhesions to the nucleus where it fine-tunes the expression of a multitude of mechanosensitive gene products. Therefore, we analyzed the role of zyxin in hypertension-induced arterial remodeling.

**Methods and results:** VSMCs from zyxin-deficient (KO) mice are highly proliferative and less contractile compared to wild type VSMCs. They migrate faster in a 2D scratch assay and sprout more in a 3D spheroid invasion assay. Telemetry based blood pressure recording showed that DOCA-salt treatment failed to increase both systolic and diastolic blood pressure in old zyxin-deficient mice. Femoral arteries from these mice exhibited lower resistivity to blood flow according to high-resolution ultrasound imaging. Moreover, a reduced network of collagen-I fibers was observed in the femoral arteries of those animals possibly due to the reduced expression of its crosslinking enzyme, transglutaminase-2. This zyxin knockout phenotype becomes more evident with age arguing for a possible functional redundancy mediated by other zyxin family members such as lipoma-preferred partner (LPP) due to its sequence similarity with zyxin and high abundance in VSMCs. Zyxin and LPP co-localize at the focal adhesions of VSMCs. Unlike in wild type animals, an age-dependent rise in LPP mRNA in the vessel wall of zyxin-deficient mice together with a modest change in LPP protein suggest that the newly synthesized LPP might be rapidly consumed to fill in for the lacking zyxin. Additionally, in vitro overexpression of LPP in zyxin-deficient VSMCs normalizes their pro-migratory phenotype.

**Conclusion:** In pathologic i.e. hypertension-induced vascular remodeling, zyxin is a novel regulator for the phenotypic shift of VSMCs from the contractile to the synthetic phenotype and LPP might support zyxin in preventing this phenotypic shift to occur.

**OS5-01**

**IL-9 producing T helper-9 (Th-9) cells are distinct from Th1/Th2/Th17 cells subsets and iTregs in sodium hydrogen exchanger 1 (NHE1) activity and pH regulation**

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IL-9 producing T helper 9 (Th9) cells are newly characterised cell types and their role has been described in protection against cancer and helminths infections. Previously, it has been described in cancer and other cell types that sodium hydrogen exchanger 1 (NHE1) plays an important role in the maintenance of intracellular pH and cell volume regulation, thus influencing various physiological cellular functions such as cell proliferation, cell migration and cell death. However, whether NHE1 regulates the development of Th cells especially of Th9 cells has not been defined yet. Therefore, we have characterised Th cells by Flow cytometry and measured the intracellular pH and NHE1 activity using 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein-Acetoxyethyl Ester (BCECF-AM) dye in different Th cells subsets. Surprisingly, we found that Th9 cells have significantly higher NHE1 activity and significantly higher intracellular pH compared to other Th cells (Th1/Th2/Th17 and iTregs). In addition to this, we found that Th2 cells have significantly lower NHE1 activity compared to other Th cells and iTregs. Th1 cells have significantly less intracellular pH among all Th cells and iTregs. Th1 cells have significantly less intracellular pH among all Th cells and iTregs. When we characterised Th9 cells for NHE1 at protein and mRNA level, we found significantly higher NHE1 expression among all Th cells and iTregs. Th1 cells have significantly less intracellular pH among all Th cells and iTregs. Th1 cells have significantly less intracellular pH among all Th cells and iTregs. When we characterised Th9 cells for NHE1 at protein and mRNA level, we found significantly higher levels of NHE1 expression among all Th cells. Further, inhibition of NHE1 activity by cariporide affected the Th9 cells development. Thus, our data reveals that NHE1 could be an important physiological regulator of Th9 cells functions.

**OS5-02**

**Estimation of biomechanical parameters for P-Selectin-mediated leukocyte rolling using experimental data and model simulations**

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Invasion of leukocytes from the blood stream into tissue proceeds as coordinated spatial-temporal sequence called leukocyte adhesion cascade and is indispensable for an efficient immune response. Numerous proteins realize the interaction between leukocytes and endothelial cells under in-vivo conditions allowing rolling, adhesion, crawling and transmigration towards the tissue surrounding the vessel. In this study we focus on the dynamics of P-Selectin-mediated leukocyte rolling. In an in-vitro experimental approach, THP1
myelomonocytic cell rolling on P-Selectin coated surfaces in flow chambers is observed using phase contrast microscopy. Coating densities and shear rates are varied. Contours of cells are extracted automatically by self-developed image-segmentation algorithms delivering the position of the center of leukocytes for each observation time point. The resulting positions of THP1 cells show a mean drift superimposed by intermittent fluctuations indicating faster and slower movements. Assuming that the origin of these fluctuations is due to the biomechanical properties of the bonds between P-Selectins of the coated surface and PSGL-1 receptors on the cell surface, this should allow assessing biomechanical properties of these interaction bonds. Therefore, we construct a simplified biomechanical model taking into account coupling of shear stress to THP1 cells, a viscoelastic behavior of the bonds modeled as Kelvin element, force-dependent rupture kinetics, and variations of the density and heterogeneity of receptors and ligands. Initial simulations are performed using parameter values from literature to obtain cell positions as a function of time similar to our experimental data. In a second step the model is used to extract the corresponding biomechanical parameters from position curves of simulated data with probability theoretical methods (Bayesian data analysis). This approach unveils the possibilities and limitations of direct parameter extraction from observed position curves. In summary, our combination of tailored experiments and modeling allows the estimation of biomechanical properties of the nano-scale from microscopic experimental observations of a whole cell.

OS5-03
Preimplantation factor (PIF) impairs leukocyte recruitment in TNF-α induced inflammation in vivo

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Preimplantation factor (PIF) is a small peptide that is secreted only by viable embryos and can be detected in maternal circulation. It promotes trophoblast invasion into maternal decidua and seems to modulate the maternal immune system. Furthermore, it has been shown that the treatment with PIF has beneficial effects in autoimmune diseases such as type I diabetes mellitus or neuroinflammation. However, so far it is unclear, how PIF interferes with the immune system; especially under in vivo conditions. Therefore, the effects of PIF on the leukocyte recruitment cascade were investigated using intravital microscopy of postcapillary venules in the mouse cremaster muscle 2h after intrasural injection of TNF-α. Here we show that administration of 1µg of PIF into the scrotum 3h prior to investigation alters leukocyte recruitment into inflamed mouse cremaster muscles. We observed a significant reduction in leukocyte rolling along the venular vessel wall in PIF-pre-treated mice, reduced leukocyte adhesion and a decrease in the number of extravasated leukocytes compared to control mice. Interestingly, FACS analysis revealed that neutrophil surface markers relevant for leukocyte recruitment, such as PSGL-1, MAC-1 or LFA-1 were not altered in the presence of PIF. Taken together, this study does not only elucidate anti-inflammatory effects of PIF during the inflammatory response, it also indicates that PIF might be an interesting drug for the treatment of autoimmune diseases.

OS5-04
GlucoCEST MRI-based non-invasive detection of acute renal allograft rejection

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Methods: Adult, uni-nephrectomized, allogeneically kidney transplanted rats (Brown Norway to Lewis) without immunosuppression after surgery served as the renal transplant model. Four days after transplantation, a time point at which the renal graft usually shows marked signs of acute cellular rejection, glucoCEST MRI was performed. The MRI images were acquired at 9.4 T on a Bruker BioSpec 94/20 using a 72 mm volume coil and a RARE sequence. For constant blood glucose levels during MRI measurements, the animals received two time-displaced glucose boluses i.p.

Results: To assess the renal graft rejection by glucoCEST, a glucose infusion protocol was successfully developed and validated. Through time-displaced i.p. application of two glucose boluses (1.0 M glucose followed by 1.5 M glucose after 30 min), the blood glucose level was raised by about 120 mg/dl and could be kept constantly over the required total MRI scan time of approximately 50 min. Moreover, in vivo glucoCEST contrast could successfully be calculated, whereas, compared to the native kidney, the renal allograft undergoing rejection showed increased CEST-contrast in both, the pelvis (native: 0.077 ± 0.039, transplanted: 0.149 ± 0.054, p-value: 0.043) and the cortex (native: 0.026 ± 0.011, transplanted: 0.060 ± 0.027, p-value: 0.033, n=6) at day four after transplantation. Conclusion: Our results show that glucoCEST MRI is a feasible method for the detection of kidney...
rejection, providing a versatile tool to non-invasively identify and differentiate zones of (renal) inflammation in vivo.

**OS5-05**

Analysis of interaction between hypoxia-inducible transcription factor (HIF) proteins using Fluorescence Resonance Energy Transfer (FRET) microscopy

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Conclusions: Applying FRET microscopy, we confirmed that the PAS domain is required for heterodimerization of HIF-2α and ARNT. Also, no interaction between HIF-2α with the deleted PAS domain and HIF-1α could be demonstrated. The result of the FRET analysis between HIF-1α and HIF-2α could indicate a weak interaction between the two proteins. The reduction of HIF-1α target genes also suggests an interaction between the two subunits.

**OS5-06**

FIH regulates cellular metabolism through hydroxylation of the deubiquitinating enzyme OTUB1


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The asparagine hydroxylase, factor inhibiting HIF (FIH) confers oxygen-dependent regulation of transcriptional activity upon the hypoxia-inducible factor (HIF), a master regulator of the cellular adaptive response to hypoxia. Studies aimed at investigating whether asparagine hydroxylation is a more general regulatory post-translational modification identified multiple non-HIF targets for FIH including ankyrin repeat domain (ARD) containing proteins. However, the functional consequences of these modifications remain unclear. Here, we demonstrate that the deubiquitinating enzyme ovarian tumor domain containing, ubiquitin aldehyde binding protein 1 (OTUB1) is a substrate for hydroxylation by FIH at Asn22 which is located in a region important in the regulation of its activity. Mutation of the OTUB1 hydroxylation site leads to a profound increase in its interaction with proteins important in the regulation of cellular metabolism. Furthermore, overexpression of mutant OTUB1 (which lacks the here reported hydroxylation site) impairs cellular metabolic processes when compared to wild type OTUB1. Based on these data, we hypothesize that OTUB1 is a target for FIH-dependent hydroxylation, and that this provides new insight into the regulation of cellular metabolism under conditions of hypoxia.

**OS5-07**

Neuronal HIF-1α and HIF-2α deficiency impairs sensorimotor function in the subacute phase after ischemic stroke

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Question: Hypoxia-inducible factor (HIF) mediates adaptive responses to ischemia by induction of e.g. anti- and pro-apoptotic genes. However, the impact of HIF on neuronal survival upon stroke is controversial. Thus, we aimed to clarify its role during ischemic stroke by using neuron-specific knockout mice deficient for Hif-1α and Hif-2α.
Methods: Mice were exposed to global hypoxia to analyze HIF-α protein stability and HIF target gene expression in brain tissue. Stroke was induced by transient occlusion of the middle cerebral artery (MCAo) for 60 min (severe) and 30 min (mild). Upon reperfusion for 24 or 72 h, infarct and edema size were quantified and functional outcome was evaluated using a neurological deficit score and sensorimotor tests.

Results: In comparison to wild type, both Hif-1α- and Hif-2α-deficient mice showed no altered infarct and edema size upon mild or severe stroke, suggesting that HIF-1α compensates the loss of neuronal HIF-2α and vice versa. Accordingly, HIF target gene expression was only marginally compensated by Hif-1αdeficient mice not only exhibited significantly reduced expression of vascular endothelial growth factor (Vegf) and Bnip3, but also showed an overall trend of decreased HIF target gene expression under hypoxic conditions. Although we did not find any difference regarding global cell death or edema formation irrespective of mild or severe injury, behavioral assessment clearly indicated that Hif-1α/Hif-2α-deficient mice performed better than wild type littermates 24 h after MCAo, but subsequently became worse and were significantly more impaired 72 h after onset of reperfusion.

Conclusions: Our findings suggest that in neurons HIF-1 and HIF-2 have redundant functions for cellular survival under ischemic conditions. Moreover, the lack of proapoptotic Bnip3 and antiapoptotic erythropoietin (Epo), whose expression was mainly unaffected with exception of proapoptotic Bnip3 and antiapoptotic erythropoietin (Epo), whose expression was mainly induced by HIF-1 and HIF-2, respectively. In contrast, Hif-1α/Hif-2α double knockout mice showed no altered infarct and edema size upon mild or severe stroke, suggesting that HIF-1α compensates the loss of neuronal HIF-2α and vice versa. Accordingly, HIF target gene expression was only marginally compensated by Hif-1αdeficient mice not only exhibited significantly reduced expression of vascular endothelial growth factor (Vegf) and Bnip3, but also showed an overall trend of decreased HIF target gene expression under hypoxic conditions. Although we did not find any difference regarding global cell death or edema formation irrespective of mild or severe injury, behavioral assessment clearly indicated that Hif-1α/Hif-2α-deficient mice performed better than wild type littermates 24 h after MCAo, but subsequently became worse and were significantly more impaired 72 h after onset of reperfusion.

OS5-08
The unfolded protein response controls phosphorylation of eIF4E by ER stress
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The cap-binding protein eIF4E is upregulated in tumor cells and may contribute to malignancy. eIF4E provides the critical interface between mRNA, recruitment of eIF4A and eIF4G, and the 40S ribosomal subunit through binding to the 7-methyl guanosine cap structure. eIF4E is regulated at multiple levels, including transcriptionally, by phosphorylation of serine 209, and through inhibitory interactions with binding proteins. During tumorigenesis, the high proliferation rate of cancer cells and heterogenous microenvironmental conditions require increased activities of protein folding, assembly, and transport in the endoplasmic reticulum (ER), which can result in ER stress and activation of the unfolded protein response (UPR). This results in global translational arrest via eIF2α and selective induction of UPR genes by ATF4, ATF4 or XBP1. However, a link between eIF4E and the UPR is pending. We found that induction of ER stress by thapsigargin or tunicamycin did not substantially affect the overall levels of eIF4E, but increased eIF4E phosphorylation in a time-dependent manner in different tumor cell lines. eIF4E phosphorylation was mediated by MAPK-interacting kinases MNK. ER stress induced MNK expression via ATF4 and ATF6, but not XBP1. ER stress also increased MNK phosphorylation by p38 MAP kinase via IRE1 and by ERK via PERK and Raf1, but not by PI3 kinase/Akt. Subsequently, increased phosphorylation of eIF4E was instrumental for tumor cell survival and colony formation under ER stress. Collectively, these data show that MNK integrate various arms of the UPR to enhance phosphorylation of eIF4E resulting in stress resistance and progression of tumor cells.

OS6
Ion channels 2

OS6-01
Characterization of the modulatory CNGB1b subunit of olfactory CNG channels
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Olfactory cyclic nucleotide-gated (CNG) channels play an essential role in the sensory transduction of the olfactory and visual system. Native CNG channels are heterotetramers composed of three different subunits: two CNGA2, one CNGA4 and one CNGB1b. The presence of CNGA4 and CNGB1b subunits in heterotetrameric channels leads to an increased apparent affinity for cyclic nucleotides and accounts for the fast inhibition of native channels by Ca2+-CaM. The CNGB4 subunit is able to bind cGMP and actively participates in channel activation. Although the CNGB1b subunit has also an intracellular binding domain, the existence of ligand binding to this subunit is still unclear. We studied ligand binding to the CNGB1b subunit and the subsequent channel activation by means of mutagenesis and confocal patch-clamp fluorometry. The CNGB1b subunit was coexpressed with either CNGA2 only or with both, CNGA2 and CNGA4 in Xenopus oocytes and studied in inside-out patches. Our results show that CNGB1b subunit needs the presence of both CNGA2 and CNGA4 for ligand binding, indicating complex interactions of the subunits within the channel. The amount of total fluorescence per CNGA2:A4:B1b channel corresponds to that of the homotetrameric CNGA2 channel,
further confirming ligand binding to all four subunits. When coexpressing CNGB1b with disabled CNGA2 and CNGA4, channel activation induced by the CNGB1b subunit alone was only weak compared to CNGA4 coexpressed with disabled CNGA2. The determined apparent affinity \( EC_{50} \) of the CNGB1b subunit under the same conditions is fourfold higher than that determined for CNGA4 and 14 fold higher than that of one CNGA2. In addition, the kinetics of ligand binding and unbinding as well as channel activation and deactivation for each of the three different subunits in the respective environment of disabled subunits, substantiate the role of CNGB1b and CNGA4 in speeding up channel closure.

In conclusion we show that native olfactory CNG channels are activated by ligand binding to all four subunits and that the binding to the CNGB1b subunit initiates channel activation.

OS6-02
A novel HCN2 subunit modulates cardiac and thalamic pacemaker currents

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Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels encode cardiac and neuronal \( I_{h} \), \( I_{n} \), or \( I_{q} \) pacemaker currents. Trafficking and gating of HCN channels are modulated by cAMP, PIP2, and the auxiliary subunit Trip8b. Using a split-ubiquitin yeast-two-hybrid screen with HCN2 and a human brain cDNA library, we have identified the vesicle-associated membrane protein-associated protein B (VAPB) as a novel HCN channel subunit. Yeast-two-hybrid direct-interaction assays and GST-pull-down experiments confirmed the protein-protein interaction with HCN2. Co-expression experiments and TEVC recordings in Xenopus oocytes revealed a concentration dependent modulation of HCN1 and HCN2 current amplitudes. In contrast, using yeast-two-hybrid direct-interaction assays no interaction between VAPB and HCN4 was observed and VAPB modulation of current amplitudes was restricted to HCN1 and HCN2 isoforms. In situ hybridization experiments of mouse brain tissue and immunohistochemistry experiments with neonatal cortical neurons showed a co-expression of HCN2 and VAPB in many neuronal tissues. Spontaneous beating rate of HL-1 cardiomyocytes increased after VAPB transfection, while shRNA mediated knock-down of VAPB slowed the action potential frequency. Patch clamp recordings of HL-1 cells after VAPB transfection or after shRNA knock-down showed an influence of VAPB on the voltage-dependence of activation and the activation kinetics of \( I_{h} \). Morpholino antisense-oligonucleotide knock-down of VAPB in embryonic zebrafish and VAPB knock-out in mice resulted in reduced heart rates. Patch Clamp recordings of neurons in the ventrobasal thalamus (VB) indicate that VAPB modulates neuronal \( I_{h} \) current densities and voltage dependence. Thus, we propose that VAPB is a HCN1 and HCN2 channel subunit of native cardiac and thalamic pacemaker currents.

OS6-03
Disease-causing TRPV4 mutations affect the PAR-\( \gamma \)-mediated sensitisation of the channel

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Introduction: Three adjacent mutations (G270V, R271P, F273L) of the non-selective cation channel TRPV4 (transient receptor potential vanilloid 4) are associated with familial digital arthropathy-brachydactyly (FDAB). Interestingly, an adjacent TRPV4 mutation (R269H) leads to a completely different sensory nerve phenotype characterized by peripheral neuropathy. This suggests that these TRPV4 mutations affect channel activity in different ways. Mutations may not only alter baseline channel function but may also affect channel regulation. One important regulator for TRPV4 activity is the protease-activated receptor 2 (PAR\( \gamma \)) which mediates TRPV4 sensitisation. The aim of this study was to investigate whether TRPV4 mutations affect PAR\( \gamma \)-mediated sensitisation of the channel.

Material and Methods: Whole-cell currents were measured in Xenopus laevis oocytes expressing wild type (WT) or mutant TRPV4 with or without PAR\( \gamma \). The TRPV4 activator GSK1016790A (50 and 100 nM) was used to stimulate TRPV4 currents. PAR\( \gamma \) was activated by trypsin (8 nM) or human neutrophil elastase (3 \( \mu \)M). A biotinylation approach was used to investigate TRPV4 expression at the cell surface.

Results: Whole-cell current recordings demonstrated a loss-of-function effect of the three FDAB-mutations. Moreover, the FDAB-mutations reduced channel expression at the cell surface. In contrast, the adjacent R269H mutation had a gain-of-function effect on TRPV4 currents without detectable increase in channel surface expression. Interestingly, PAR\( \gamma \)-mediated TRPV4 sensitisation was significantly reduced and delayed by the three loss-of-function mutations. Notably, PAR\( \gamma \) activation did not further increase TRPV4 currents in oocytes expressing the gain-of-function mutation.
**Conclusion:** These findings demonstrate that three FDAB-causing TRPV4 mutations (G270V, R271P, F273L) have a loss-of-function effect which may be attributed at least in part to reduced channel expression at the cell surface. Moreover, reduced responsiveness to PAR2-mediated sensitisation may contribute to the loss-of-function effect of the FDAB mutations. Interestingly, PAR2-mediated TRPV4 sensitisation was not observed in oocytes expressing the R269H mutant channel suggesting that the gain-of-function effect mimics PAR2-mediated sensitisation. In summary, our results demonstrate that disease-causing TRPV4 mutations affect channel regulation by PAR2 (and possibly other GPCRs), which may contribute to the pathophysiology of TRPV4 channelopathies.

**Methods:** We profiled the expression of TRPC1-7 in PSCs isolated from TRPC6 channels expressed at the highest level. We then investigated the role of TRPC6 channels in PSCs isolated from WT mice in comparison with TRPC6-/- mice. Using qPCR, revealing TRPC6 channels to be one of the major effector proteins in an autocrine stimulation pathway triggered by hypoxia.

**Results:** We show that TRPC6 channels play a prominent role in PSC migration in response to hypoxia. Due to reduced autocrine stimulation, TRPC6-/- PSCs failed to increase their migration velocity and translocation to the same level as WT PSCs, when migrating on a 2D surface or within a 3D matrix under hypoxic and acidic conditions. This was also observed in the presence of PDGF. However, TRPC6-/- PSCs were still able to chemotax in a gradient of PDGF. We can therefore rule out a role of TRPC6 channels in the “steering” mechanism of PSCs response to PDGF. In line with these results WT but not TRPC6-/- PSCs showed an increased calcium influx under hypoxia, which is consistent with TRPC6-mediated Ca2+ signaling in PSC migration stimulated by hypoxia.

**Conclusions:** We can conclude that PSCs are activated by hypoxia. Loss of TRPC6 channels prevents activation of PSCs because they are major effector proteins in an autocrine stimulation pathway triggered by hypoxia.
Acid sensing ion channels (ASICs), Na⁺ permeable ion channels gated by protons, are members of the DEG/ENaC family. They are known to form homo- and heterotrimers with different functional properties depending on the subunits involved. Recent molecular imaging revealed that the ASIC1a/2a heteromer has a flexible 1:2/2:1 stoichiometry. However, the functional characteristics of the two heteromers remained unknown.

In this work we examined the electrophysiological properties of the two ASIC1a/2a heteromers. We covalently connected ASIC1a and ASIC2a subunits to a fixed stoichiometry of either ASIC1a-2a-1a or ASIC1a-2a-2a and investigated the functional properties of the two concatamers in Xenopus laevis oocytes.

The two concatamers had significantly different EC50 values and Hill coefficients for their apparent H⁺-affinity. Similarly, pH values and Hill coefficients of half-maximal steady-state desensitization were significantly different. In each case, the concatamer containing two ASIC1a subunits had higher apparent proton affinity, suggesting that both subunits in the heteromer determine proton affinity, such that a heteromer with two ASIC1a subunits is closer to ASIC1a wt and the one with two ASIC2a subunits closer to ASIC2a wt. Tachyphylaxis is a phenomenon characterized by reduced response to successive proton stimulations. So far, tachyphylaxis had been reported only for homomeric ASIC1a. Both concatamers did not show tachyphylaxis, indicating that indeed three ASIC1a subunits in the trimeric complex are necessary for this phenomenon. Millimolar concentrations and increased inward cation currents. Analyzing TRPC6-gene deficient podocytes we found no differences in the response to mechanostimulation compared to control podocytes, suggesting that mechanically induced currents were not mediated by TRPC6. Instead, mechanically induced currents were significantly decreased by the specific P₂X₄ blocker 5-BDBD indicating an involvement of P₂X₄ channels. Moreover, mechanical P₂X₄ channel activation was cholesterol and podocin dependent, but independent of the actin cytoskeleton. Since P₂X₄ channels are not intrinsically mechanosensitive, we investigated whether mechanical stimulation of podocytes leads to ATP release using a fluorimetric approach. Indeed, a mechanically induced ATP release from podocytes was observed. To determine the physiological relevance of P₂X₄ channels as mechanotransducing elements in podocytes we monitored reorganization of the actin cytoskeleton in the presence and absence of 5-BDBD. Interestingly, 5-BDBD could rescue stretch-induced disorganization of the actin cytoskeleton pointing to a significant involvement of P₂X₄ channels in the adaption of podocytes to mechanical stress. Altogether our findings reveal a new role of P₂X₄ channels as mechanotransducers in podocytes leading to mechanically-induced podocyte injury that may contribute to hypertensive nephropathy.
**OS6-08**

**Regulation of voltage gated K⁺ channel Kv1.5 by the Janus kinase JAK3**

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**Background/aims:** The tyrosine kinase Janus kinase 3 (JAK3) participates in the regulation of cell proliferation and apoptosis of lymphocytes and tumour cells. The kinase has more recently been shown to participate in the regulation of ion channels and transport proteins. The present study explored whether JAK3 contributes to the regulation of the voltage gated K⁺ channel Kv1.5, which participates in the regulation of diverse functions including atrial cardiac action potential and tumor cell proliferation.

**Methods:** cRNA encoding Kv1.5 was injected into Xenopus oocytes with or without additional injection of cRNA encoding wild-type JAK3, constitutively active A568VJAK3, or inactive K851AJAK3. Voltage gated K⁺ channel activity was quantified utilizing dual electrode voltage clamp and Kv1.5 channel protein abundance in the cell membrane utilizing chemiluminescence of Kv1.5 containing an extracellular hemagglutinin epitope (Kv1.5-HA).

**Results:** Kv1.5 activity and Kv1.5-HA protein abundance were significantly decreased by wild-type JAK3 and A568VJAK3, but not by K851AJAK3. Inhibition of Kv1.5 protein insertion into the cell membrane by brefeldin A (5 µM) resulted in a decline of the voltage gated current, which was similar in the absence and presence of A568VJAK3, suggesting that A568VJAK3 did not accelerate the retrieval of Kv1.5 protein from the cell membrane. A 24 hours treatment with ouabain (100 µM) significantly decreased the voltage gated current in oocytes expressing Kv1.5 without or with A568VJAK3 and dissipated the difference between oocytes expressing Kv1.5 alone and oocytes expressing Kv1.5 with A568VJAK3.

**Conclusion:** JAK3 contributes to the regulation of membrane Kv1.5 protein abundance and activity.

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**OS7**

**Renal function and transporters**

**OS7-01**

**Inducible glomerular erythropoietin production in the adult kidney**

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Hypoxia-inducible factor-2 (HIF-2) triggered erythropoietin production in renal interstitial fibroblast-like cells is considered as the physiologically relevant source of erythropoietin for regulating erythropoiesis. These cells convert into myofibroblasts during renal fibrosis and lose their capability to produce sufficient amounts of EPO leading to renal anemia. In order to find out if potential reserve cells for EPO production in the kidney might exist, we aimed to test the capability of non-epithelial glomerular cells to produce erythropoietin in vivo.

For this purpose HIF transcription factors were stabilized by cell specific deletion of the von Hippel-Lindau (VHL) gene via the Cre-loxP-system. On the one hand we used an inducible Cre deleter under the control of the connexin 40 (Cx40) promoter, which affects juxtaglomerular, mesangial and endothelial cells simultaneously. On the other hand we had mice expressing a Cre deleter only in one of the above mentioned cell types.

The inducible deletion of VHL in glomerular Cx40 expressing cells comprising endothelial, renin and mesangial cells, significantly increased glomerular EPO mRNA expression levels, plasma EPO concentrations and hematocrit values. These changes were mimicked by inducible cell specific VHL deletion in renin and in mesangial cells but not in endothelial cells. In mice with a co-deletion of VHL and HIF-2 these increases of erythropoietin production were absent. Moreover, we found that the induction of glomerular EPO expression was associated with a down-regulation of juxtaglomerular renin expression, again in a HIF-2 dependent manner.

These findings indicate that renin and mesangial cells have the capability to produce relevant amounts of erythropoietin and to suppress renin expression in the adult kidney when HIF-2 is stabilized.
We studied the effects of cholesterol and aquaporin-1 (AQP1) on CO₂ permeability (PCO₂) of lipid membranes by a stopped-flow technique. We measured the kinetics of CO₂ uptake of artificial phospholipid vesicles composed of PC/PS (molar ratio 8:2), to which either 0 or 50% cholesterol had been added. The change of intravesicular pH was followed spectrophotometrically using a pH dye present inside the vesicles. With a computer model, we investigated the effect of CO₂ partial pressure and intravesicular carbonic anhydrase (CA) activity on CO₂ uptake. These simulations show that i) CA activity has a critical influence on the kinetics of CO₂ uptake and has to be known exactly to obtain reliable CO₂ permeability values (PCO₂) from the experimental kinetics, and ii) CO₂ partial pressure has to be as low as possible to make the uptake process slow enough to be measurable by the stopped-flow technique. The mathematical model shows that even with optimal parameters the stopped-flow technique cannot distinguish between membrane permeabilities of 0.1 cm/s and 1 cm/s or higher, because with these PCO₂ values the kinetics becomes too fast and occurs mostly within the dead time of the instrument. In our experiments, we used a CO₂ partial pressure of 18 mmHg and CA activities between 15,000 and 66,000. In one experimental series, cholesterol reduced the rate constant of CO₂ uptake from 279 s⁻¹ ± SD=18 s⁻¹ (PC:PS:Chol = 8:2:0) to 155 s⁻¹ ± SD=7 s⁻¹ (PC:PS:Chol = 8:2:10) by about 45 %, which corresponds to an increase of the half time of CO₂ uptake from 2.5 to 4.5 ms. We used our computer model to determine PCO₂ from these half times and found a reduction of PCO₂ by cholesterol from ≥ 0.1 cm/s (PC:PS:Chol = 8:2:0) to 0.010 cm/s (PC:PS:Chol = 8:2:10). In another series, reconstitution of AQP1 into vesicles composed of PC:PS:Chol = 8:2:10 accelerated the CO₂ uptake process and increased the rate constant from 143 to 204 s⁻¹. This corresponds to a decrease of the half time from 4.8 to 3.4 ms. Thus, incorporation of AQP1 restored high membrane permeabilities, increasing PCO₂ from 0.01 cm/s by at least one order of magnitude to ≥ 0.1 cm/s.

**Conclusions:** CO₂ uptake by phospholipid vesicles can be followed by a stopped-flow technique. Cholesterol reduces PCO₂ of lipid membranes from high values of ≥ 0.1 cm/s to about 0.01 cm/s. Incorporation of AQP1 restores PCO₂ to its value in the absence of cholesterol. These data are in full agreement with own previous mass spectrometric measurements.

Furosemide is a loop diuretic that inhibits Na⁺ and Cl⁻ reabsorption in the thick ascending limb (TAL). Besides massive diuresis, furosemide also induces a pronounced urinary acidification. This is commonly explained by the increased Na⁺ delivery to the distal tubule, which is believed to stimulate H⁺ secretion by the V-type H⁺ ATPase in the α-intercalated cells. The direct role of the TAL on urinary acidification has never been investigated directly. We have previously reported that furosemide causes a NHE3-dependent H⁺ secretion in isolated perfused medullary TAL and here we wanted to investigate if this H⁺ secretion contributes to furosemide-induced urinary acidification in whole animals. Mice were anesthetized and the bladders were catheterized. Urinary net acid excretion (NAE) and urinary pH was determined during furosemide administration with or without the NHE3 specific inhibitor #4167.

In control animals, urine pH and NAE remained unaltered for the duration of the experiment. Furosemide administration caused a rapid onset of urinary acidification and reached the maximum H⁺ secretion after 20 minutes (14.89 ± 9.86 μM), which was reflected by a significant increase in NAE (ΔNAE 6.52 ± 2.02 μmol/h). When #4167 was given, a reduction in H⁺ secretion was observed (i.e. an alkalisation) but this did not cause a change in NAE. When both drugs were given together, the furosemide-induced H⁺ secretion and NAE were much attenuated compared to furosemide alone (H⁺ peak 2.93 ± 1.15 μM; ΔNAE 1.87 ± 0.66 μmol/h).

In conclusion, we show that inhibition of the apical NHE3 causes a significant drop in furosemide induced H⁺ and NAE excretion. These results indicate that furosemide stimulates H⁺ secretion in the TAL via the apical NHE3 and revise the current understanding of loop diuretic-induced urinary acidification.

**Modulation of NKCC2 function by phosphorylation**

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**Question:** The furosemide-sensitive Na⁺-K⁺-2Cl⁻-cotransporter (NKCC2) of the thick ascending limb (TAL) is modulated by N-terminal phosphorylation and dephosphorylation. It has been postulated that serine-threonine kinases activate, and that the phosphatase calcineurin deactivates the transporter. We investigated the effect of the calcineurin inhibitor cyclosporine A (CsA) on the equivalent short circuit current
The Na+/H+ exchanger (NHE1) determines the mechanical strength of melanoma cell-cell adhesion

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An early step in tumor cell metastasis is the detachment of cells from the primary tumor caused by loss of cell-cell adhesion. The strength of cell-cell adhesion might be an important indicator of tumor malignancy since low interactive adhesion forces might promote cell detachment, migration, invasion and metastasis. Tumor cell migration and invasion require a well-balanced cell-matrix adhesion. In this context, the Na+/H+ exchanger NHE1 was shown to affect cell-matrix adhesion via two independent mechanisms: (i) cytoskeletal anchoring and (ii) local pH-regulation. Based on this observation we raised the question whether the presence and/or the function of NHE1, represented by a change in the proton concentration at the cell surface, can modulate cell-cell adhesion.

Experiments were performed either on human melanoma cells (MV3) transfected with an NHE1 expression vector or on NHE1-deficient MV3 cells. As the respective controls empty vector and NHE1-deficient cells rescued by NHE1 transfection were included. The absolute adhesion force between single melanoma cells was quantified by mechanically separating individual cells using Atomic Force Microscopy (AFM). In addition, cell aggregation assays were performed to study the adhesive potential of the different cell clones in a cell cluster.

Our AFM experiments show that the cell-cell adhesion force was high in NHE1-deficient cells and low in NHE1-overexpressing cells compared to the respective control. This is consistent with the cell aggregation assays, where NHE1-overexpressing cells were no longer able to form stable tumor spheroids. Furthermore, an acidification of the extracellular medium also lowered cell-cell adhesion. Melanoma cell adhesion molecule, a prognostic marker of melanoma progression, was increased in NHE1-overexpressing cells. Thus, in melanoma cells, both NHE1 expression and extracellular pH affect the stability of cell-cell junctions. The observed effects are in contrast to the already known cell-matrix interactions in that here NHE1 and acidification reduce cell-cell adhesion. We therefore conclude that NHE1 promotes metastasis by first facilitating the cells’ detachment from the primary tumor. It then modulates cell-matrix interactions to promote cell migration and invasion.

Background: Genetic deletion or inflammation-associated downregulation of the PDZ-adaptor PDZK1/NHERF3 results in decreased transport activity and loss of cAMP-mediated regulation of the Na+/H+ exchanger NHE3 in the murine intestine, accompanied by increased enterocyte NHE3 mRNA expression levels, but a borderline reduced NHE3 protein expression in or near the apical membrane. This suggested to us that PDZK1 may play a role in targeting/stabilizing NHE3 in the enterocyte brush border membrane, as well as in cAMP-dependent signalosome formation.

Aim and Methods: To clarify the molecular details, we searched for an intestinal cell line that endogenously expresses PDZK1, and found the spontaneously differentiating cell line Caco-2BBBe to do so. VSVG-tagged NHE3 was stably expressed in Caco-2BBBe cells, and PDZK1 was stably knocked down by using recombinant lentiviral ShRNA followed by antibiotic selection. We then studied the effect of PDZK1 knockdown on NHE3 half life, brush border membrane expression, and transport function of NHE3.

Results: Biotinylation of membrane proteins, followed by Western analysis and immunocytochemistry revealed a significantly decreased amount of apical membrane NHE3, as well as mislocalization of NHE3 to endosomal/cyttoplasmic...
and basolateral pools, in PDZK1 knock down (KD) Caco2BBE/NHE3-VSVG. This correlated with a significantly decreased acid-activated NHE3 transport activity in the PDZK1 KD compared to control cells. Membrane half life studies for NHE3 were performed by following the degradation of biotinylated NHE3 over time. This revealed that control cells have a biphasic time course for NHE3 membrane loss, with a rapid phase, leading to approx. 50% loss of membrane NHE3 in the first 5 hours, followed by a slower phase with linear loss in the next 48 hours. In PDZK1 KD cells, the initial percentage of NHE3 that was expressed in the membrane was significantly lower than in control cells, and the early phase of rapid loss of membrane NHE3 was completely ablated while the second, slower phase was significantly more rapid than in control cells. At different NHE3 membrane expression levels (achieved by repeated acid-suicide selection cycles), application of the adenylate cyclase activator forskolin only inhibited NHE3 activity in controls, not in the PDZK1 KD cells.

Conclusions: The PDZ-adaptor protein PDZK1 is important for the targeting and/or retention of NHE3 to the brush border membrane of intestinal epithelial cells. In addition, PDZK1 plays a role in cAMP-mediated NHE3 inhibition, which requires the formation of the multiprotein complex. We speculate that PDZK1 binds to and targets PKA anchor proteins (AKAPs) to NHE3, either directly and/or via binding AKAP binding proteins such as NHERF1/2.

OS7-07
Towards the physiological role of the Cl/H+ exchanger CIC-3 in the brain
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CIC-3, a member of the CLC gene family, localizes to endosomes and was also proposed to localize to synaptic vesicles (SV) and synaptic-like microvesicles. Its close homology to the CI/H+-exchanger CIC-4 and CIC-5 and the presence of the ‘proton glutamate’ strongly argue for CIC-3 being an exchanger as well. Loss of CIC-3 in mice leads to severe neurodegeneration of the hippocampus and the retina. Synaptic vesicle-enriched fraction (LP2) from Clcn3-/- mice showed a decreased ATP-driven acidification compared to their WT counterparts suggesting that CIC-3 mediates vesicular acidification, but it has to be considered that LP2 fractions were isolated from brain with incipient degeneration. Indeed, recent work by others attributed impaired vesicle-acidification to a loss of VGLUT1 at that time and proposed the presence of CIC-3 on less than 0.05% of SV. However, the lack of CIC-3 antibodies suitable for immunohistochemistry and high resolution microscopy precluded the unambiguous characterization of its subcellular localization. Thus, the physiological role of CIC-3 in synaptic function remains obscure.

We now generated new knock-in mice in which CIC-3 was N-terminally tagged with Venus, a brightly fluorescent variant of GFP. Homozygous Clcn3Venus/Venus mice were born at expected Mendelian ratio and were phenotypically indistinguishable from their WT littermates suggesting normal expression and function of the Venus-CIC-3 protein. Immunolabeling of brain sections revealed intense signals throughout the cortex, cerebellum and the stratum pyramidale, stratum lucidum and hilus of the hippocampus. Closer examination revealed vesicular expression of Venus-CIC-3 in cell bodies of CA1 pyramidal neurons whereas the immunoreactivity in CA3 was also strong in the stratum lucidum, a region that harbors extensive synaptic contacts. In the cerebellum Venus-CIC-3 was expressed exclusively in somata of Purkinje cells. Co-immunolabeling of primary hippocampal neurons showed that Venus-CIC-3 does neither localize to the postsynaptic, nor to excitatory or inhibitory SV but was largely found on EEA1 and TIR positive endosomal compartments within cell bodies and apical dendrites. Additionally, overexpressed CIC-3fusion in hippocampal neurons did not undergo exocytotic fusion after electrical field stimulation.

Furthermore, we confirmed that ATP-dependent acidification of Clcn3-/- LP2 fraction was indistinguishable from wild-type controls at two weeks of age whereas it became progressively impaired with increasing age and progressing neurodegeneration. These new findings question the proposed function of CIC-3 on SV. Explaining the mechanism by which loss of CIC-3 function causes neurological pathologies are challenging tasks for future investigations.

OS7-08
Molecular basis of voltage-dependent gating in CLC transporters
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The CLC family encompasses CI channels and coupled CI/H+ exchangers. CLC channels and transporters both exhibit voltage-dependent gating, the physiological importance of which is illustrated by multiple disease-causing mutations that specifically result in altered voltage sensing. Despite a large body of available functional and structural data, the molecular mechanisms underlying voltage gating in CLCs still remain unclear. Using electrophysiological admittance measurements, we recently decomposed voltage-dependent activation of the human CIC-5 transporter into multiple discrete electrogenic transitions (Grieschat M & Alekov AK (2014) Biophys J 107, L13-L15). A key player of the gating machinery appears to be the so-called gating glutamate (Glu148 in EcCIC), which is thought to move upon changes in voltage. To better understand the basis of voltage-dependent gating, we conducted molecular dynamics simulations of the bacterial homologue EcCIC and developed a novel method for gating charge calculations. Using a double-biayer simulation system, we applied small ionic concentration gradients across the membrane that resulted in charge imbalances ΔQm and computed the resulting potential differences Vm. Analysis of the dependence of Vm,
on $\Delta q_{\text{ion}}$ for various conformations that differed in $\text{Cl}^-$ binding site occupancy and the protonation state of Glu ext was used to determine charge displacements of the transporter along the electric field. Multiple processes, including protonation and conformational changes of Glu ext, binding of $\text{Cl}^-$ ions and refocusing of the electric field triggered by changes in water accessibility are associated with charge transfer across the membrane and therefore exhibit intrinsic voltage dependence. We calculated gating charges that underlie these conformational transitions of the CLC transport cycle. Based on these calculations, gating charge recordings and capacitance measurements on ClC-5, we propose a molecular description of CLC voltage sensing which attributes fractional gating charges to these partial reactions of the transport cycle.

OS8
Sensory and motor systems

OS8-01
High stimulus-related information in inhibitory interneurons of the adult rat barrel cortex in vivo

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A fundamental goal in neuroscience is to understand how sensory information is represented in the central nervous system. In this regard, the manner in which populations of inhibitory (INH) and excitatory (EXC) neocortical neurons collectively encode stimulus-related information is a central, yet still unresolved question. Here we address this question by simultaneously recording with large-scale multi-electrode arrays (of up to 128 channels) the activity of cell ensembles (of up to 74 neurons) upon sensory stimulation in the anesthetized adult rat somatosensory cortex in vivo. The recorded cells were distributed along all layers of 3-4 neighboring cortical columns, and further classified as putative INH and EXC neurons according to their distinct extracellular spike waveforms. Using two different whisker stimulus modalities (of up to 74 neurons) upon sensory stimulation in the anesthetized adult rat somatosensory cortex in vivo. The recorded cells were distributed along all layers of 3-4 neighboring cortical columns, and further classified as putative INH and EXC neurons according to their distinct extracellular spike waveforms. Using two different whisker stimulus modalities (of up to 74 neurons) upon sensory stimulation in the anesthetized adult rat somatosensory cortex in vivo. The recorded cells were distributed along all layers of 3-4 neighboring cortical columns, and further classified as putative INH and EXC neurons according to their distinct extracellular spike waveforms.

OS8-02
Exercise-induced hypoalgesia differentially affects deep pain, cutaneous pain and innocuous somatosensation

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Questions: Aerobic exercise has a generally accepted clinical relevance in terms of pain relief. However, it remains unclear whether exercise-induced hypoalgesia affects superficial skin pain and deep muscle pain, and whether tactile perception is compromised as well. Hence, this study focused on putative differential modulation of cutaneous vs. deep pain as well as differential modulation of non-nociceptive vibratory and tactile processing.

Methods: 68 healthy volunteers participated in a randomized study design. It consisted of an exercise session and a control session on a cycle ergometer against resistance and no resistance of 20 min each on two different days. Both sessions were performed under prerequisite of heart rate controlled exercise intensity. Under control conditions, exercise intensity was adjusted to heart rate increase by 15-25% of the individual resting pulse. Under exercise conditions, exercise intensity was adjusted to heat rate increase by 65-77% according to clinically relevant exercise intensities. Psychophysical testing was performed on the dominant hand 10 min before, 10 and 20 min during and 10 and 20 min after exercise. Psychophysical parameters were pressure pain threshold (thenar muscle), mechanical pain (skin of hand dorsum), mechanical detection threshold (skin of hand dorsum) and vibration detection threshold (stloyd process of radius). Borg CR10 ratings were collected for documenting both exercise intensity and heart rate controlled cycling conditions. Psychophysical parameters underwent 2-way repeated measures statistical analysis with condition time and interaction as factor A and factor B as well as interaction between factors with Holm-Sidak post-hoc testing. Borg CR10 ratings underwent Wilcoxon signed rank-t-test analysis.

Results: Muscle pain threshold increased in the exercise session (interaction: $p<0.001$) exclusively during cycling (5 min: $p<0.001$; 15 min: $p<0.001$). There was no significant interaction between factors for both cutaneous, mechanical...
detection and mechanical pain perception thresholds. Vibratory perception threshold was impaired in the exercise session (p<0.001) during cycling (5 min: p<0.001; 15 min: p<0.001) and after cycling (5 min: p<0.05). Borg CR10 ratings were higher in the exercise session (p<0.001).

Conclusions: Aerobic exercise induced differential, modulation of somatosensation and pain in healthy volunteers. Myofascial hypoalgesia could reflect a right-shift of stimulus-response function. This provides further arguments for implementation of aerobic exercise in musculoskeletal disorders.

**OS8-03**

**TRPA1-protein complexes: tuning a noxious stimuli detector**

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**Question:** The transient receptor potential A1 (TRPA1) channel is essential for vertebrate pain. TRPA1 plays an essential role as a polymodal detector for potentially harmful chemicals, noxious cold and tissue damage. Even though TRPA1 activation modalities have been studied extensively, the network of protein interactions regulating TRPA1 is only poorly understood. Considering the crucial role of TRPA1 in pain signaling, we are in urgent need to shed light on the elusive molecular machinery regulating TRPA1 channels in sensory neurons, which might represent a key regulator of TRPA1 membrane abundance and function. To test this hypothesis we used an unbiased proteomics-based approach aimed to uncover and characterize a novel TRPA1 binding partner and study its role for nociceptive signaling.

**Methods:** We first identified candidate TRPA1 interactors by affinity purification of native TRPA1 channels from mouse sensory neurons. Whole-cell lysates were obtained by detergent solubilization and endogenous TRPA1-protein complexes were affinity purified using specific TRPA1 antibodies. Bound proteins were then analyzed by tandem mass-spectrometry (MS/MS) to obtain a list of potential interacting partners. A selected candidate was subsequently validated using a combination of immunohistochemistry, live labeling of surface TRPA1 channels, electrophysiology, calcium imaging, and mouse behavior.

**Results:** We uncovered the physical association of Annexin A2 (AnxA2) with native TRPA1 in mouse sensory neurons. AnxA2 is enriched in a subpopulation of sensory neurons and co-expressed with TRPA1. Furthermore, we observed an increase of TRPA1 membrane levels in cultured sensory neurons from AnxA2-deficient mice. This was reflected by our calcium imaging experiments revealing higher responsiveness upon TRPA1 activation in AnxA2-deficient neurons. In vivo these findings were associated with enhanced nociceptive behaviors specifically in TRPA1-dependent paradigms of acute and inflammatory pain, while heat and mechanical sensitivity as well as TRPV1-mediated pain were preserved in AnxA2-deficient mice.

**Conclusions:** We identified AnxA2 as a novel TRPA1-associated protein that regulates TRPA1 channels in vitro and in vivo. These results demonstrate a role for AnxA2 as an endogenous modulator of TRPA1 activity and shed light on a mechanism capable of controlling TRPA1-mediated nociception in vertebrates. These findings underscore the idea that TRPA1 surface availability is controlled by protein-protein interactions and influences nociceptive signaling in sensory neurons. Future studies regarding dynamic changes of TRPA1-associated proteins in different pain states (e.g. acute vs inflammatory) could therefore open the possibility to develop targeted therapeutics for TRPA1-related pain disorders.

**OS8-04**

**Visualizing dopamine mediated signaling in the retina on the basis of second messengers**

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The retina enables vision in different light conditions: from bright sunlight to a starry night. The retina manages this task by adapting to different light conditions. These adaptation processes are mediated by a variety of neurotransmitters and second messengers. One neurotransmitter mainly involved in these adaptation processes is the catecholamine dopamine (DA). In the retina, DA is released from a single cell type: the tyrosine hydroxylase (TH) positive amacrine cell. Downstream signaling of DA involves cAMP mediated pathways through regulation of adenyl cyclases. It is also discussed whether dopaminergic signaling is connected to changes in [Ca\(^{2+}\)]. Here, we want to contribute to a better understanding of DA’s role in light adaptation on the basis of the second messengers cAMP and Ca\(^{2+}\).

Using immunohistochemistry, we first investigated the expression of different DA receptors in vertical cryo-sections of adult C57BL/6 retina. We found that D1 receptors are expressed in two types of ON-bipolar cells (type 5 and type 7). D2 receptors are mainly expressed in rod bipolar cell terminals in the inner plexiform layer. For both receptors we could show expression on ganglion cell dendrites. We, therefore, investigated whether the activity of retinal ganglion cells is influenced by DA. For this purpose, we use a transgenic mouse line expressing the FRET-based Ca\(^{2+}\)-sensor TNL-15 in retinal ganglion cells. The mouse retina comprises 12-15 types of ganglion cells. We found that different ganglion cells respond to application of DA in different ways, showing either an increase in [Ca\(^{2+}\)], a decrease in [Ca\(^{2+}\)], a biphasic response or no response.

In order to monitor DA triggered cAMP signaling, we utilize the genetically encoded and FRET-based cAMP-sensor EPAC (Nikolaev et al., 2004). We transiently expressed EPAC in HEK 293 cells and in cultures of mouse retinal dissociated neurons. EPAC visualized increases in the internal cAMP concentration upon direct pharmacological activation of adenyl cyclases and upon stimulation of D1 receptors by DA. In future experiments we plan to utilize EPAC to further unravel dopaminergic signaling via the cAMP pathways in retinal neurons.
Plectin is a large ubiquitous versatile cytoskeletal linker protein. In muscle cells, four different plectin isoforms exist, which, together with desmin, form lateral linkages between myofibrils, mitochondria, the outer endoplasmic reticulum-nucleus system and the sarcolemma. Due to its interactions with actin and the intermediate filament desmin and its localization near the muscle Z-discs, the plectin 1d isoform has been proposed to connect individual myofibrils to each other by transversal linkages via the desmin network. Structural models depict plectin 1d to perform this connection by encircling the Z-discs of individual myofibrils. However, because of the small diameter (<1µm) of individual Z-discs, this notion was rather obtained from plectin biochemical interactions and histological features of plectin-deficient muscle than from direct structural visualizations. This is because diffraction-limited confocal microscopy merely reveals a close localization of plectin, desmin and a-actinin but does not resolve their distinct spatial distribution. Here we take use of the super resolution provided by STED microscopy (lateral resolution: 50nm) and GSD microscopy (lateral resolution: 20 nm, axial resolution: 50 nm) for visualizing the three-dimensional distribution of the three proteins by indirect immunofluorescence within thin (~3µm diameter) myofibrillar bundles isolated from mouse psoas muscle. 3D-GSD-images show that in axial direction of the bundles, plectin is located in thin layers which are well aligned with a-actinin, meaning that plectin is specifically aligned with the Z-disc and not with I bands. In transversal direction, a-actinin neither overlaps with plectin nor with desmin. Instead, both plectin and desmin are located in transversal gaps between the single, less than 1µm wide a-actinin discs, confirming the role of plectin and desmin as linkers located between the individual myofibrils. 3D-STED images visualize that plectin encompasses single a-actinin discs at their periphery as has been postulated in the structural models derived from the biochemical interactions of plectin. Our work establishes super resolution microscopy as a valuable tool for resolving the architecture of sarcomeric and intermyofibrillar proteins in the muscle structure. In combination with corresponding mechanical force measurements of myofibrillar bundles from plectin-knockout and wildtype mice, the information from 3D super resolution microscopy will advance the understanding of the biomechanical function of plectin for muscle stability and muscle contraction.
ORAL SESSIONS

OS8-07
Skeletal muscle fiber type shift depends on extracellular matrix remodeling in rat model of physiological loading
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**Question:** The extracellular matrix (ECM) is a structural entity that comprises a variety of different molecule classes and families. In skeletal muscle tissue the ECM is critically involved in muscle integrity regulation, since the ECM serves as a critical sensor of mechanical stresses able to transduce these stimuli into biochemical signals that mediate muscle adaptations. In this context it has been shown that physical exercise, as a main source of mechanical stress production in skeletal muscle tissue, bears the potential to alter the composition of the ECM, while it remained unspecified what physiological consequences are connected to changes in the ECM.

**Methods:** We used Sprague Dawley rats (age 7wks at the experimental start) that were assigned to the following groups: age-matched control (Con, sedentary, n=8), concentric muscle contractions (Level, 6wks level running at 0° on a treadmill, v=18m*min⁻¹, 30min*d⁻¹, 5d*wk⁻¹), eccentric muscle contractions (Dh, 6wks downhill running at -20° on a treadmill, v=18m*min⁻¹, 30min*d⁻¹, 5d*wk⁻¹). ECM components were analyzed by qPCR, western blot (WB) and immunofluorescence (IF) in M. gastrocnemius. Signaling pathways were also analyzed by WB. Myosin heavy chains were analyzed by WB and ATPase/immunohistochemistry.

**Results:** While qPCR did not indicate significant changes in ECM components, e.g. collagen types I and III, WB data showed significant reductions in collagen types I and III protein levels after Level and Dh compared to Con conditions. Interestingly, type II myosin heavy chains were increased, while type I decreased in both Level and Dh compared to Con conditions with additionally significant differences between Level and Dh. Related signaling pathways, e.g. MAP kinases, were regulated along with changes in myosin heavy chains.

**Conclusion:** These data show that chronic concentric, but specifically chronic eccentric muscle contractions result in significant modifications of ECM collagen compositions. Interestingly, these changes go along with severe alterations in myosin heavy chain compositions and related signaling cascades in M. gastrocnemius. Our data thus indicate that changes in the ECM exert important physiological functions in skeletal muscles and hence are critical for muscle function, also under physiological circumstances. These coherences will be studied in more detail using different knockout mouse models in the future.

OS8-08
Speed of shortening is not altered in the alpha-actinin-3 (ACTN3) 'gene of speed' knock-out mutation in fast-twitch skeletal muscle
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~20 % of the world population shows a null polymorphism in the ACTN3 gene (R577X) with absence of the cytoskeletal protein alpha-actinin-3 in fast-twitch (IIx) muscle. Although not presenting with any known disease phenotype, this mutation is associated with a shift in metabolic profiles of Actn3 null fast fibres from a predominantly anaerobic to an aerobic production of ATP, predisposing mutation carriers to endurance over sprint performance. However, it does not necessarily mean that biomechanical properties of fast-twitch fibres switched to slow-twitch profiles as evident in muscles from Actn3 KO mice, e.g. histochemical fiber typing along with isometric contractions in fast-twitch muscle (Chan et al. 2011, Am J Physiol Cell Physiol). Although those studies showed no change in myosin isoform expression, pCa-force curve or isometric twitch kinetics, direct confirmation of unchanged unloaded speed of shortening was still missing. Here, we recorded unloaded speed of shortening in single intact fast twitch FDB muscle fibres from wt and Actn3 KO mice using a CMOS high speed camera upon external field-stimulation in response to single twitch and between 10 Hz and 100 Hz tetanic contractions at frame rates up to 4,000 fps. Minimum shortening length and maximum speed of shortening did not significantly differ between wt and Actn3 KO fibres at any stimulation frequency (e.g. ~5.5 mm/s @ single twitches, ~6.5 mm/s @ 100 Hz stimulations). Thus, lack of alpha-actinin-3 in fast-twitch muscle results in normal contraction kinetics despite a metabolic shift towards slow-twitch fibre aerobic ATP production pathways.
Both, cancer progression and immune disease are associated with aberrant glycosylation. Store-operated Ca\(^{2+}\) entry (SOCE) is impaired in immune cell malfunction and different types of cancer.

This study demonstrates that Orai1 is N-glycosylated with a cell type specific band pattern and variable degree of alpha 2-6 linked sialic acids. Sialylation of Orai1 by sialyltransferases (ST6Gal) regulates Ca\(^{2+}\) entry in Jurkat T-cells. The glycodeficient mutant of Orai1 (N223A) mediates increased SOCE and Ca\(^{2+}\) release activated Ca\(^{2+}\) current (I\(_{\text{CaSO}}\)) compared Orai1 wild type. This phenotype is absent in a glycodeficient cell system. Ca\(^{2+}\) signaling during formation of an immune synapse also depends on glycosylation of Orai1. Sialic acid-binding immunoglobulin-type lectins (Siglecs) regulate SOCE Ca\(^{2+}\) signaling in human mast cells as knockdown of siglec-6 and siglec-8 significantly increases SOCE.

**Background:** Platinum-based drugs are a common use in cancer therapy but linked to various side effects like neurotoxicity, which is often accompanied by neuropathic pain. It has been suggested that death of dorsal root ganglion (DRG) neurons by oxaliplatin is promoted by a critical increase of the density of VGCC currents, suggesting changes in expression or function of ion channel proteins. This provides evidence of a crucial role in oxaliplatin-based neurotoxicity.

**Methods:**
- Live cell calcium imaging & functional analysis
- Western blotting
- qRT-PCR
- Delivery of Orai1

**Results:** Oxaliplatin modulates VGCC currents in small DRG neurons.

**Conclusion:** In contrast to our hypothesis, oxaliplatin reduces VGCC currents during acute treatment. Nevertheless, incubation of DRG neurons with oxaliplatin led to an increase of the density of VGCC currents, suggesting changes in expression of ion channel proteins.
Conclusions: We provide evidence for LEFTY-A activity in the endometrium of human and mice. LEFTY-A impairs embryonic implantation by perturbing regulation of pregnancy associated genes and/or down-regulation of Orai1, SOCE and COX2. The LeftyA/Orai1 axis is a promising target for fertility control as well as for the prevention of implantation failure, especially in the context of assisted reproductive technologies.

P004 Subtype-dependent modulation of voltage-gated calcium channels in small dorsal root ganglion neurons by cisplatin
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Background: Platinum-based chemotherapy is essential for the treatment of various types of cancer. In addition to their high effectiveness, cisplatin leads to various side effects during chemotherapy.

Methods: Isolated DRG neurons (<25 µm) from 3 weeks old Wistar rats were cultured for 24 h. Using the whole-cell patch-clamp technique, VGCC currents were isolated. By using specific ion channel modulators, isolation of L-, N- T- and P/Q-type VGCC currents was performed.

Results: Acute treatment with cisplatin on small DRG neurons led to dose-dependent (0.01µM - 50 µM) reduction of VGCC currents (13-77 %) in a voltage range of -60 mV to +50 mV. Subtype isolation of VGCC currents showed differential effects on VGCC subtypes. N-type current densities were increased by 26.1±2.99 % during a depolarization to 0 mV. Incubating DRG neurons with cisplatin (0.5 and 5 µM) for 24-48 h led to a significant increase in current density.

Conclusion: These results provide evidence for a subtype specific effect of platinum-based drugs on VGCC in DRG neurons suggesting a crucial role in causing neurotoxic side effects during chemotherapy.

P005 Orai2 regulates store-operated Ca²⁺ entry and hypoxia-induced Ca²⁺ accumulation in cortical neurons
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Question: Store-operated Ca²⁺ entry (SOCE) is a Ca²⁺ influx mechanism that is triggered by depletion of intracellular Ca²⁺ stores. In neurons, the molecular composition and biological function of SOCE is not fully understood. Previously, we found a major contribution of the endoplasmatic reticulum Ca²⁺ sensor STIM2 to SOCE and ischemia-induced Ca²⁺ overload in cultured cortical neurons. However, the role of putative SOCE channels in the plasma membrane of neurons remained to be clarified.

Methods: The expression of the putative SOCE channel subunits Orai1-3 in neuronal and glial cultures was analyzed by quantitative PCR. We performed fura-2 Ca²⁺ imaging in neuronal and glial cultures from cortical tissue of wildtype and Orai2−/− mice. SOCE was induced by application of cyclopiazonic acid and ischemia-like conditions by oxygen-glucose deprivation (OGD).

Results: Orai2 is the dominant isoform in neurons but not in astrocytes (Orai3) and microglia (Orai1). SOCE as well as OGD-induced Ca²⁺ accumulation was significantly reduced in Orai2−/− neurons. In astrocytes and microglial cells, ablation of Orai2 did not affect SOCE.

Conclusions: Our data show that Orai2 contributes to SOCE in cortical neurons but not in glial cells. As previously shown for STIM2, Orai2 is required for hypoxic Ca²⁺ load suggesting a role in neuronal death during cerebral ischemia.

P006 A concerted action of L-type and T-type calcium channels regulates pacemaking in neurons of the locus coeruleus
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Loss of noradrenergic locus coeruleus (LC) neurons is an early hallmark of Parkinson’s disease (PD). LC neurons are rhythmic pacemakers responsible for a sustained release of noradrenaline to different structures, as for instance the substantia nigra pars compacta. The analysis of ion channels underlying the electrical activity of LC neurons, which is ultimately coupled to cell survival signaling pathways, can lead to a better understanding of the vulnerability of these neurons during PD. In LC neurons the pacemaking is accompanied by somatodendritic calcium oscillations which are attributed to the opening of L-type calcium channels (e.g. Cav1.3). However, Cav1.3 channels do not directly influence the spiking frequency. This activity dependent calcium influx is linked to mitochondrial oxidant stress. However, the functional impact of low threshold activated T-type calcium channels was not studied yet. In our current study we
Tumor necrosis factor-α (TNF) is a proinflammatory cytokine, which is involved in the development and maintenance of inflammatory and neuropathic pain. Its effects are mediated by two types of receptors, TNF-receptor 1 (TNFR1) and TNF-receptor 2 (TNFR2). TNFR1 plays a crucial role in the sensitization of tetrodotoxin (TTX) resistant sodium channels by TNF while TNFR2 is not involved in this process. Using sensitization of tetrodotoxin (TTX) resistant sodium channels by TNF-receptor 1 and 2 (TNFR1 and TNFR2), TNF promotes excitation in primary afferent neurons and might help to explain the sensitization mechanisms after nerve injury in neuropathic and inflammatory pain.

**P007**

**Modulation of voltage-activated sodium channels by activation of tumor-necrosis factor receptor 1 and 2**

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Tumor necrosis factor-α (TNF) is a proinflammatory cytokine, which is involved in the development and maintenance of inflammatory and neuropathic pain. Its effects are mediated by two types of receptors, TNF-receptor 1 (TNFR1) and TNF-receptor 2 (TNFR2). TNFR1 plays a crucial role in the sensitization of tetrodotoxin (TTX) resistant sodium channels by TNF while TNFR2 is not involved in this process. Using the whole-cell patch-clamp technique we examined the influence of TNFR1 and TNFR2 on voltage activated sodium channels (VASC) and TTX resistant NaV1.8 channels in isolated rat dorsal root ganglion neurons by using selective TNFR agonists. The TNFR1-agonist D145 (10pg/ml) led to an increase of normalized currents of VASC ($I_{NaV1.6}$) of 40%, while the TNFR2 Agonist R32W (10pg/ml) increased the current by 30%. Isolating NaV1.8 R32W application (100pg/ml) resulted in an increase of $I_{NaV1.8}$ by 19% while D145 application (100pg/ml increased the current by 17%). In current-voltage relation application of 10pg D145 or R32W led to an increase of the current in a bell-shaped voltage dependent manner with a maximum effect at -30 mV. The effects of TNFR on VASC promote excitation in primary afferent neurons and might help to explain the sensitization mechanisms after nerve injury in neuropathic and inflammatory pain.
**P009**
Expression of alternative transcripts of the cardiac L-type Ca\(^{2+}\) channel in primary cultured rat left ventricular cardiomyocytes

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Cacna1c, the gene encoding the α\(_{1c}\) subunit of the cardiac L-type Ca\(^{2+}\) channel, is subjected to extensive alternative splicing. In addition, transcription can be initiated at different promoters leading to the generation of alternative Cacna1c transcripts differing in their 5'-region: one containing exon1a which is predominantly expressed in the heart and the other one containing the more ubiquitously expressed exon 1b (accounting for ~10% of cardiac Cacna1c). Moreover, alternative transcript expression might participate in the regulation of L-type Ca\(^{2+}\) current properties. In the present study we therefore investigate the expression of alternative transcripts or splicing variants of Cacna1c during primary culture of isolated cardiomyocytes.

Isolated rat left ventricular myocytes were plated on poly-L-Lys covered six-well tissue culture dishes and incubated at 37°C. Expression levels of Cacna1c mRNA were investigated using quantitative real-time RT-PCR with exon-specific intron-overspanning primers (for exons 1a, 1b, 5, 8, 8a, 9*) after 0, 24h and 48h incubation and normalized to the expression of the house-keeping gene β-actin.

The expression level of exon1a remained stable during 24-48 h of incubation (n=9) as well as the expression level of exon 5, which is not subject to alternative splicing and can therefore be considered as marker for total Cacna1c mRNA. The expression of exon1b substantially increased after 24 h to 403±61% of its initial value (p<0.001) and after 48h to 550±53% (p<0.001, n=8). The incubation did not increase the expression levels of alternative non-cardiac specific splicing variants of Cacna1c containing exon 8 or exon 9*. The expression of two fibroblast markers (vimentin and discoidin domain receptor 2) was either stable or decreased during the incubation indicating that the increased expression of exon 1b is not caused by an increased number of fibroblasts. Continuous pacing of cardiomyocytes at 1Hz for 48h attenuated the increase of exon1b expression (301±58% vs. 517±59%, p<0.05, n=8).

In conclusion, non-paced primary culture of isolated cardiac myocytes leads to a substantial alteration in the expression pattern of alternative transcripts of Cacna1c.

**P010**
Function of the Ca\(_{\beta}\) subunit of voltage-gated Ca\(^{2+}\) channels in intracellular trafficking

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Voltage-activated calcium channels (VACC) are hetero-multimers formed by an α, pore-forming subunit (Ca\(_{\alpha}\)) that associates with one or more accory subunits, including the β-subunit (Ca\(_{\beta}\)). VACC open by membrane depolarization and allow the selective entry of calcium that acts as second messenger triggering a variety of cellular functions. In order to coordinate all these processes while preventing calcium toxic buildup VACC are tightly regulated in time and space. The amount of calcium entering into the cell can be controlled by altering the effectiveness of the ion permeation through the channel pore and/or the number of channels in the surface membrane. Ca\(_{\alpha}\) facilitates the voltage-dependent opening of the channel and its delivery to the plasma membrane.

Here we investigated the trafficking pathway of the channel complex toward the plasma membrane using live-cell conventional and spinning disk confocal microscopy, and single molecule localization microscopy (SMLM) in fixed cells. We observed that the L-type cardiac Ca\(_{\alpha}\), isoform, namely Ca\(_{1\,2}\), expressed alone in HEK293 cells is retained in the endoplasmic reticulum (ER) while coexpression with Ca\(_{\beta}\) restores ER export. Pharmacological disruption of the actin as well as of the tubulin-based cytoskeleton inhibits targeting of Ca\(_{\alpha}\) to the plasma membrane. We found that the release of the channel from the ER requires an intact tubulin cytoskeleton and that inhibition of actin polymerization results in channels distributed throughout the intracellular trafficking machinery. SMLM images using fluorophore-conjugated antibody staining show that Ca\(_{1\,2}\) distributes along tubulin filaments in HL-1 cardiac derived tumor cells.

Our results suggest that during their itinerary toward the plasma membrane VACCs appear to travel over long distances along tubulin filaments while, prior to membrane insertion, short-range movements take place along actin filaments.

**P011**
The regulation of mature dentate gyrus granule cells intrinsic excitability by voltage-gated T-type calcium channels

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T-type calcium channels are key regulators of neuronal excitability due to their sensitivity to subthreshold voltage changes and to their permeability to calcium which can act as a depolarizing drive on membrane potential as well as an intracellular messenger. The dentate gyrus is the main gateway for neocortical sensory inputs to hippocampus and one of the very few regions in mammalian brain where adult neurogenesis takes place. Intriguingly, T-type calcium channels are broadly expressed in the dentate gyrus and have been reported to be one of the main determinants of the higher excitability of immature granule cells compared to their mature counterparts. In this study, we aimed to characterize the role of T-type voltage-gated calcium channels in regulating intrinsic excitability of mature granule cells, which compose the vast majority of the global population of granule cells.

For this, we performed electrophysiological recordings of mature granule cells using patch-clamp technique and imaging of calcium signals using two-photon microscopy on
Methods: CaV1.3 subunits and Rab27a were heterologously expressed in mammalian cell cultures and co-cultained with VEGF-A secretion was analyzed by ELISA. CaV1.3 channel and Rab27a over-expression at the basolateral side of the RPE. Rab27a over-expression at the basolateral side of the RPE. Rab27a co-expression reduced surface expression of CaV1.3 subunit. After deleting the MyRIP homologous domain between the cytosolic loop and the first homologous repeat of the 2nd transmembrane domain of the CaV1.3 subunit, co-immunoprecipitation was lost. Our results suggest that T-type calcium channels are present in mature granule cells, are non-uniformly distributed among dendritic and axonal membrane and contribute to the control of neuronal firing patterns. Further experiments will explore the specific functions of dendritic and axonal T-type calcium channels, as well as their modulation by plasticity phenomena.

Results: Co-localization of both Rab27a and CaV1.3 was lost. We detected co-immunoprecipitation of Rab27a and CaV1.3 pore-forming subunit. After deleting the MyRIP homologous domain between the cytosolic loop and the first homologous repeat of the 2nd transmembrane domain of the CaV1.3 subunit, co-immunoprecipitation was lost.

Conclusion: We conclude that Rab27a modulates activity of CaV1.3 channels via direct interaction with the pore-forming subunit at a MyRIP homologous domain resulting in changed voltage-dependence and surface expression. We show the first evidence of the direct functional modulation of an ion channel by a Rab27a protein suggesting a new mechanism in the control of exocytotic processes such as VEGF-A secretion in the RPE.

Question: Exocytosis and endocytosis are regulated by the combined activity of the small GTPase Rab27a and L-type Ca2+ channels of the CaV1.3 subtype. Both, CaV1.3 channels and Rab27a are expressed in the retinal pigment epithelium (RPE) which is able to secrete a variety of factors such as VEGF-A. Thus we investigated whether direct modulation of CaV1.3 channel/Rab27a interaction modulates secretion.

Methods: CaV1.3 subunits and Rab27a were heterologously expressed in CHO-K1 cells. Immunoprecipitation and Western Blot were carried out to detect protein interaction. CaV1.3 currents in CHO-K1 and ARPE-19 cells (endogenous CaV1.3 expression) and Rab27a were recorded by the whole-cell configuration of the patch-clamp technique. Localization of CaV1.3 subunits and Rab27a was assessed by confocal microscopy. VEGF-A secretion was analyzed by ELISA. CaV1.3 sequence was modified by site directed mutagenesis.

Results: Immunohistochemical analysis of mouse retina sections revealed co-localization of both Rab27a and CaV1.3 at the basolateral side of the RPE. Rab27a over-expression in ARPE19 cells resulted in decreased VEGF-A secretion, decreased L-type channel current density and positive shift of their voltage dependence. Current density of heterologously expressed CaV1.3 channels was also decreased when co-expressed with Rab27a. Under these conditions a negative shift of voltage dependence was observed. Confocal microscopy with heterologously expressed CaV1.3 and Rab27a revealed co-localization of both proteins in CHO-K1 cells. However, Rab27a co-expression reduced surface expression of CaV1.3. We detected co-immunoprecipitation of Rab27a and CaV1.3 pore-forming subunit. After deleting the MyRIP homologous domain between the cytosolic loop and the first homologous repeat of the 2nd transmembrane domain of the CaV1.3 subunit, co-immunoprecipitation was lost.

Conclusion: We conclude that Rab27a modulates activity of CaV1.3 channels via direct interaction with the pore-forming subunit at a MyRIP homologous domain resulting in changed voltage-dependence and surface expression. We show the first evidence of the direct functional modulation of an ion channel by a Rab27a protein suggesting a new mechanism in the control of exocytotic processes such as VEGF-A secretion in the RPE.
Activation of the cardiac mineralocorticoid receptor (MR) increases the L-type Ca\(^{2+}\) current (\(I_{\text{CaL}}\)), a key player in Ca\(^{2+}\) homeostasis and hence, regulation of cardiac contractility. Within the left ventricle a gradient of action potential (AP) waveforms is present, with short AP duration (APD) in epicardial (epi) and long APD in endocardial (endo) myocytes. As a consequence, the AP-induced Ca\(^{2+}\) influx is larger in endo than in epi myocytes. We demonstrated that after 24h incubation, \(I_{\text{CaL}}\) is similar in endo and epi myocytes (n.s.) and that MR activation increases \(I_{\text{CaL}}\) to a similar extent in both layers (each p<0.001). Since the epi AP was markedly more prolonged by MR activation than the endo AP, AP-induced Ca\(^{2+}\) influx was increased 4-fold (p<0.001) in epi myocytes, while in endo myocytes only a 1.8-fold increase (n.s.) was noted. Here we investigate region-specific effects of these changes in the transmembrane Ca\(^{2+}\) flux on the Ca\(^{2+}\) transient and fractional shortening (FS) in left ventricular rat endo and epi myocytes. Ca\(^{2+}\) transients were assessed simultaneously with sarcomere length using fura2-AM. Despite longer APD and higher AP-induced Ca\(^{2+}\) influx in endo myocytes, the amplitude of the Ca\(^{2+}\) transient, its decay time constant and FS were similar in endo and epi myocytes after 24h under control conditions, as were baseline Ca\(^{2+}\) levels and sarcomere length (each n.s.). After 24h of MR stimulation, Ca\(^{2+}\) transients and FS were affected in a similar way in epi and endo myocytes; in epi myocytes the Ca\(^{2+}\) transient amplitude increased by 43% (p<0.001) and by 70% in endo myocytes (p<0.001). The decay of the Ca\(^{2+}\) transient was significantly accelerated in endo myocytes (p<0.001). Baseline Ca\(^{2+}\) levels were not affected. FS was increased by 48% (p<0.05) in epi and by 74% (p<0.001) in endo myocytes. To examine whether the increase in the Ca\(^{2+}\) transient was due to an increased filling of the SR, its Ca\(^{2+}\) content was assessed using a 10mM caffeine pulse after steady-state pacing. SR filling was similar in endo- and epicardial myocytes under control conditions after 24h (n.s.). MR activation slightly tended to increase SR filling (endo: +5%, n.s., epi: +15%, n.s.). These data indicate that the increase in \(I_{\text{CaL}}\) transients upon MR activation in endo- as well as in epicardial myocytes is predominantly caused by the increase in \(I_{\text{CaL}}\) density and consequently an increase in fractional Ca\(^{2+}\) release from the SR and to a far lesser extent by an increased filling of the SR.
**POSTER PRESENTATIONS**

**Methods:** Serum FGF23 was determined by ELISA. Gene expression was analysed in UMR106 osteoblast-like cells by qRT-PCR and SOCE by Fura-2 fluorescence.

**Results:** DOCA treatment and salt depletion of mice elevated the serum FGF23 concentration. Orai1 was expressed in UMR106 cells and immortalized primary osteoblasts (IPO). The expression was decreased by NFκB inhibitor wogonin. In UMR cells store depletion by sarcoendoplasmatic Ca2+ ATPase (SERCA) inhibitor thapsigargin triggered SOCE, which was significantly blunted by Orai inhibitors 2-APB and YM58483, Orai1 silencing, and wogonin. FGF23 transcript levels in UMR cells were increased by thapsigargin, Ca2+ ionophore ionomycin, and decreased by Orai inhibitors 2-APB, and SK&F96365, Orai1 silencing as well as NFκB inhibitors wogonin and withaferin. Aldosterone increased and spironolactone decreased SOCE in UMR cells. Moreover, aldosterone increased FGF23 transcript levels in UMR cells, an effect reversed by spironolactone, YM58483, and withaferin. FGF23 transcript levels in UMR cells were increased by thapsigargin, Ca2+ ionophore ionomycin, and decreased by Orai inhibitors 2-APB, and SK&F96365, Orai1 silencing as well as NFκB inhibitors wogonin and withaferin. Aldosterone increased and spironolactone decreased SOCE in UMR cells. Moreover, aldosterone increased FGF23 transcript levels in UMR cells, an effect reversed by spironolactone, YM58483, and withaferin.

**Conclusions:** Aldosterone enhances FGF23 expression, an effect sensitive to NFκB-dependent up-regulation of Orai1 and subsequent increase in store-operated Ca2+ entry.

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**Oxygen, hypoxia and cell death**

**P017**

**Study of a new rhenium complex for carbon monoxide photorelease in human intestinal epithelial (Caco-2) cells**

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**Introduction:** Carbon monoxide (CO) is a signal molecule which regulates vital cellular processes. The gas is generated naturally in the human body and can exert antiapoptotic, vasodilatory and anti-inflammatory effects. New substances which release CO after exposure to light (= CO-releasing molecules Photoactivatable (PhotoCORMs) are therefore highly interesting for new biomedical and pharmacological applications.

**Question:** Before CO-release studies in biological systems can be performed, the question arises, how the new water-soluble organometallic rhenium-derivative RE (BIAN) (CO) 3 Cl complex (PhotoCORM 3) affects human cells?

**Methods:** The biological compatibility was tested on intestinal epithelial (Caco-2) cells. Differentiated Caco-2 cells were treated with different PhotoCORM 3 concentrations, followed by analysis of cell morphology (light microscopy) and cytotoxicity (lactate dehydrogenase assay, LDH). Additional, the cellular uptake of PhotoCORM 3 was determined spectrophotometrically.

**Results:** Cytotoxicity could not be determined colorimetrically using LDH assay, because the LDH assay (absorption wavelength 490 nm) interfered with the red coloured PhotoCROM 3 (absorption wavelength 500 nm). Caco-2 cell morphology did not change markedly after 24 h treatment with the PhotoCROM 3 and high biologically compatibility is expected. Interestingly, regions of intense cellular uptake of the red-coloured PhotoCORM 3 were located dose-dependently within the cell monolayer.

**Conclusions:** Selective absorption of PhotoCORM 3 into subpopulations of intestinal epithelial cells should be investigated more in detail, e.g. with regard to a new colorectal cancer therapy. The LDH assay was not applicable, therefore an alternative cytotoxicity method should be used.

**Keywords:** Carbon monoxide (CO), intestinal epithelial cell, photoactive CO-releasing moieties (PhotoCORMs)

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**P018**

**Endogenous Nox4 limits fibrosarcoma development in the murine 3-methylcholanthrene model**

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Through the constitutive production of H2O2 the NAPDH Oxidase Nox4 promotes differentiation of cells and contributes to cellular quiescence. Our previous observations in the mesenchymal tissue of the vasculature and the bone suggest that the absence of Nox4 promotes inflammation and
de-differentiation. Chronic inflammation and lack of differentiation are pre-carcinogenic states. We therefore hypothesized that lack of Nox4 promotes malignant transformation and tumor development.

The process of inflammation-dependent tumor formation was studied in response to the chemical carcinogen 3-methylcholanthrene (MCA) which was administered subcutaneously to mice and induces sarcoma formation. Initial fibrosarcoma formation was massively enhanced in Nox4-deficient mice and in average their tumors were bigger as compared to those of wildtype mice. Nox1y/- mice, in contrast, developed fewer tumors. Genetic deletion of Nox4 resulted in an increased number of F4/80 positive myeloid cells within the tumor. Simultaneously, the activity of NFkB, one of the most important pro-inflammatory transcription factors, was significantly increased in tumor cells cultured from Nox4-/-/ mice as compared to WT control mice. As a consequence, pro-inflammatory cytokines and adhesion molecules e.g. IL-1 beta, TNF-alpha and VCAM were expressed at higher levels in the tumors of Nox4 knockout mice. Thus, a higher degree of inflammation is present in the absence of Nox4 which may promote tumor development. Mechanistically, the abundance of the tumor suppressor p53 was significantly reduced in the tumor tissue as well as in the cells of Nox4-/- . This was accompanied by an increased phosphorylation of AKT which is presumably responsible for the reduction of p53 through its ability to activate the E3 ubiquitin-protein ligase MDM2 which promotes p53 degradation. Additionally, induction of the AKT pathway in the Nox4-deficient tumor cells may lead to a pro-survival phenotype which contributes to tumor growth.

Taken together these results suggest that anti-inflammatory properties of endogenous Nox4 attenuate inflammation-induced tumor formation in the murine 3-methylcholanthrene model.

P019
Non-catalytic enhancement of H+‐coupled lactate transport by carbonic anhydrase IX in MCF‐7 breast cancer cells
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Local hypoxia, which arises from elevated respiration and/or inadequate blood supply is a hallmark of most solid tumors and is associated with poor prognosis. Hypoxia affects many important processes in tumor metabolism and promotes malignant progression by alteration of glycolysis, followed by increased acid extrusion.

The influence of hypoxia on lactate flux and pH regulation was examined in MCF-7 breast cancer cells cultured under normoxic (21% O2) or hypoxic (1% O2) conditions for 3 days. Expression levels of proteins involved in metabolite transport and acid/base regulation were measured by quantitative real-time PCR and Western blot analysis. Changes in H+ and lactate flux were measured on the single cell level with H+-sensitive dyes and a FRET-based lactate sensor, respectively. The impact of carbonic anhydrase (CA) on H+/lactate flux and cancer cell survival was investigated by addition of the CA inhibitor 6-ethoxy-1,3-benzothiazole-2-sulfonamide (EZA, 30 μM) and knockdown of CAIX. Under hypoxia, expression of the cancer-specific carbonic anhydrase CAIX was significantly increased, while expression of the monocarboxylate transporters MCT1 and MCT4 remained unchanged. Hypoxia caused a significant increase in MCT-mediated H+/lactate flux, both in the presence and absence of CO2/HCO3−, which was insensitive to inhibition of CA catalytic activity with EZA. However, knock-down of CAIX with siRNA abolished the hypoxia-induced increase in MCT activity. These results indicate that hypoxia-induced CAIX enhances transport activity of MCT by a non-catalytic mechanism. This ‘non-catalytic transport metabolon’ between MCT1 and CAIX plays a significant role in cancer cell proliferation: Knockdown of CAIX reduced proliferation of hypoxic MCF-7 cells to a similar level as did inhibition of MCT1 transport function with AR-C155858, while inhibition of CA catalytic activity had no effect on cell proliferation. Taken together, these results suggest that hypoxia-induced CAIX facilitates H+/lactate cotransport by non-catalytic interaction with MCT1, and that this interaction plays a significant role in cancer cell proliferation.

P020
Fumaric acid esters promote neuronal survival upon ischemic stress through activation of the Nrf2 pathway
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**Question:** Oxidative stress is a hallmark of ischemic stroke pathogenesis causing neuronal malfunction and cell death. Up-regulation of anti-oxidative genes through activation of the NF-E2-related transcription factor 2 (Nrf2) is one of the key mechanisms in cellular defense against oxidative stress. Fumaric acid esters (FAEs) represent a class of anti-oxidative and anti-inflammatory molecules that are already in clinical use for multiple sclerosis therapy. Purpose of this study was to investigate whether FAEs promote neuronal survival upon ischemia. We further analyzed putative underlying molecular mechanisms (e.g. Nrf2) in neurons.

**Methods:** Murine organotypic hippocampal cultures, and the neuronal cell lines HT-22 (mouse) and SH-SY5Y (human) were treated with the FAEs dimethyl fumarate (DMF) and monomethyl fumarate (MMF). Ischemic conditions were generated by exposing cells and slice cultures to oxygen-glucose deprivation (OGD). Cell death was determined through propidium iodide fluorescence staining. The Nrf2 knockdown in neurons was performed by RNA interference technique. Gene expression in cells was analyzed by real-time PCR and Western Blotting. Immunofluorescence technique was used to determine subcellular localization and expression of Nrf2 protein.

**Results:** DMF treatment either before or after OGD strongly reduced cell death in hippocampal cultures ex vivo. Both DMF and MMF promoted neuronal survival in HT-22 and...
Apoptosis, the programmed cell death, is thought to play an important role in cardiovascular development and disease. However, the exact role of apoptosis in particular for origin and progression of heart disease is still poorly understood. We are aiming to address this question by establishing a novel system for the in vivo detection of apoptosis.

Our assay is based on a secreted human Annexin V (sA5) protein fused to yellow fluorescence protein (YFP) under control of the ubiquitous CAG promoter. The sA5-YFP protein is secreted into the extracellular space and binds to phosphatidylserine residues, which are exposed on the outer leaflet of the membrane once a cell undergoes apoptosis. In this way, apoptotic cells are labeled and identified based on sA5-YFP fluorescence signals. We have established a transgenic mouse line derived from stable transfecting CAG-secA5-YFP mouse embryonic stem cells (mESCs) and tested the system in vivo. Apoptosis is present during embryonic development and we therefore analyzed transgenic embryos at developmental day 7.5 (E7.5). Very bright YFP signals could be observed within the yolk sac, brain and heart as has been reported before, proving the functionality of the system. Next, we assessed apoptosis during different stages of mouse heart development (E7.5 - E17.5), apoptotic signals were also confirmed by co-staining for cleaved Caspase 3 (cCasp3). Co-localization of sA5-YFP+ cells and cCasp3 was detected in different cardiac regions, mainly close to the aortic region and ventricular trabeculae. In addition, to analyze CMs apoptosis ex vivo, we isolated embryonic CMs from the transgenic sA5-YFP mouse line and induced apoptosis with Etoposide. Accumulation of sA5-YFP was observed at the cell membrane of apoptotic cells by an increase in YFP-fluorescent intensity over time, thereby proving the feasibility of the system.

Our novel in vivo assay for the identification of apoptotic cells will provide better insight into the incidence and relevance of cardiac apoptosis during heart development, its role in heart disease, and how its modulation can be potentially used as a treatment approach for cardiovascular disease.

**Conclusions:** Our results may provide the basis for a new therapeutic approach to treat ischemic pathologies such as stroke with a drug that already has a broad safety record in humans.

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**P022**

**Role of cadmium and sphingolipids in autophagy and cell death of renal proximal tubule cells**

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The kidney proximal tubule (PT) is the major target of cadmium (Cd²⁺) toxicity where it causes cell stress and death via apoptosis that partly involves sphingolipid signaling. Autophagy is the basic catabolic process by which organelles and long-lived proteins are delivered to the lysosome for degradation. Autophagy can also be induced by cell stress and contributes to stress-induced cell survival or death depending on the cellular context. Recent studies indicate sphingomyelin (SM) and acid sphingomyelinase (ASM) mediate autophagy (E. Gabande-Rodriguez et al., Cell Death Differ, 21:864-875, 2014). It has been reported in renal PT that autophagy induced by Cd²⁺ is a pro-survival mechanism and the autophagy inducer rapamycin prevented Cd²⁺-induced PT death. We have further investigated Cd²⁺-induced autophagy in cultured rat kidney PT cells (PTCs) (WKPT-0293 Cl.2, NRK-52E) and its role in Cd²⁺-induced cell death. Immunoblotting and immunofluorescence of autophagy markers LC3 and p62 indicate rapid induction of autophagy flux by Cd²⁺ (5-10µM) exposure for 1h that was accompanied by the formation of autophagic vacuoles labelled with monodansylcadaverine (MDC). Cd²⁺ exposure exceeding 3h resulted in the accumulation of p62 and LC3 as well as an increase of extra-lysosomal cathepsins, indicating decreased autophagy flux and lysosomal dysfunction and damage. Cd²⁺ (6h) also increased cellular SM that was detected by lysenin staining and correlated with Cd²⁺-induced inhibition of ASM activity. These processes were associated with decreased cell viability, as measured by MTT assay, that could be attenuated by an inhibitor of autophagic vacuole formation 3-methyladenine. However, the autophagy inducer rapamycin (100 nM) did not prevent Cd²⁺-induced decrease of cell viability observed with longer Cd²⁺ exposure. In conclusion, the data suggest a dual and temporal impact of Cd²⁺ on autophagy in PTCs: It rapidly activates autophagy (possibly via reactive oxygen species signaling to counteract PTC stress and damage). Prolonged Cd²⁺ exposure blocks autophagy flux and increases SM possibly resulting in lysosomal disruption and increased cell death.
**P023**

**Androglobin, the fifth mammalian oxygen-binding globin contributes to male fertility**
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Hemoglobin and myoglobin represent the two most-established and well-studied heme-containing oxygen binding proteins. The post-genomic era revealed the presence of additional mammalian globin types, including neuroglobin and cytoglobin. We recently reported the identification of a new metazoan globin lineage, consisting of large chimeric proteins with an N-terminal cysteine protease domain and a central circular permuted globin domain, named androglobins (Adgbs), because of their specific expression in testis tissue in human, mice and zebrafish. Intriguingly, this new member of the globin family is evolutionarily ancient and extremely conserved, being present in mammals, vertebrates, more basal animal clades and even unicellular organisms. Hexacoordination of the Adgb heme iron and lack of transcriptional induction in hypoxia suggest a function independent of classical oxygen supply. Immunofluorescence studies illustrate Adgb localization in the cytoplasm in *vitro*. Analysis of a newly generated Adgb knock out mouse model indicates a crucial role of Adgb in reproduction. Male Adgb-deficient mice are infertile with a developmental defect during later stages of spermatogenesis. Round haploid postmeiotic spermatids of Adgb-deficient mice do not differentiate into elongated spermatids, underscoring the functional importance of Adgb during spermatogenesis.

**P024**

**The transcription factor ARNT is regulated by hypoxia and the von Hippel-Lindau tumour suppressor protein in a cell-specific manner**

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Oxygen deprivation (hypoxia) is a common feature of solid tumours due to excessive cellular proliferation and often associated with poor prognosis. Cellular adaptation to this micro-environmental stress is mediated by the Hypoxia-inducible factor (HIF) pathway leading to e.g. metabolic alterations, invasion/metastasis and angiogenesis. The transcription factor HIF-1, itself composed of HIF-1α and the Aryl hydrocarbon receptor nuclear translocator (ARNT; also designated as HIF-1β) is the major player within this cascade. In normoxia, HIF-1α is subjected to proteasomal degradation in a von Hippel-Lindau (pVHL)-dependent manner, but accumulates under low oxygen tension followed by the expression of target genes. In contrast to its binding partner HIF-1α, ARNT is regarded to be constitutively expressed, meaning to be unaffected by hypoxia on both mRNA and protein levels (despite the name HIF-1β). However, there is accumulating evidence that ARNT is upregulated in response to hypoxic conditions in a number of cell lines (reviewed by Mandl & Depping 2014). Therefore this recently initiated project aims to elucidate the mechanism responsible for this rare cellular capability. First data obtained from *vhl*-deficient renal carcinoma cell lines (786-O, RCC4) and stably transfected *vhl* positive counterparts, point towards pVHL as a contributor mediating this effect. ARNT mRNA and protein levels were profoundly increased in Hep3B hepatocellular carcinoma cells cultured in hypoxia (3% O₂) for eight hours. Taken together, this model will enable us to study the mechanisms underlying the hypoxia-dependent upregulation of ARNT.

**P025**

**Loss of myeloid hypoxia inducible factor-1 ameliorates Dextran Sodium Sulfate induced colitis in mice**

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Hypoxia is a hallmark of chronically inflamed tissue when compared to healthy tissue. Under oxygen deficiency, hypoxia, the transcription factor complex hypoxia inducible factor (HIF) regulates expression of genes potentially also involved in the pathogenesis of Inflammatory Bowel Disease (IBD). Among others HIF-1 is essential for myeloid cell life span and function during inflammation. The complex interplay between hypoxia and inflammation during IBD prompted us to investigate the role of HIF-1 in the pathogenesis of IBD and especially its role in myeloid cells during inflammation.

Mice with a conditional knockout of HIF-1α in myeloid cells (LysMcre/HIF-1α*−/−*) were examined *in vivo* in a Dextran Sodium Sulfate (DSS) induced model of IBD. Furthermore, bone-marrow-derived macrophages (BMDM) lacking HIF-1α were analyzed under inflammatory and/or hypoxic conditions. First experiments provide evidence that LysMcre/HIF-1α*−/−* mice deficient in functional HIF-1 in myeloid cells show a milder disease with less weight loss and lower inflammatory parameters when compared to HIF-1α*−/−* control mice. This could indicate that HIF-1 could act as a proinflammatory regulator of myeloid cells during the course of IBD.
Endocytosis is an important mechanism of cells for diverse cellular functions such as signaling and nutrient uptake. Apart from this, it enables cells to control the homeostasis of their membrane enclosed compartments. To this end cells incorporate proteins that follow the endocytotic retrograde transport pathways. This enables cells to respond flexibly to changes in stimulation or cellular context. The availability of oxygen is an important factor in the cellular context. Therefore, we performed endocytosis assays using the lipophilic styryl dye FM®1-43 (N-(3-Triethylammoniumpropyl)-4-(4-(Dibutylamino) Styryl) Pyridinium Dibromide), which labels the plasma membrane and allows the visualization of vesiculation during the endocytotic trafficking, in normoxia and hypoxia. The exposure to hypoxia resulted in an enhanced staining with the FM®1-43 dye pointing to an increase in endocytotic events, which was reversible upon reoxygenation. This observation was not restricted to the primary cardiac fibroblast but was also found in other cell types like primary tail fibroblasts and the epithelial mamma carcinoma cell line MDA-MB-231. Hypoxia-induced endocytosis was independent from the hypoxia-inducible factor (HIF) as demonstrated in HIF-1α knock out cells or after transfection of HIF-1α shRNA. Interestingly, not only the intensity of the staining with FM®1-43 was altered in hypoxia, also the morphology of the stained vesicles was clearly distinct from the normoxic samples. In cells pre-incubated under hypoxic conditions the FM®1-43 staining was found in tubular like structures whereas in normoxic cells mainly vesicular structures were found. By taking advantage of the novel membrane probe mCLING (membrane-binding fluorophore-cysteine-lysine-palmitoyl group), we further analyzed the nature of these structures. By using specific markers for the different compartments involved in the endocytotic pathway we were able to show that these structures are related to the trans-Golgi network, whereas cis-Golgi and early endosome markers were significantly less co-localized. This suggests that under hypoxic conditions the retrograde transport from endosomes to the trans-Golgi network is increased.

**P026**

**Hypoxia alters trans-Golgi associated endocytosis independent from the Hypoxia-inducible factor-1**

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Downregulation of the HIF-α signaling pathway by the importin α/β-specific inhibitor Ivermectin

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Hypoxia-inducible transcription factors (HIFαs) regulate more than 200 genes involved in cellular adaptation to reduced oxygen availability. HIFs consist of one out of three different O2-labile α-subunits (primarily HIF-1α and HIF-2α) and a constitutive HIF-1β subunit. In normoxia the HIF-α subunit is hydroxylated by members of a family of prolyl-4-hydroxylase domain (PHD) proteins, PHD1, PHD2 and PHD3, resulting in recognition by von Hippel-Lindau protein (pVHL), ubiquitination and proteasomal degradation. In contrast, reduced oxygen availability inhibits PHD activity resulting in HIF-1α stabilization and nuclear accumulation. Nuclear import of HIF-1α depends especially on a classical nuclear localization signal (NLS) and involves importin α/β heterodimers. Recently, Ivermectin has been identified as a specific inhibitor of importin α/β-dependent nuclear import with no effects on a range of other nuclear transport pathways involving members of the importin protein family. In this study we evaluated the biological and physiological activity of this importin α/β-inhibitor in the hypoxia response pathway. Treatment with Ivermectin decreases binding activity of HIF-1α to the importin α/β heterodimer. Moreover, HIF-1α nuclear localization, nuclear HIF-1α protein levels, HIF-target gene expression, as well as HIF-transcriptional activity are reduced upon Ivermectin treatment. For the first time, we demonstrate the effect of specific importin α/β-inhibition on the hypoxic response on the molecular level. The modification of nuclear translocation of HIF could provide a novel tool in anti-cancer therapy or the treatment of ischemic diseases.

**P028**

**Establishment and validation of recombinant cell lines for high-throughput DNT testing**

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The increase of neurological diseases like attention deficit hyperactivity disorder (ADHD) and autism is mainly associated with an exposure to enriched pollutants like heavy metals and pesticides but is not necessarily restricted to these chemicals. Everyday products contain chemicals that are potentially harmful for the developing central nervous system. Developmental neurotoxicity (DNT) is typically evaluated by animal experimentation including the test No. 426: a high-throughput DNT testing approach to identify chemicals which disturb the differentiation process. A cell line that is increasingly considered as a suitable model for DNT testing is the NT2 cell line. Upon a chemical stimulus NT2 cells differentiate into neurons of the central nervous system. Based on NT2 pluripotent stem cells we have established two recombinant cell lines stably expressing DsRed2 and a halosensitive YFP (yellow fluorescent protein) variant which will be expressed under the control of the constitutive active ubiquitin promoter and enables a fluorescent based functional analysis of ligand gated chloride channels.
like GlyR and GABAAR and an identification of chemicals which disturb the function of these proteins.

We present data demonstrating that the cell lines are suitable for in vitro based high-throughput DNT testing.

**P029**

**Hypoxia-inducible factor 1 in dendritic cells is crucial for the activation of protective regulatory T cells in murine colitis**

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Dendritic cells (DCs) serve as a bridge between innate and adaptive immunity and help to maintain intestinal homeostasis. Inflammatory bowel disease (IBD) is associated with dysregulation of the mucosal immune response and hypoxic inflammation when gene expression by DCs is regulated by the transcription factor hypoxia inducible factor (HIF)-1. Recent studies have described a protective role for epithelial HIF-1 in mouse models of IBD.

We investigated the role of HIF-1 in DC function in a dextran sodium sulfate (DSS)-induced model of murine colitis. Wild-type and dendritic cell-specific HIF-1α knock-out mice were treated with 3% DSS for 7 days. Knock-out of HIF-1α in DCs led to a significantly larger loss of body weight in mice with DSS-induced colitis than in control mice. Knock-out mice exhibited more severe intestinal inflammation with increased levels of proinflammatory cytokines and enhanced production of mucin. Induction of regulatory T cells (Tregs) was impaired, and the number of forhead box P3 (Foxp3) Tregs was diminished by dendritic HIF-1α knock-out.

Our findings demonstrate that in DCs HIF-1α is necessary for the induction of sufficient numbers of Tregs to control intestinal inflammation.

**P030**

**Neuronal HIF prolyl 4-hydroxylase 2 deficiency age dependently improves cognitive abilities in mice**

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**Question:** Dementia is often associated with impaired spatial learning and memory. Previous studies indicated a favorable effect of the hypoxia-inducible factor (HIF) targets VEGF (vascular endothelial growth factor) and erythropoietin (Epo). We investigated whether activation of the whole adaptive HIF pathway by inhibition of HIF prolyl 4-hydroxylase 2 (PHD2) function can improve cognitive abilities in young and aged mice.

**Methods:** Cognitive function in young (3 months) and aged (12-24 months) mice with neuron-specific deficiency for PHD2 (nPHD2) were assessed using the Morris water navigation task. Global cerebral oligemia was induced by permanent occlusion of the left common carotid artery. Molecular biology and immunofluorescent techniques were applied to study gene expression and neurogenesis in the hippocampal subgranular zone.

**Results:** Young nPHD2 mice showed an enhanced cognitive performance in the Morris water maze under both normoxia and cerebral hypopfusion as compared to wild type littermates. In contrast, no significant difference was observed in aged nPHD2 mice. We found increased protein levels of Epo and VEGF in the hippocampus, the essential region for memory formation and learning, of young nPHD2 mice, while only VEGF levels were increased in aged mice. BDNF and Egr1, known to be implicated in learning and memory formation, were not differentially expressed, irrespective of genotype or age. However, histological analysis revealed that neuronal PHD2 ablation increased proliferation of neural precursor cells within the hippocampal subgranular zone, while precursor differentiation into mature neurons was not affected.

**Conclusions:** Our pre-clinical findings suggest that PHD enzymes might be a promising therapeutic target for the treatment of dementia-related disorders.

**P031**

**Pre- and post-conditional inhibition of prolyl-4-hydroxylase domain enzymes protects the heart from an ischemic insult**

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Several genetically modified mouse models implicated that Prolyl-4-hydroxylase domain enzymes (PHD) are critical mediators for protecting tissues from an ischemic insult including myocardial infarction by affecting the stability and activation of the Hypoxia-inducible factor (HIF)-1 and 2. Thus, the current efforts to develop small molecule PHD inhibitors open a new therapeutic option for myocardial tissue protection during ischemia.

Therefore, we aimed to investigate applicability and efficacy of pharmacological HIFs stabilization by a small molecule PHD inhibitor in the heart. We tested for protective effects in the acute phase of myocardial infarction after pre- or post-conditional application of the inhibitor. Application of the specific PHD inhibitor 2-((1-chloro-4-hydroxysoquinoline-3-carboxamido) acetate (ICA) resulted in HIF-1α and HIF-2α accumulation in heart muscle cells in vitro and in vivo. The rapid and robust responsiveness of cardiac tissue towards ICA was further confirmed by induction of the known HIF target genes heme oxygenase-1 and PHD3. Pre- and
post-conditional treatment of mice undergoing myocardial infarction resulted in a significantly smaller infarct size. Tissue protection from ischemia after pre- or post-conditional ICA treatment demonstrates that there is a therapeutic time window for the application of PHI post-myocardial infarction, which might be exploited for acute medical interventions.

**P032**

Genotoxicity of hyperbaric oxygen and reversible DNA-protection adaptation induced by repetitive exposure

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**Question:** There is evidence for genotoxicity of hyperoxia, but adaptive protective processes seem to be be induced, too. Aim of the study: 1. To determine the amount of double-strand breaks in PBMCs (peripheral mononuclear blood cells) after a single exposure to high-dose hyperbaric oxygen in vivo. 2. To monitor the decline of the protective adaptation after a phase of repetitive exposures to hyperbaric oxygen in professional closed-circuit divers in a prospective approach.

**Methods:** 1. DNA-damage was determined in PBMCs from subjects (n=12) immediately before and after a 30 minutes mandatory oxygen tolerance test (pO₂=280kPa). All subjects were males of the same age and personal situation who have never been exposed to hyperbaric oxygen before. 2. Likewise by determining DNA-damage in freshly isolated PBMCs the baseline of DNA-damage was assigned in professional divers (n=7) 24 hours after a three-month period of about 85 repetitive mainly physically strenuous closed-circuit dives (50 to 120 min, pO₂=160kPa). The same PBMCs were then exposed to hyperoxia (400kPa, pO₂=100%) ex vivo and DNA-damage was determined hourly for 6 hours. The same experiment was performed weekly at PBMCs of the same subjects over a five weeks period without any further exposition to hyperoxia. All subjects were males of the same age taking part in the same schooling program. The DNA-damage of either investigation was determined by means of the alkaline Comet Assay calculating the percent- aged amount of DNA-damage by a visualized binary scoring system (Yes/No DNA-damage) as well as analyzing the computerized estimation of “Tailmoment” and “% Tail DNA” of 200 PBMCs, respectively.

**Results:** 1. PBMCs after oxygen tolerance test showed high significant (p < 0.02). The prospective approach revealed that baseline DNA-damage increases significantly (p_{4h+5w}=0.022; p_{2h+5w}=0.049) in an almost linear way. Slopes of graphs from data raised 24 hours after last oxygen dive were significantly (p_{2h+5w}=0.022; p_{4h+5w}=0.049). The ArhGAP29 knock down results in RhoA activation and changes in cell morphology, cell polarization and migration in hypoxia and normoxia. We hypothesize that ArhGAP29 has a prominent role in the fine tuning of actin cytoskeleton dynamics in hypoxia.

**Conclusion:** Hyperbaric oxygen is genotoxic to PBMCs. Repetitive exposures to hyperoxia lead to in vivo adaptation of PBMCs with less susceptibility towards hyperoxic stress afterwards. This DNA-protecting effect of adaptation decreases with time, whereas up to one week after last exposure protection is still high.

**P033**

Remodeling of the actin cytoskeleton in hypoxia: an emerging role for ArhGAP29

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Cells can adapt to hypoxia by various mechanisms. We observed that L929 fibroblasts cultivated in hypoxia (1% O₂) showed striking morphological differences as compared to cells cultivated under normoxic conditions (20% O₂) including the appearance of prominent actin filaments. It has been shown in different cell types that hypoxia influences members of the Rho family of GTPases which are master regulators of the actin cytoskeleton. However, the mechanisms of hypoxia-specific Rho regulation are largely unexplored. Here we show that ArhGAP29 (Rho GTPase activating protein 29), a regulator of RhoA, is strongly induced in hypoxia in a HIF-1α dependent manner. The ArhGAP29 knock down results in RhoA activation and changes in cell morphology, cell polarization and migration in hypoxia and normoxia. We hypothesize that ArhGAP29 has a prominent role in the fine tuning of actin cytoskeleton dynamics in hypoxia.
P034
The influence of norepinephrine on the cardiopulmonary reaction to hypoxia in rats
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Question: Hypoxia is associated with sympathetic activation, which may improve tissue oxygenation in this condition. However, both strong sympathetic activation and hypoxia can induce pulmonary edema. The aim of the present study was to investigate whether the sympathetic transmitter norepinephrine (NE) may contribute to the formation of pulmonary edema under conditions of hypoxia.

Methods: Rats were exposed to normobaric hypoxia in a chamber with 10% O2 in nitrogen over time intervals of 1.5 and 6 h and were infused with either 0.9 % NaCl (H+NaCl) or with NE (0.1 mg kg⁻¹ h⁻¹; H+NE). Animals kept under normoxic conditions received the same types of infusion (N+NaCl, N+NE) and served as controls. At the end of the experiment, heart catheterization was performed for examination of hemodynamic function. Finally, lung tissue was obtained for histology.

Results: After 6 h of hypoxia, left ventricular (LV) systolic pressure (LVSP) and contractility (LV dP/dt max) were significantly reduced by 25% and 35%, respectively. Right ventricular systolic pressure (RVSP) remained at control level. Lung histology showed moderate interstitial edema. Additional NE infusion (H+NE) improved LVSP and LV dP/dt max to or even above normoxic control level (N+NaCl). In contrast, pulmonary edema was aggravated under this condition.

Conclusions: These results indicate that sympathetic activation under hypoxic conditions restores cardiac function but may promote formation of pulmonary edema. Further experiments are designed to investigate whether adrenergic blockers may serve as a therapeutic option in the prevention or treatment of hypoxic pulmonary edema.

Transporters (Transmitters)

P035
Potassium binding to excitatory amino acid transporters
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Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. After release from presynaptic nerve terminals, glutamate is taken up into glial and neuronal cells by glutamate transporters belonging to the excitatory amino acid transporter (EAAT) family. EAATs harness the energy stored in [Na⁺] and [K⁺] gradients for secondary active glutamate transport. After binding of glutamate, three Na⁺ ions and one proton, the transporter isomerizes from the outward- to the inward-facing conformation. Substrates are released into the cytoplasm, and K⁺-dependent relocation terminates the transport cycle. The K⁺-associated steps are rate-limiting for glutamate transport and represent promising pharmacological targets to modify EAAT transport rates in various human disease conditions. EAATs are not only secondary active transporters, but also anion-selective channels. EAAT anion channels open from intermediate translocation conformations, and anion currents thus permit detection of transitions in the glutamate transport cycle.

We here combined extensive molecular dynamics simulations of prokaryotic EAAT homologs and patch-clamp recordings of mammalian EAAT2 to identify the molecular mechanisms of K⁺-coupled glutamate uptake. Our simulations show spontaneous association of K⁺ to four distinct K⁺ binding sites (K1-K4), which partly overlap with already known Na⁺ binding sites. Simulations suggest that association to K3 is a precondition for binding at K1. K⁺ binding at K1 weakens binding at the other sites, while a K⁺ ion remains stably bound at K1. These results suggest that K1 serves as the final binding site that is occupied during the isomerization between inward- and outward-facing conformations.

We next sought to experimentally validate the predicted K⁺ binding sites by site-directed mutagenesis, heterologous expression and whole-cell patch-clamp recordings. Mutations in the K1 and K3 site that preserved function of the transporter altered K⁺-dependent transitions. Some mutations even resulted in transporters that were unable to perform the K⁺-dependent relocation, but retained the glutamate-dependent translocation.

In conclusion, the combination of experimental and computational methods successfully resolved the K⁺ binding sites in EAATs thereby expanding our knowledge about Na⁺- and K⁺-coupled glutamate uptake.
Autosomal dominant polycystic kidney disease (ADPKD) is the most prevalent inherited cause for end-stage renal disease. It is defined by the age-dependent massive enlargement of both kidneys, which is characterised by dilated or cystic renal tubular segments. The two causative genes PKD1 and PKD2 encode for a sensory receptor/ion-channel complex that is required for renal tubular morphogenesis. Yet, the molecular function of the Polycystin-1/TRPP2 signalling module has remained elusive. To investigate the polycystin complex on a molecular level, the establishment of suitable orthogonal in vitro model systems is essential. Loss of function cell lines from knockout mice or RNAi-mediated depletion of PKD-genes have provided valuable insights, but are compromised by technical challenges including lack of cellular differentiation in vitro, poor scalability, incomplete knockdown, and putative RNAi off-target effects.

Here we present the site-specific genome engineering-mediated depletion of Pkd2 using Transcription Activator-Like Effector Nucleases (TALENs). Mouse inner-medullary collecting duct (mIMCD3) cells retain core differentiation characteristics of the collecting duct, e.g. apico-basolateral polarisation, epithelial transport, and tight junctions. mIMCD3 cells deficient for TRPP2 (mIMCD3 Pkd2−/−), on the other hand, show a distinctive morphology characterised by impaired cellular differentiation and cytoskeletal aberrations. This marked TRPP2-dependent defect of proper epithelial organisation is even more pronounced in a complex three-dimensional extracellular matrix environment as highlighted by break down of the epithelial monolayer. The mIMCD3 cells retain core differentiation characteristics of the collecting duct, e.g. apico-basolateral polarisation, epithelial transport, and tight junctions. The mIMCD3 Pkd2−/− phenotype is prominent and easy to score and might serve as readout for impaired tubular morphogenesis in vitro.

In this proof of concept study we establish that the non-segregating mIMCD3 Pkd2−/− cells deficient for TRPP2 (mIMCD3 Pkd2−/−), on the other hand, show a distinctive morphology characterised by impaired cellular differentiation and cytoskeletal aberrations. This marked TRPP2-dependent defect of proper epithelial organisation is even more pronounced in a complex three-dimensional extracellular matrix environment as highlighted by break down of the epithelial monolayer. The mIMCD3 Pkd2−/− phenotype is prominent and easy to score and might serve as readout for impaired tubular morphogenesis in vitro; and that the directed manipulation of PKD genes provides novel opportunities to model polycystin protein function.

**Background:** Lefty A, a powerful regulator of stemness and embryonic differentiation, reprograms cancer cells, thus counteracting cell proliferation and tumor growth. Key properties of tumor cells include enhanced glycolytic flux with excessive consumption of glucose and formation of lactate. As glycolysis is highly sensitive to cytosolic pH, maintenance of glycolysis requires export of H+ ions and lactate, which is accomplished by Na+/H+ exchangers (NHE), such as NHE1. An effect of Lefty A on transport processes has, however, never been reported. The present study thus elucidated an effect of Lefty A on cytosolic pH (pH), NHE1 expression, NHE activity, glucose consumption and lactate release.

**Methods:** Experiments were performed in Ishikawa cells without or with prior Lefty A (5ng/ml) treatment. NHE1 transcript levels were determined by qRT-PCR, pH estimated utilizing (2’,7’-bis-[2-carboxyethyl]-5-(and-6)- carboxyfluorescein [BCECF] fluorescence, Na+/H+ exchanger activity from Na+-dependent realalkalinization after an ammonium pulse and lactate concentration in the culture supernatant utilizing Lactate Dehydrogenase Enzymatic Colorimetric assay.

**Results:** A 2 hours treatment with Lefty A (5ng/ml) was followed by a significant decrease of transcript levels encoding NHE1, of pH (from 7.22 ± 0.02 to 7.05 ± 0.02, n = 3), of NHE activity (from 0.11 ± 0.02 to 0.05 ± 0.02 a.u., n = 3), and of lactate release (from 12.06 ± 2.17 to 7.14 ± 1.25 ng/μl, n = 3–4).

**Conclusions:** Lefty A markedly down-regulates NHE1 expression, Na+/H+ exchanger, pH, as well as lactate release in Ishikawa cells. Those effects presumably contribute to reprogramming and growth inhibition of tumor cells.

**Acute regulation of human multidrug and toxin extrusion protein 1 (hMATE1)**

**Background:** Inactivation of multidrug resistance (MDR) transporters by overexpression of MDR transporters is a common resistance mechanism of cancer cells. The hMATE1 is expressed in the apical membrane of renal proximal tubule cells and of hepatocytes. Together with organic cation transporters (OCT) expressed on the basolateral plasma membrane, hMATE1 mediates the vectorial transepithelial transport (mainly resulting in secretion) of endogenous and exogenous organic cations. Since some MATE1 substrates such as cisplatin are toxic, MATE1 is involved in cytotoxicity or resistance to it. Sequence analysis of hMATE1 reveals the presence of several intracellular phosphorylation sites for different kinases (PKA, PKC, calcium/calmodulin-complex (CaM), tyrosine kinase and caseine kinase II (CKII)), which may be important for the acute regulation of the transporter.

The aim of this work was to characterize the function and acute regulation of hMATE1 stably expressed in HEK293 cells. The cellular influx and in some cases also the efflux of the fluorescent substrate 4-((4-((dimethylamino) styryl)-N-methylpyridinium (ASP+) was monitored by using a microtitre plate based fluorescence reader (excitation at 450 nm, emission at 590 nm). The efflux characteristics of hMATE1 were studied by measuring the decrease of cellular

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Mitochondrial diveral metal transporter 1 (DMT1) transports manganese

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Mitochondria contain a major fraction of the cells’ iron, required in particular for heme and iron-sulfur cluster synthesis. Yet, the mechanism of iron translocation from the cytosol into mitochondria is still incompletely resolved. We have previously obtained evidence for mitochondrial localization of the diveral metal transporter 1 (DMT1) by a variety of methods, including cyto-immunogold electron microscopy, partial colocalization of DMT1 with mitochondrial markers detected by confocal immunofluorescence microscopy, and DMT1 co-purification with mitochondrial markers during mitochondrial isolation as determined by immunoblotting. Moreover, in a split ubiquitin yeast-two-hybrid screen, the mitochondrial protein cytochrome C oxidase subunit II (COXII) was identified as an interaction partner of DMT1. This interaction was supported by co-immunoprecipitation of COXII with DMT1 from cell lysates. (Wolff N.A. et al. FASEB J. 28:2134-45, 2014)

Again using the split-ubiquitin yeast two-hybrid system, we determined that, at least when expressed in yeast, DMT1 associates with COXII through hydrophobic interactions. DMT1 localization was supported by DMT1 immunoreactivity of isolated rat kidney cortex mitochondria detected by confocal immunofluorescence microscopy, with the identity of mitochondria confirmed by MitoTracker® staining. Consistently, mitochondria isolated from HEK293 cells that could overexpress DMT1 under the control of a TET-ON promoter stained positive for DMT1 without induction while assessed by flow cytometry. Upon induction by doxycycline, DMT1 immunoreactivity in mitochondria increased. In line with previous data, partial mitochondrial localization of heterologously expressed DMT1 was not isoform-dependent. Most importantly, functional studies support a role of DMT1 in mitochondrial metal ion uptake. Thus, mitochondria isolated from stably DMT1-transfected HEK293 cells exhibited substantially higher uptake of the known DMT1 substrate manganese when the cells had been pretreated with doxycycline. Moreover, manganese uptake into mitochondria from induced cells was sensitive to a specific DMT1 inhibitor, with relative sensitivities of different isoforms similar to what had been observed for uptake into whole cells.

Taken together, these data further substantiate a role of DMT1 in mitochondrial uptake of metal ions, including manganese for antioxidant defense.
by the anti-inflammatory drugs indomethacin with high and salicylate with low potency. Pre-incubation of OAT2-transfected HEK293 cells with PGF$_{20}$ increased cGMP uptake by 21.5 ± 7.5 %.

**Conclusion:** Unlike OAT1 and OAT3, human OAT2 does not interact with dicarboxylates. In addition, human OAT2 is not affected by monocarboxylates as was observed earlier for the rat and mouse ortholog. As cGMP is taken up without any preincubation, OAT2 may function as an uniporter or may exchange against an - so far unknown - intracellular substrate. In contrast to OAT1 and OAT3, OAT2 is independent of $pH_{in}$. However, similar to OAT1 and OAT3, OAT2 interacts with prostaglandins and facilitates influx as well as efflux of PGF$_{20}$.

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**P042 Interaction partners of the SLC45 sucrose transporters**

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The members of the mammalian SLC45 family exhibit significant sequence similarities to plant sucrose transporters and have been implicated with the regulation of glucose homeostasis in the brain (SLC45A1), skin and hair pigmentation (SLC45A2), prostate cancer and myelination (SLC45A3). Recently we have shown that the three members A2, A3 and A4 are indeed proton-coupled sucrose transporters. These findings challenged existing concepts of mammalian sugar transport, as the members of the SLC45 family i) transport a disaccharide and ii) perform secondary active transport in a proton-dependent manner (Bartlóke et al., Biochem. J., 2014; 464(2):193-201).

In mouse tissues the expression of SLC45A2 mRNA appeared to be restricted to eyes and skin, that of SLC45A3 was found primarily in the prostate, but also in other tissues, whereas that SLC45A4 showed a predominantly ubiquitous expression. The broad distribution of SLC45 members suggests their possible involvement in various processes in animals.

To get some hints for possible functions and regulation mechanisms in the SLC45 family, we looked for interaction partners using a yeast two-hybrid screen as well as co-precipitation assays. In this way we found candidates involved in diverse cell processes such as carbohydrate metabolism, protein degradation, and signaling. The interaction of one signaling molecule identified by the yeast two-hybrid assay, 14-3-3$\gamma$, could be verified by Far-Western and direct pull-down assays for SLC45A3 and SLC45A4 and may link sugar uptake by the SLC45 members to metabolism. Furthermore, phosphorylation by protein kinase A was demonstrated for some SLC45 proteins in vitro. The physiological consequence of these interactions still needs to be elucidated, but already suggests a highly regulated mechanism of sugar transport by the SLC45 members.

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**P041 Negative regulation of the Creatine transporter CreaT SLC6A8 by SPAK and OSR1**

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**Background/Aims:** Transport regulation involves several kinases including SPAK (SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1), which are under control of WNK (with-no-K$[$Lys$]$) kinases. The present study explored whether SPAK and/or OSR1 participate in the regulation of the creatine transporter CreaT (SLC6A8), which accomplishes Na$^+$ coupled cellular uptake of creatine in several tissues including kidney, intestine, heart, skeletal muscle and brain.

**Methods:** cRNA encoding SLC6A8 was injected into Xenopus laevis oocytes with or without additional injection of cRNA encoding wild-type SPAK, constitutively active T233ESPAK, WNK insensitive T233ASPAK, catalytically inactive D212ASPAK, wildtype OSR1, constitutively active T185EOSR1, WNK insensitive T185AOSR1 and catalytically inactive D164AOSR1. Transporter activity was determined from creatine (1 mM) induced current utilizing dual electrode voltage clamp.

**Results:** Coexpression of wild-type SPAK and of T233ESPAK, but not of T233ASPAK or of D212ASPAK was followed by a significant decrease of creatine induced current in SLC6A8 expressing oocytes. Coexpression of SPAK significantly decreased maximal transport rate. Coexpression of wild-type OSR1, T185EOSR1 and T185AOSR1 but not of D164AOSR1 significantly down-regulated SLC6A8 activity. OSR1 again decreased significantly maximal transport rate.

**Conclusions:** Both, SPAK and OSR1, are negative regulators of the creatine transporter SLC6A8.

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**P043 Effect of the absorption enhancer sodium caprate on transport and barrier function in lung epithelia**

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Inhalation of compounds became an emerging strategy to administer drugs with avoidance of the digestive system. In this case, the respiratory epithelium forms the limiting barrier for compound uptake. Amphiphilic compounds and in particular sodium caprate are known to enhance the resorption and bioavailability of inhaled compounds. However, little is known about effects of those enhancers on the function of the respiratory epithelium. Thus we investigated the effect of sodium caprate on transepithelial water transport and barrier function in air/liquid interface cultivated NCI-H441 epithelia,
which are commonly used as a suitable model of the lung epithelium.
Sodium caprate decreased the transepithelial electrical resistance (TEER) in a time and dose dependent manner. 2 h after exposure, a significant decrease of TEER was found for concentrations of 300 µM sodium caprate and above. Addition of liquid to the apical surface of the epithelium resulted in fluid resorption. In the presence of sodium caprate, water transport rates remained unaffected. Investigation of the transepithelial osmotic water permeability ($P_{\text{osm}}$) revealed, that osmotically driven water flux was slightly reduced by the aquaporin (AQP) inhibitor copper in control epithelia. However, treatment with sodium caprate resulted in an increase of copper sensitive $P_{\text{osm}}$, whereas total $P_{\text{osm}}$ was not altered. In an expression screening of AQP transcripts, AQP 3, 4 and 10 were observed to be the most abundantly expressed AQP in NCI-H441 epithelia.

Our results demonstrate that sodium caprate induces an opening of the paracellular transport route without disturbing epithelial function. However, sodium caprate seems to modify the osmotically driven paracellular water flux and facilitate a copper sensitive water pathway, which probably depends on AQP.

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**P044**
Role of intracellular pH and Ca$^{2+}$-concentration on the pH-dependent endocytosis of macromolecules in tumor cells

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The abnormal vascular network is characteristic of solid tumors and can be utilized for improving chemotherapy by passive accumulation of nano-scaled macromolecules as drug carriers (enhanced permeability and retention effect). These carriers are then taken up into the cells by endocytosis and the cytotoxic drugs are cleared intracellularly. In parallel tumors often show pronounced glycolytic metabolism leading to extracellular acidosis. However, at present it is unclear whether the low extracellular pH affects the endocytotic uptake of macromolecules into tumor cells. The aim of the study was to determine macromolecule endocytosis under acidic conditions and to analyze the role of intracellular pH (pH$_i$) and Ca$^{2+}$-concentration [Ca$^{2+}$].

Rat AT-1 prostate carcinoma and the Walker-256 mammary carcinoma cell lines were investigated for endocytotic uptake of an Oregon Green 488 labeled 70 kDa dextran (as a marker substance for macromolecular drug carriers). Experiments were performed in bicarbonate buffered HEPES- and MES-Ringer adjusted to different pH. To investigate, whether endocytosis depends on intracellular or extracellular acidity cells were incubated with lactate in combination with the anion exchange inhibitor DIDS in order to solely acidify the intracellular compartment. A reduction of the [Ca$^{2+}$] was achieved by incubating cells with 0.005 mM Ca$^{2+}$. The pH$_i$ and [Ca$^{2+}$] was measured by BCECF or Fura-2 fluorescence, respectively. Extracellular acidosis had opposite effects on endocytosis in both cell lines investigated. In Walker-256 cells the dextran uptake increased with decreasing pH in an almost linear manner whereas in AT-1 cells the uptake linearly decreased. Since extracellular acidosis led to an intracellular acidification as well as a reduction of [Ca$^{2+}$] in both cell lines, the intracellular pH was reduced solely to separate the effects of intra- and extracellular acidosis. These experiments showed that in both cell lines intracellular acidification reduced the endocytotic dextran uptake. When lowering the [Ca$^{2+}$] (with constant pH 7.4) by 30-40% endocytosis was significantly increased in AT-1 cells but was slightly reduced in Walker-256 cells.

These results indicate that the endocytotic uptake of macromolecular drug carriers is markedly pH-dependent. But even more importantly this pH effect is cell line specific and cannot be attributed to changes of the pH$_i$. However, the (pH-dependent) change in [Ca$^{2+}$] could play an important role for the altered dextran uptake under acidic conditions.

**P045**
GABA transporters-mediated modulation of synaptic transmission in the subplate

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In the developing neocortex subplate neurons (SPns) form a transient layer between the cortical plate and intermediate and thalamo-cortical axons establish temporary synapses on SPns before innervating the cortical plate. Activity of these transient circuits is of critical importance for the formation of thalamo-cortical axons. It has been demonstrated that GABA transporters (GATs) modulate synaptic transmission in the superficial layers of the immature neocortex. Here we elucidate the role of GATs in the subplate. GABAergic and glutamatergic postsynaptic currents (GPSCs and EPSCs) were recorded from SPns using whole-cell patch-clamp technique. Baclofen, a specific GABA$_B$ receptor (GABA$_B$R) agonist, reduced evoked GPSC (eGPSC) amplitudes and increased the paired-pulse ratio (PPR). This effect of baclofen was inhibited by CGP55845, a selective GABA$B_R$ blocker. Similar effects of baclofen and CGP55845 were observed for evoked EPSCs. Thus presynaptic GABA$_B$Rs control the strength of GABAergic and glutamatergic synapses. NNC-711, a selective GABA$B$_1 blocker, increased eEPSC decay time, PPR and decreased eGPSC amplitudes. NNC-711-induced effects, except decay time change, were blocked by CGP55845, SNAP-5114, a specific GABA transporter-2/3 (GAT-2/3) blocker, decreased PPR, increased eGPSC amplitudes and did not change eGPSC decay time. We conclude that GABA$_B$Rs are tonically activated by extracellular GABA, which is, in turn released by GAT-2/3 operating in the reversed mode, whereas GAT-1 functions in the uptake mode.
**Vascular functions and circulation**

**P047**

**Importance of vascular smooth muscle cell EGFR for the contractile response to vasoactive substances in mouse aorta**

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The epidermal growth factor receptor (EGFR) plays an important role in cell proliferation, differentiation, survival, is involved in cardiovascular diseases and can be activated either by its classical ligands or by vasoactive substances, like endothelin-1 or epinephrine, a process called transactivation. Here we determine the contribution of vascular smooth muscle cells (VSMC) EGFR to vasoconstriction/dilation induced by vasoactive substances.

We used two models of mice with a deletion of EGFR in VSMC: (a) conditional inducible KO (iEGFRΔ/ΔVSMC, deletion in adult animal) (b) a conditional constitutive KO (EGFRΔ/ΔVSMCΔCM, prenatal deletion) and compared force generation of aortic rings from knockout and wildtype animals using a wire myograph.

Force generation or relaxation in response to KCl, serotonin, carbachol or the NO-donor SNAP was not affected by EGFR deletion. Force generation in response to endothelin-1 was not affected in the inducible model but significantly attenuated in mice with constitutive deletion of VSMC-EGFR. By contrast, the responses to norepinephrine and phenylephrine were significantly reduced in iEGFRΔ/ΔVSMCΔCM animals and in female animals of the EGFRΔ/ΔVSMCΔCM model. Histo-morphometry revealed no major alterations in aortae from knockout animals.

Our data show for the first time the stimulus-specific functional importance of VSMC-EGFR for the regulation of vascular tone via transactivation. We propose that VSMC-EGFR is functionally required for a full alpha1-adrenergic response but not for endothelin-1 or serotonin. Furthermore, VSMC-EGFR is required for the physiological development of vessel reactivity to endothelin-1.

**P048**

**Pregnancy hormones induce an increase of capillary density in the mouse heart**

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During pregnancy the maternal organism undergoes a variety of adaptive physiological changes. The cardiovascular system is strongly affected and its response is characterized by cardiac hypertrophy. The molecular mechanisms underlying these adaptations are still poorly understood. The recent postulation of resident organotypic stem cells in the cardiovascular system prompted us to explore the potential involvement of such cells and also the related signaling...
cascades regulating the adaptive process in the heart. We have analysed cell proliferation using immunostainings for pH3 and Ki-67 in mouse hearts at different stages during pregnancy. The number of Ki-67+ nuclei in hearts increased from virgin controls to gestational day 3 (GD3), peaked at GD14, and immediately stopped after delivery. Co-staining for cell-type specific markers revealed that approximately 2/3 of the proliferating cells were fibroblasts and 1/3 endothelial cells (ECs). The proliferation of ECs and fibroblasts reflects angiogenesis, which was underscored by an increase of capillary density, and extracellular matrix remodeling known to occur during pregnancy induced hypertrophy, respectively. In order to mimic the action of pregnancy hormones, female as well as male mice were injected subcutaneously with a mixed solution of progesterone and estrogen for periods of 3, 7, or 14 days. Capillary density in the harvested hearts was significantly increased after 3 days of treatment without induction of hypertrophy. We have analysed cardiac cell and EC proliferation using immunostainings for pH3 and Griffonia simplicifolia lectin I in heart sections of hormone treated and control mice. The portion of proliferating cardiac cells and ECs were significantly increased after 14 days of treatment. This indicates that increase in capillary density as well as proliferation of ECs is directly caused by pregnancy hormones. By quantitative RT-PCR we measured the mRNA levels of angiogenic factors in hearts of pregnant and hormone-treated mice. We observed a significant upregulation of pro-angiogenic factors at GD14 and 14 days after hormone treatment corresponding to an increase of proliferation. We are currently investigating the mechanisms underlying pregnancy hormones induced proliferation in cardiac ECs and fibroblasts.

P049
Nuclear factor of activated T-cells 5 controls the phenotype of stretch-activated vascular smooth muscle cells
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Background: During the onset of hypertension-induced vascular remodeling processes, vascular smooth muscle cells (VSMCs) are exposed to elevated wall stress or biomechanical stretch, which promotes a phenotypic switch of these cells from contractile to synthetic. Nuclear factor of activated T-cells 5 (NFAT5) has recently been reported to control their phenotype in vascular remodeling processes but its regulation and effects in stretch-stimulated VSMCs are unknown and thus was focus of this study.

Methods & Results: Cultured human arterial VSMCs were exposed to biomechanical stretch (13%, 0.5 Hz) and analyzed by immunofluorescence and protein-biochemical techniques. Stretch led to an increase of NFAT5 mRNA and protein within 24 hours and promoted its rapid translocation into the nucleus. Stretch-mediated NFAT5 protein synthesis but not mRNA expression was decreased by inhibition of c-Jun N-terminal kinase (JNK) through SP600125. Nuclear NFAT5 translocation was dependent on palmitoylation as evidenced by interfering with this post-translational modification through 2-bromopalmitate. Carnitine palmitoyl transferase family 1 (CPT1) was identified to be rate-limiting as their specific inhibition by Etopomor prevented NFAT5 from entering the nucleus in stretch-stimulated VSMCs. RNA microarray and CHIP analyses revealed that NFAT5 controls the expression of tenascin C (TNC) and ACTBL2 (K-Actin) under these conditions. Both gene products play an important role in VSMC migration. Consequently, siRNA-mediated knockdown of either NFAT5 or TNC inhibited planar migration compared to control siRNA-treated HUASMCs whereas siRNA-mediated knockdown of ACTBL2 led to a disturbed directional migration, which was analyzed by time-lapse live-cell imaging.

Conclusion: Biomechanical stretch of VSMCs triggers NFAT5 expression and nuclear translocation of this transcription factor. By controlling the expression of gene products orchestrating VSMC migration, this mechanism may be crucial for coordinating the migratory activity of VSMCs during maladaptive vascular remodeling processes as they occur as a consequence of hypertension.

P050
The histone demethylase JHDM1D is upregulated by oscillatory blood flow
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The Jumonji-C-domain containing histone demethylase 1D (JHDM1D) binds to histone 3 lysine 4 (H3K4) and demethylates H3K9me1/2 and H3K27me1/2 to activate gene expression in early development. Given that cellular dysfunction frequently recapitulates embryonic programs, we hypothesized that JHDM1D is induced in certain situations to alter gene expression.

PCR expression screening identified JHDM1D to be largely down-regulated in human umbilical vein endothelial cells. However, after cytokine treatment with TNFα and IL1β JHDM1D was induced on mRNA and protein level. Furthermore, application of oscillatory shear (15dyn/cm²), which is associated with atherosclerosis, to endothelial cells similarly induced JHDM1D whereas JHDM1D expression to laminar flow was unaffected. This finding was supported by in-vivo data, which showed a significant upregulation of JHDM1D in aortic endothelial cells with disturbed flow versus stable flow after partial ligation surgery. In line with this, the expression level of JHDM1D was also increased in human atherosclerotic plaques.

Therefore, JHDM1D is induced upon cellular activation. The data suggest that the enzyme plays a role in atherosclerotic development by regulating endothelial gene expression in response to inflammatory conditions.
The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that can be activated i) by its ligands, e.g. EGF or HB-EGF or ii) by intracellular signaling cascades induced by e.g. vasoactive substances binding to their canonical receptors. In the last years we demonstrated that in vascular smooth muscle cells (VSMC) the EGFR is involved in physiological blood pressure and arterial tissue homeostasis. The EGFR in VSMC has also been implicated in the induction and regulation of VSMC migration. Therefore we aimed to analyze the impact of EGFR on insulin like growth factor-1 (IGF-1), phenylephrine (PE), endothelin-1 (ET-1), thrombin and ATP induced VSMC migration.

ERK-1/2 phosphorylation in VSMC from wildtype animals (WT) was increased upon incubation with either IGF-1, PE, ET-1, thrombin or ATP. Only for PE, ET-1 or ATP a significant difference between WT cells or KO cells (deletion of EGFR) could be observed.

Cell proliferation was analyzed via a cell counting technique. FCS increased the proliferation of WT and KO cells. EGF increased proliferation in WT but not in KO cells. Proliferation was increased by thrombin and IGF-1 and in tendency by ET-1 (p=0.076) in WT but not in KO animals. PE and ATP had in both genotypes no effect on proliferation.

Cell migration was analyzed by wound closure assay and a modified Boyden chamber assay. Wound closure was slower in control KO cells compare to WT cells. There was no significant difference between WT or KO if wound closure was stimulated by FCS. Stimulation with EGF resulted in an accelerated wound closure by WT but not by KO cells. Wound closure was stimulated by ET-1 in WT but not in KO cells. PE and ATP had no effect on wound closure. IGF-1 increased wound closure in WT and in KO cells to the same extend. Thrombin stimulates wound closure in the WT but not in KO cells.

FCS increased transmigration in the Boyden chamber by WT and KO cells to the same extend. EGF increased transmigration by WT but not by KO cells. WT cells showed a higher transmigration when incubated with ATP compared to control. ATP had no effect on KO cells. PE and ET-1 had no effect, neither in WT nor in KO cells. Incubated with IGF-1 or thrombin resulted in a slight reduction in transmigration of KO but not of WT cells.

Our data show the differential importance of VSMC-EGFR for pathological relevant effects of vasoactive substances.

**P052 Regulator of G-protein signaling 5 balances smooth muscle cell activation during arteriogenesis**

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**Introduction:** Progressive occlusion of conduit arteries has inevitable consequences for the local tissue perfusion and may result in organ failure as it occurs during myocardial infarction. In this context, the growth of pre-existing collateral arteries may serve as a physiological response to evade the gradual loss of organ perfusion – a process referred to as arteriogenesis. During this biomechanically induced complex vascular remodeling process, collateral arteries are transformed to conductance arteries bypassing the occluded artery. While activation of smooth muscle cells is a prerequisite for the architectural alteration of the vessel wall, not much is known about the molecular determinants controlling their phenotype under these conditions. Here, we report that the onset of arteriogenic remodeling is accompanied with the expression of regulator of G-protein signaling 5 (RGS5) in smooth muscle cells (SMCs).

**Methods and Results:** Considering the relevance of flow-induced NO-release and wall stress or biomechanical stretch for arteriogenesis-dependent collateral growth, consequent in vitro experiments with cultured SMCs revealed that both determinants robustly elevates RGS5 mRNA expression and protein abundance in SMCs. In vivo, elevated RGS5 levels in SMCs of growing collateral arteries were associated with increased vascular SMC proliferation and RhoA activity. This turned out to be functionally relevant as arteriogenesis was reduced by 80% in RGS5-deficient mice as a result of reduced VSMC proliferation and RhoA activity. This was enhanced upon RGS5-over-expression and blocked by inhibiting Gαq/11-dependent signaling. Concurrently, RGS5 overexpression abolished the bradykinin-induced Gαq/11-dependent intracellular calcium release in SMCs.

**Conclusions:** All in all, our results point out that RGS5 inhibits Gαq/11-dependent signaling on the one hand and presumably indirectly promotes the Gα12/13/RhoA pathway on the other hand. This reveals a novel regulatory mechanism which is likely to be rate-limiting for the activation of vascular smooth muscle cells during the onset of arteriogenesis.
**P053**

The histone demethylase PHF8 is an epigenetic facilitator of the angiogenic phenotype

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Epigenetic modifiers by controlling the histone code are important regulators of cellular phenotype and expression maintenance. Although much progress has been made in understanding the histone code, the function of the H3K9me1 histone mark is incompletely understood. The jmj-domain containing histone demethylase PHF8 (Plant Homeodomain Finger 8) is an H3K9 demethylase of unknown vascular function. We hypothesized that PHF8 by demethylating H3K9 is required to maintain the endothelial angiogenic program. PHF8 protein was found to be highly expressed in the nucleus of human umbilical vein endothelial cells (HUVECs). siRNA-mediated knockdown of PHF8 in HUVECs impaired angiogenic sprouting and tube formation, whereas overexpression of PHF8, but not of a catalytically inactive mutant, facilitated angiogenic sprouting. To uncover the underlying mechanism of PHF8 in the vascular system, an Illumina gene array and subsequent RT-qPCR validation were carried out. Among others, the expression of the small nuclear RNA (snRNA) U1 was found to be dependent on PFH8: down-regulation of PFH8 significantly decreased snRNA U1. Likewise, overexpression of active PHF8 but not of a catalytically dead mutant augmented U1 snRNA expression. In keeping with this, chromatin immunoprecipitation revealed that knockdown of PHF8 induced the H3K9me1 histone mark at the U1 transcriptional start site (TSS), whereas a global reduction of the H3K9me1 was observed after PHF8 overexpression. U1 snRNPs is an essential member of a spliceosomal complex involved in recognition and binding onto the 5’-splice site of an intron of pre-mRNA, and it has not been described so far in endothelial cells. Thus, by maintaining U1 snRNP expression, PHF8 most likely governs endothelial mRNA splicing, which is essential for the maintenance of the endothelial angiogenic phenotype.

**P055**

Regulation of venous smooth muscle cell phenotype switch during the development of varicose/spider veins

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**Introduction:** Varicose veins are commonly associated with swollen and painful legs which may potentially develop complications including skin ulcers, superficial thrombophlebitis, bleeding and inability to stand or to walk. Although not comparable in the extent of their severity, spider veins are defined as dilated, intradermal venules less than 1 mm in diameter which resemble varicose veins on a much smaller scale. We have previously shown that an increase in venous filling pressure is *per se* sufficient to support varicose-like vein formation in mice by activating venous smooth muscle cells and promoting venous remodelling. In this context, we revealed that stretch-induced activation of venous smooth muscle cells was dependent on activator protein-1 (AP-1), a leucine zipper heterodimeric transcription factor composed of proteins belonging to the c-Fos, c-Jun, ATF and JDP families.

**Methods and results:** To investigate whether spider veins are directly connected to the deeper vein plexus and thus exposed to elevated venous pressure levels, blood perfusion of a spider vein (left leg, knee level) was measured from a healthy female volunteer using the PeriCam PSI blood perfusion imager. An increase in blood perfusion of a spider vein was observed when the volunteer was asked to actively contract the muscles of the leg as opposed to the low perfusion levels after prolonged standing without any muscle contraction. We therefore assume that superficial veins may also be affected by chronic venous hypertension. Consequently, the aim of this study is the identification and manipulation of mechanisms which control the phenotype switch of venous endothelial and smooth muscle cells upon exposure to elevated levels of biomechanical stretch, specifically: (i) the role of ROS and NAD(P)H oxidase in venous remodelling processes and (ii) the role of AP-1 complex and its upstream regulators. We have utilized *in vitro, ex vivo* and *in vivo* models to better understand the underlying mechanisms of stretch-induced vein remodelling and varicose/spider vein development. To this end, human umbilical vein smooth muscle cells were exposed to biomechanical stretch for up to 48 hours, which increased their MMP2/9 expression and gelatinase activity. In order to investigate the role of ROS production in stretch-dependent venous smooth muscle cell remodelling, superoxide production was measured utilizing dhydroethidium, a superoxide indicator.

**Conclusions:** Collectively, our findings support the hypothesis that spider veins are directly connected to the deeper venous plexus and may thus be affected by an increased filling pressure in the underlying venous network, thereby promoting ROS production and an increased proteolytic activity in their vessel wall.

**P056**

JARID1B represses HOXA5 and thereby maintains angiogenesis

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Epigenetic regulation involves histone methylation and the methylation state is dependent on histone methyl-transferases but also by demethylases. Histone H3 is one of the most frequently modified histones. Methylation at H3-lysine-4 (H3K4me3) if located in promoter region, increases gene expression whereas removal of this histone mark silences transcription. JARID1B is a H3K4me3 demethylase and...
highly expressed in endothelial cells (ECs), but its function has not been studied in this cell type. We hypothesized that JARID1B is required for endothelial gene repression and thereby maintaining EC functions. Knockdown of JARID1B by shRNA in human umbilical vein endothelial cells (HUVECs) attenuated angiogenic sprouting, tube formation and cell migration in the scratch wound assay. Importantly, also 2-4(4-methyl/phenyl)-1,2-benzisothiazol-3-(2H)-one, a pharmacological JARID1B inhibitor, diminished the angiogenic capacity of ECs. To clarify the importance of JARID1B function in the vascular system, JARID1B knockout mice were studied. As global knockout of the gene is embryonic lethal, tamoxifen-inducible conditional knockout mice were generated. Genetic deletion of JARID1B attenuated retina angiogenesis and endothelial sprout outgrowth from aortic segments. To uncover the underlying mechanism, exon arrays were performed. Depletion of JARID1B in HUVECs and in mouse lung ECs altered HOX gene expression. Importantly, the anti-angiogenic HOX5, also a marker for quiescent ECs, was induced by knockout of JARID1B on RNA and protein level. Consistently, chromatin immunoprecipitation revealed that JARID1B occupied and changed the H3K4me3 mark in the HOX5 promoter, suggesting a role of JARID1B in endothelial HOX gene regulation.

**Conclusion:** JARID1B, by suppressing HOX5, maintains the endothelial angiogenic phenotype.

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**P057 Mimicking angiogenesis in vitro**


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Compensatory repair processes as they occur during wound healing and upon tissue ischemia is accompanied with the formation of new capillaries from pre-existing capillary networks which is referred to as angiogenesis. To mimic this process in vitro, we developed a three dimensional in vitro angiogenesis assay that is based on collagen gel-embedded, size-defined spheroids generated from cultured human umbilical vein endothelial cells (HUVECs). By utilizing time-lapse analyses, we show that tip cells lead the formation of capillary-like and partially lumenized sprouts originating from the spheroids. Angiogenic sprouting was inducible in spheroids from five different primary cultured human endothelial cell types and stimulated by less than 1 ng/ml FGF-2 or VEGF 165. However, while this assay allowed for a rapid and reproducible determination of functional IC₅₀ values of anti-angiogenic determinants such as the small kinase inhibitors SU5402 and SU5614, the sprouting results were partially affected by the HUVEC passage number and lot variability. To overcome this limitation, immortalized HUVEC were used which show an enhanced stability in both their phenotype and sprouting response over multiple population doublings as evidenced by detecting the abundance of VE-Cadherin and Angiopoietin-2 as well as determining the VEGF-responsiveness of these cells. Collectively, the spheroid-based angiogenesis assay provides a sensitive and versatile tool to study the impact of pro- and anti-angiogenic determinants on multiple steps of the angiogenic cascade. It is compatible with different endothelial cell types and allows usage of immortalized HUVEC to improve its overall robustness.

**P058 Impact of epidermal growth factor receptor deletion on antidiuretic hormone effects in vascular smooth muscle cells**

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**Introduction:** The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that can be activated not only by its cognate ligands but also transactivated by e.g. stress, radiation, GPCR-ligands or vasoactive substances, like components of the renin-angiotensin-aldosterone-system (RAAS) or vasopressin (ADH). Therefore the EGFR acts as an integrator for various signalling cascades and modulates cellular functions like migration, proliferation and matrix homeostasis. Unfortunately, little is known about the role of EGFR for the expression and action of receptors for vasoactive substances.

**Methods and Results:** We generated a mouse model with a deletion of the EGFR in vascular smooth muscle cells (VSMC). The mRNA expression of the receptors for endothelin-1, norepinephrine, angiotensin II and vasopressin were analysed in freshly isolated aortas and in cultivated aortic VSMC. Furthermore, the effect of ADH on VSMC proliferation and migration was investigated. In aortas of knockout animals the expression of vasopressin receptor was elevated, while the expression of adrenoreceptor alpha-1d and beta-2 was reduced. In primary aortic VSMC in culture these differences were no longer present. We then analysed the impact of the deletion of EGFR on ADH mediated effects on VSMC migration and proliferation. 100nM ADH increased ERK 1/2 phosphorylation in cells from wildtype but not in cells from knockout animals. Regarding proliferation, no effect of ADH on VSMC could be observed. In a wound closure assay ADH stimulated migration after 4, 8, 24 and 48 h in wildtype but not in knockout cells. In a modified Boyden chamber assay ADH had no effect on transmigration neither in wildtype nor in knockout animals.

**Conclusions:** EGFR-expression is required for the promigratory effect of ADH in VSMC. This is possibly linked to the EGFR-dependence of ADH-induced MAPK activation, but not a result of altered ADH receptor expression. However EGFR expression impacts ADH receptor expression in aortic tissue. Yet, this effect is lost in primary culture. In conclusion, EGFR impacts on ADH-receptor expression but also on VSMC migration upon ADH stimulation.
A role for cytochrome P450 2c44 and the soluble epoxide hydrolase in tumour formation and metastasis

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**Question:** Polyunsaturated fatty acids (PUFAs) are metabolized by cytochrome P450 (CYP) epoxygenases to bioactive epoxides and then further metabolized by the soluble epoxide hydrolase (sEH) to their respective diols. The deletion of the sEH and supra-physiological arachidonic acid epoxide levels have been linked with tumour formation and metastasis but the role of endogenously generated epoxides and diols is not completely understood. The aim of this project was to determine the role of Cyp2c44 and the sEH in tumour formation, tumour angiogenesis as well as metastasis.

**Methods:** sEH-/- and Cyp2c44-/- mice were crossed with polyoma middle T oncoprotein (PyMT +/-) mice to generate animals that spontaneously develop breast cancer. Female mice were sacrificed at 20 weeks, and total tumour weight, volume and metastasis formation were studied. Angiogenesis and lymph angiogenesis were determined by immunohistochemistry for CD31 (endothelial cell marker) and lymphatic vessel endothelial hyaluronan receptor (LYVE1; lymph endothelial cell marker). Lung and lymph node metastasis were analysed by H&E staining.

**Results:** There was a significant increase in the weight and volume of tumours from sEH-/-xPyMT+/- and Cyp2c44-/-xPyMT+/- mice versus the control PyMT +/- group. However, tumours from Cyp2c44-/-xPyMT+/- mice showed more prominent LYVE1 than CD31 staining in PyMT+/- mice while tumours from sEH-/-xPyMT+/- mice demonstrated a higher level of CD31 staining. The more prominent lymph angiogenesis in animals lacking Cyp2c44 was coincident with a significantly increased rate of pulmonary metastasis versus sEH-/-xPyMT+/- and PyMT+/- mice. An in vitro confrontation assay in which murine ES cells were cultured in spheroids impregnated with tumour cells revealed that epoxides of arachidonic acid and linoleic acid potentiated the differentiation of CD31 expressing cells while the corresponding diols increased the number of LYVE1-expressing cells.

**Conclusions:** The deletion of Cyp2c44 versus the sEH has differential effects on lymph angiogenesis and metastasis. From the confrontation assay it seems that the balance between epoxides and diols has marked consequences for endothelial cell versus lymph endothelial cell development and it is tempting to speculate that alterations in lymphangiogenesis can account for the differences in metastasis. It will be important to identify the PUFA epoxides and diols altered in the primary tumours and to determine whether altering dietary lipids can affect these responses.

**Fig 1** Cyp2c44 and sEH role in tumour growth and vasculization

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**P060**

Influence of the catalytic subunit beta of calcineurin on vascular signaling and function

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**Introduction:** Calcineurin (PPP3C) is a serine/threonine-dependent phosphatase consisting of a catalytic and a regulatory subunit. The catalytic subunit CnAα und CnAβ are expressed ubiquitously. By dephosphorylation, PPP3C can modulate the activity of important transcription factors like CREB and NFAT and thereby influencing immune response, cell proliferation, antioxidant capacity and composition of extracellular matrix. The beta isoform has been shown to be involved in cardiac hypertrophy. Cell culture data suggest it is regulated by components of the renin-angiotensin aldosterone system known to elicit pathological effects in the vascular system whereat only little is known about the effect of PPP3C in vascular system. Enhanced activation of CnAβ is known to harbor pro-inflammatory properties in vascular smooth muscle cells (VSMC) and hypertrophic as well as apoptotic characteristics in cardiomyocytes. Therefore, we investigate the relevance of CnAβ subunit for vascular signaling and function.

**Methods and Results:** To investigate the role of CnAβ, we compare mice carrying a ubiquitous deletion of CnAβ (KO) and wildtype littermates (WT) under basal conditions. We could confirm deletion of CnAβ in real time PCR and Western blot experiments without compensatory upregulation of CnAα. Concerning organ weights, no differences were found between the different genotypes except a slight reduction in left ventricle weight in KO animals compared to WT. Basal systolic and diastolic blood pressure were identical in WT and KO mice. But after long term stimulation (4 weeks) with angiotensin II, known to induce hypertension in mice, gender-specific differences in systolic blood pressure could be detected. Under basal conditions no differences in gene expression of extracellular matrix proteins (e.g. collagen I, III,
IV and fibronectin) and inflammatory markers (e.g. MCP1, osteopontin, TGFβ, PAI-I and CCL5) could be detected between KO and WT mice. However, primary VSMC of KO mice had a significantly higher activity in glucose-6-phosphate-dehydrogenase and were less prone to necrosis measured by LDH release, suggesting that deletion of CnAβ exerts protective effects in VSMCs.

**Conclusion:** Overall, our results suggest that CnAb is of relevance for vascular signaling and function and future studies will look at the effects and underlying mechanisms in more detail.

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**P061**

**Characterization of endothelial cell-specific PECAM/eGFP expression in a transgenic mouse model**

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**Question:** In order to monitor vascular development and to test potential pro- and antiangiogenic treatments in vitro and in vivo, endothelial-specific reporter gene models are highly valuable tools. However, available mouse models have limitations in regard to their expression pattern (e.g. expression exclusively at the embryonic stage or only in small vessels of adult animals). Therefore new endothelial-cell specific reporter gene models are required. We have chosen to create a reporter gene model where eGFP is driven by the platelet endothelial cell adhesion molecule (PECAM) promotor. This promotor was selected because PECAM is known to be highly expressed at all developmental stages and almost all types of vessels.

**Methods:** We have used bacterial artificial chromosomes (BACs) for the establishment of a PECAM/eGFP ES cell line and this line was used to generate transgenic mice via the diploid aggregation method. PECAM/eGFP expression was characterized in ES cells, mouse embryos at different developmental stages and adult mouse organs using fluorescence microscopy. The endothelial-specific expression pattern of PECAM/eGFP was assessed in cryosections of tissues using immunohistochemical stainings.

**Results:** In differentiating ES cells eGFP+ vascular sprouts could be detected. Mouse embryos at embryonic day 9.5 showed strong eGFP expression in the developing vasculature such as branchial arteries, outflow tract of the heart, dorsal aorta and intersomitic vessels. In adult mice eGFP expression was found in vascular networks of various organs and tissues (e.g. heart, brain, kidney, liver, spleen, lung, intestine, skeletal muscle) and vessels of all calibres. When comparing the expression patterns of PECAM/eGFP mice and flt-1/eGFP mice, where the endothelium of large arteries was not labelled, we found in PECAM/eGFP mice that the endothelium of aorta, carotid and coronary arteries displayed strong eGFP expression.

**Conclusion:** Thus, transgenic PECAM/eGFP ES cells and transgenic mice display prominent eGFP expression throughout the vasculature and are very good models to monitor vessel formation during embryonic development and in the adult stage.

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**P062**

**Influence of epidermal growth factor receptor on thromboxane receptor mediated effects in vascular smooth muscle cells**

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**Aim:** In vascular smooth muscle cells (VSMC) both epidermal growth factor receptor (EGFR) and thromboxane A2 receptor (TPR) are involved in physiological - e.g. regulation of vascular tone or vascular homeostasis - and pathophysiological processes like vascular remodeling. As the EGFR can be transactivated by various vasoactive stimuli, we aimed to analyze if part of the TXA2 effects are mediated by transactivation of the EGFR.

**Methods and results:** We employed immortalized aortal VSMC of rats (A7r5) and primary aortal VSMC (aVSMC) of mice with (KO) or without (WT) EGFR-deletion, as well as aortic rings from this animal model. In aVSMC from WT mice and A7r5 cells U46619 - a TXA2 analogue - induced phosphorylation of EGFR and an increase in ERK1/2 phosphorylation. This effect could be inhibited by AG1478, an EGFR antagonist, in A7r5 cells. In aVSMC from KO animals no increase in ERK1/2 phosphorylation could be observed during incubation with U46619. Intracellular calcium increase was analyzed via single cell Fura-2 measurements. U46619 induced an increase in intracellular calcium in A7r5 cells. This effect could be reduced by preincubation of the cells with AG1478. Migration was measured by in vitro wound closure assay. In aVSMC, U46619 reduced the gap closure compared to vehicle treated cells derived from wildtypes. This effect was abolished in cells with a deletion of the EGFR.

**Conclusion:** The TPR mediates a range of its cellular effects through transactivation of the EGFR, like ERK 1/2 phosphorylation, Ca2+-signaling, inhibition of VSMC migration and proliferation. But the EGFR seems not to be necessary for TXA2-induced vasoconstriction.
Blood, immune system and inflammation

P063
Detection of LPS by a Luciferase-reporter cell line expressing Toll-like receptor 4

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Numerous microbial, fungal and viral pathogens try to invade the human body and are fought by the mechanisms of innate and adaptive immunity. The innate immune system is activated within seconds after pathogens pass the skin or mucosal barriers by recognizing conserved pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) belong to the repertoire of innate immune receptors that recognize PAMPs such as the bacterial cell wall component Lipopolysaccharide (LPS). TLR activation provokes a signaling cascade resulting in the translocation of the transcription factor NF-κB from plasma to the nucleus. The activation of NF-κB target genes leads to inflammatory and antibacterial responses.

We generated a stable isogenic reporter cell line expressing the TLR4 and its cofactors MD2 and CD14. The recognition of LPS by the TLR4 receptor complex initiates the binding of NF-κB to the NF-κB-specific response element of the luciferase reporter construct.

The stimulation of this stable cell line with LPS or heat inactivated E.coli cells leads to a dose-dependent activation of the reporter gene. By using a measurement protocol without cell lysis, the luciferase activity could be observed for 24 hours or more.

An optimized version of the system may potentially be used as a biosensor for bacterial components in liquid substrates or solutions.

P064
The purinergic receptor P2X4 is involved in mucin secretion and inflammatory processes of the human upper airways

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We have recently described a “fusion-activated” Ca2+-entry (FACE) via vesicular P2X4 receptors in primary alveolar type II cells (ATII) (PNAS, 2011 Aug 30; 108(35): 14503-8). FACE regulates fusion pore expansion and facilitates pulmonary surfactant release during the post-fusion stage of lamellar body (LB) exocytosis. Mucin secretion via exocytosis of mucin granules in goblet cells in the airways shares many similarities with surfactant secretion from ATII cells. Hence, it was the aim of this study to investigate a potential role of FACE and P2X4 receptors in mucin secretion under physiologic and pathophysiologic conditions.

Primary human trachea epithelial cells (HTECs) were grown on filters at air liquid interface (ALI) for a time span of one month. Interleukin-13 (IL-13) treatment was used to induce an inflammatory response, goblet cell hyperplasia and hypersecretory phenotype. RT-PCR and immuno staining confirmed up-regulation of MUC5AC - one of the predominant mucins in the human respiratory tract and a marker for goblet cell proliferation - following IL-13 treatment. In addition, we also detected up-regulation of P2X4 in HTECs treated with IL-13 by RT-PCR, immuno staining and western blotting experiments. MUC5AC secretion was further investigated in ELISA experiments. Interestingly, stimulation of mucin secretion in the presence of ivermectin - a potent P2X4 potentiator - resulted in an increase in MUC5AC secretion.

In summary our results show that treatment of HTECs with the inflammatory cytokine IL-13 causes goblet cell hyperplasia associated with mucus hypersecretion. We found a correlation between up-regulation of MUC5AC and P2X4 receptor expression following IL-13 treatment and a stimulatory effect of P2X4 receptor activation of mucin secretion. Hence, we speculate that purinergic P2X4 receptors might play a role for mucin secretion and inflammatory lung diseases.

P065
CD44 sensitive platelet activation and in vitro thrombus formation

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**Question:** CD44 is required for signaling of macrophage migration inhibitory factor (MIF), an anti-apoptotic pro-inflammatory cytokine. MIF is expressed and released from blood platelets, key players in the orchestration of occlusive vascular disease. Nothing is known about a role of CD44 in the regulation of platelet function.

**Methods:** The present study thus explored whether CD44 modifies degranulation (P-selectin exposure), integrin activation, caspase activity, phosphatidylserine exposure on the cell surface, cell volume, Orai1 protein abundance and cytosolic Ca2+-activity ([Ca2+]i). Platelets from mice lacking CD44 (cd44−/−) were compared to platelets from corresponding wild-type mice (cd44+/+). P-selectin and dllbβ3-integrin abundance at the platelet surface were estimated with Fluorophore-labeled antibodies, caspase-3 activity with CaspGlow Fluorescein, phosphatidylserine abundance with FITC-labelled annexin-V, cell volume with forward scatter, Orai1 protein surface abundance with CFTM488A conjugated antibodies, [Ca2+], with Fluo-3 fluorescence and in vitro thrombus formation with a flow chamber.

**Results:** In resting platelets, P-selectin abundance, dllbβ3 integrin activation, caspase-3 activity and phosphatidylserine exposure were negligible in both genotypes and Orai1 protein abundance, [Ca2+], and volume were similar
in cd44-1 and cd44+ platelets. Thrombin (0.02 U/ml) increased P-selectin abundance, αIIbβ3-integrin activation, caspase-3 activity, phosphatidyserine exposure, [Ca2+]i, and surface Orai1 protein abundance, effects significantly more pronounced in cd44-1 than in cd44+ platelets. Thrombin further decreased forward scatter in cd44-1 and cd44+ platelets, an effect which tended to be again more pronounced in cd44-1 than in cd44+ platelets. Moreover, thrombosis formation under arterial shear rates was significantly augmented in cd44+ platelets.

**Conclusion:** Genetic deficiency of CD44 augments activation and pro-thrombotic potential of platelets.

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**P066**

**SHP-2 dependent modulation of IL-1β induced endothelial inflammatory responses**

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**Question:** The Src homology-2 domain containing tyrosine phosphatase (SHP-2) is known to be part of cytokine and growth factor dependent signalling processes in many cell types. Here we investigated whether changes in SHP-2 affect the endothelial response to IL-1β with regard to inflammatory dependent expression of adhesion molecules and subsequent neutrophil adhesion.

**Methods:** Wild type (WT) SHP-2, as well as the dominant negative (SHP-2 CS) or constitutively active (SHP-2 E76A) protein mutants were overexpressed in human umbilical vein endothelial cells (HUVEC) by lentiviral transduction. Expression of the adhesion molecules ICAM and VCAM upon stimulation with 10ng/ml IL-1β (4h and 24h) was assessed using flow cytometry and western blot. Adhesion of neutrophils to HUVEC under low shear (1dyn/cm²) was studied using a flow chamber assay. Effects on NFκB and the signalling molecules p38, AKT, HSP27 were studied by western blot, and, functionally, using specific pharmacological inhibitors. NFκB activation was assessed by immunofluorescence staining and detection of DNA binding activity in nuclear extracts.

**Results:** Lentiviral transduction yielded similar amounts of SHP-2 mutant protein expression. Cells overexpressing SHP-2 E76A expressed significantly less ICAM and VCAM than SHP-2 WT upon stimulation with IL-1β (p<0.05, n=5). In contrast, SHP-2 CS cells expressed significantly higher amounts of adhesion molecules on their surface (p<0.05, n=5). Likewise, adhesion of neutrophils to HUVEC was either enhanced (SHP-2 CS) or decreased (SHP-2 E76A) compared to SHP-2 WT (p<0.05, n=3). These effects could be assigned to SHP-2 dependent modulation of NFκB activity, as SHP-2 E76A expression reduced nuclear translocation of p65 (p<0.05, n=3) and SHP-2 CS expression enhanced IL-1β dependent DNA binding activity (p<0.05, n=4). In addition, the NFκB inhibitor Ro106-9920 (10µM) blocked the effects of mutants on adhesion molecule expression. SHP-2 CS expression enhanced IL-1β dependent activation of p38, Akt and HSP27, whereas SHP-2 E76A expression prevented this response (p<0.05, n=4). Pharmacological inhibition of p38 (SB203580, 10µM) also significantly reduced the IL-1β induced upregulation of adhesion molecules (p<0.05, n=6-8) as well as the activation of Akt and HSP27 (n=3-6).

**Conclusion:** SHP-2 activity (as assessed by the effects of constitutively active or dominant negative mutants) effectively modulates the IL-1β induced upregulation of adhesion molecules most likely acting upstream of a p38/Akt/HSP27 cascade which controls NFκB activity. Thus, increases in SHP-2 activity reduce the development of an endothelial proinflammatory state under the influence of IL-1β, one of the key signaling molecules in diabetes.

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**P067**

**Extracellular acidosis fosters inflammatory processes in fibroblasts via cAMP-phosphatase-MAPK-signaling**

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Various states of disease (tumor, ischemia and inflammation) are characterized by a reduced extracellular pH (acidosis) that affects disease progression via changes in gene expression and signaling of the residing cells. In our study the impact of acidosis on omnipresent fibroblasts was analyzed in concern of the expression of inflammatory markers (COX-2, iNOS, MCP-1, TNF-α) and of the signaling pathways involved. Normal rat kidney fibroblasts (NRKF) were challenged with bicarbonate-buffered Ringer’s solution pH 7.4 (control) and pH 6.6 (acidosis) for an incubation period of 3 h to 6 h, harvested and analyzed by qPCR or Western blot. Acidosis led to an increased mRNA and protein expression of COX-2, iNOS and TNF-α. Additionally, Akt, CREB, ERK1/2 and p38, but not JNK signaling was stimulated in an acidic microenvironment. Activation of p38 was critical for the induction of COX-2 and iNOS expression and was based on an inhibition of CAMP signaling and Ser/Thr-phosphatase activity at pH 6.6. Thus, acidosis affects inflammatory programs in fibroblasts by elevated expression of inflammatory markers and activation of p38 signaling that is involved in stress response. This could aggravate the pathological situation and lead to tissue remodeling and fibrosis.

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**P068**

**IL-13 impairs lung epithelial barrier function via modulating claudin 8**

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Elevated TH2 cytokine levels and in particular IL-13 levels are major characteristics of allergic asthma bronchiale. Allergic asthma is usually treated with glucocorticoids (GC),
which are subsequently introduced at increasing dosages during therapy according to disease progression. The anti-inflammatory effect of GCs is the predominant objective of this treatment. GCs are also known to modulate epithelial barrier function, but their effect on respiratory epithelia at elevated IL-13 levels has not been investigated so far. Herein we addressed the question if IL-13 affects epithelial tight junctions and if GCs may antagonize those effects. We used the epithelial cell line NCI-H441 cultivated at air/liquid interface (ALI) as a well-established model of distal lung epithelium. While the GC dexamethasone (Dex) increases the trans epithelial electrical resistance (TEER), IL-13 had almost no effect on TEER. However, IL-13 treatment resulted in a decrease of Cl- selectivity of tight junctions, which was accompanied by a down-regulation of cldn-8 expression and a decreased cldn-8 phosphorylation. RT-PCR experiments revealed the expression of IL-13Ra1 and IL-4Ra receptors, but only IL13-Ra1 expression was upregulated by Dex. Our data give evidence that the IL-13 impairs tight junction properties via interfering with cldn8 expression and phosphorylation. The observation that Dex upregulates IL13Ra1 receptors points towards the possibility, that GC treatment might sensitise lung epithelia to IL-13. Hence, GC treatment could be counterproductive with respect to epithelia barrier function in allergic asthma patients.

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P069
Endothelial cells counteract a genetically determined nitric oxide deficit by enhancing the release of the anti-inflammatory prostaglandin 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2).

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Blood flow-generated shear stress (FSS) is the major determinant of endothelial nitric oxide synthase (NOS3) expression. In humans, a promoter variant of the NOS3 gene, the C-variant of the T−786C single nucleotide polymorphism (SNP), renders the gene insensitive to shear stress, resulting in a reduced endothelial capacity to generate nitric oxide (NO). Endothelial dysfunction, commonly associated with decreased NO availability, may facilitate vascular inflammation. Consequently, individuals homozygous for the C-variant have an increased risk of developing cardiovascular and rheumatic diseases.

However, there are at least two mechanisms by which insufficient NO production can be counteracted in CC-genotype endothelium, one of which involves a multi-component pathway leading to the increased release of the anti-inflammatory prostaglandin 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2). FSS up-regulated expression of COX-2 and L-PGDS, the rate-limiting enzymes for 15d-PGJ2 synthesis, solely in CC-genotype ECs. Only these cells revealed an increased release of 15d-PGJ2 in response to FSS, and pre-treatment with 15d-PGJ2 exerted a pronounced anti-inflammatory effect on monocytes, demonstrated by its inhibitory effect on interleukin-1 beta (IL-1β) expression, a marker for monocyte pro-inflammatory activation. We found that this inhibition occurs at the transcriptional level, as 15d-PGJ2 repressed tumor necrosis factor-α (TNF-α)-induced IL-1β promoter activity in transiently transfected HEK 293 cells. In addition, exogenously added 15d-PGJ2 significantly reduced the transmigration of monocytes through EC monolayers. The anti-inflammatory activity of 15d-PGJ2 in monocytes may involve an induction of the Nrf2/anti-oxidant response element (ARE) pathway. Similar to 15d-PGJ2, constitutively activated Nrf2 attenuated the expression of IL-1β.

Despite an inadequate capacity to form NO, CC-genotype ECs reveal a robust anti-inflammatory phenotype due to an up-regulation of 15d-PGJ2 synthesis in response to FSS.

P070
The polarity protein Scribble mediates the inflammatory response of endothelial cells

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The polarity protein Scribble is required for endothelial cell angiogenesis by facilitating integrin α5 recycling to the plasma membrane. As cellular adhesion molecules are also subject of turnover, we hypothesized that Scribble is required for endothelial inflammatory signalling. Stimulation of human umbilical vein endothelial cells (HUVEC) with TNFα or LPS induced an inflammatory response as evident by the induction of VCAM-1. Down-regulation of Scribble greatly attenuated this effect in cultured cells. Moreover, in endothelial specific conditional Scribble knockout mice LPS treatment resulted in attenuated VCAM-1 induction. These effects were functionally relevant as the VCAM-1 mediated adhesion of leukocytes to TNFα-stimulated endothelial cells in culture was attenuated. Even more importantly, in vivo the VCAM-1 mediated leukocyte adhesion after TNFα stimulation was reduced after endothelial-specific deletion of Scribble, as determined by intra-vital microscopy in the cremaster model.

Subsequent mechanistic studies revealed that only Scribble but not other polarity proteins are required for inflammatory signalling. The effect, however, was matrix and integrin α5 independent suggesting that it was different from those operative in Scribble-induced angiogenesis. Moreover, deletion of Scribble had no impact on typical inflammatory pathways as NFκB- and MAP kinase. Co-immunoprecipitation/mass spectrometry uncovered the zinc finger transcription factor “GATA-like-protein” (GLP-1) as a novel Scribble interacting protein. GLP-1 represses the transcription factor GATA6, which is a positive mediator of VCAM-1 expression in TNFα-activated human endothelial cells. Indeed, deletion of Scribble reduced the cytosolic but increased the nuclear abundance of GLP-1.
Thus, scrib supports endothelial inflammatory signalling in vitro and in vivo by trapping GLP-1 in the cytosol. Inhibition of Scrib is a novel anti-inflammatory strategy.

P071
Impact of arachidonic acid and the leukotriene signaling pathway for vasculogenesis of embryonic stem cells

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Embryonic stem (ES) cells can differentiate into various kinds of cells, such as endothelial and hematopoietic cells. Previous studies have demonstrated that angiogenesis and leukopoiesis are two related mechanisms. In addition, some evidence suggested that inflammation mediators such as leukotrienes, which include the 5-lipoxygenase (5-LOX) family, can regulate endothelial cell differentiation. In the present study the eicosanoid precursor arachidonic acid (AA) dose-dependent stimulated vasculogenesis of ES cells by increasing vascular structures positive for CD31 and VE-cadherin as well as leukocyte markers. This effect was blunted upon inhibition of the rate limiting enzyme in the leukotriene signaling pathway, 5-lipoxygenase activating protein (FLAP), by AM643 and REV9501. Vasculogenesis could be significantly restored upon exogenous addition of leukotrienes (LTs). Furthermore the BLT1 receptor blocker U75302, the BLT2 receptor blocker LY255283 as well as the CYsLT blocker BAY-u9773 inhibited vasculogenesis of ES cells. Arachidonic acid treatment of differentiating ES cells increased reactive oxygen species (ROS) generation. Inhibition of ROS generation by either the free radical scavengers vitamin E or N-(2-Mercaptopropionyl)glycine (NMPG) or the NADPH oxidase inhibitor VAS2870 downregulated vasculogenesis of ES cells and decreased binding of the monocytes to the eGC. In summary our data demonstrate that pro-inflammatory arachidonic acid stimulates vasculogenesis and leukopoiesis of ES cells via the leukotriene pathway by mechanisms involving ROS generation.

P072
Salt triggers monocyte-adhesion on vascular endothelium

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Question: Inflammatory processes are hallmarkmed by binding of monocytes to adhesion molecules at the surface of endothelial cells and subsequent transmigration into the subendothelial matrix. Synergistic effects of salt (NaCl) and aldosterone may support vascular inflammation which is characterized by the recruitment of monocytes and associated with endothelial dysfunction hence contributing to the development of atherosclerosis. An important target of inflammatory processes is the endothelial glycocalyx (eGC), a negatively charged mesh of proteoglycans, covering the surface of endothelial cells. Recently, it was shown that the eGC functions as a Na⁺ buffer barrier with protective effects on the endothelium. Since high Na⁺/aldosterone leads to a collapse of the eGC, we tested the hypothesis that under such conditions the binding of monocytes to the endothelial surface is altered.

Methods: Human endothelial cells (EA.hy 926) were grown for 24 hours in low (130 mM) and high (150 mM) Na⁺ concentrations in the presence of aldosterone (1 nM), TNFα (0.57 nM) and with/without the aldosterone antagonist spironolactone (100 nM). To test the adhesion of monocytes to the endothelial surface fluorescently labeled monocytes (anti-CD14) were seeded onto confluent endothelial monolayers for 5h and quantified afterwards. To test the barrier function of the eGC, the adhesion forces between immobi- lized monocytes and the eGC were quantified with a specialized Atomic Force Microscope (CellHesion). Changes in the conformation of the eGC, in response to low and high Na⁺, were detected by using quantum dot (QD)-mediated immunofluorescence staining of heparan sulfates (HS) on the endothelial cell surface.

Results: In the presence of high Na⁺/aldosterone the number of adherent monocytes on the endothelial cell surface was significantly increased by 49% while the adhesion forces between monocyte and eGC were significantly decreased by 26%. In addition high Na⁺ diminished the number of detectable HS, the major component of the eGC, by 34%, indicating either shedding or conformational changes of the eGC. All effects could be prevented by treatment with the aldosterone receptor antagonist spironolactone. The data provide evidence for a two-step mechanism of monocyte adhesion. In a first step, monocytes bind to the eGC and in a second step to the surface of the endothelial cell. Treatment with high Na⁺/aldosterone collapses the eGC leading to a decreased binding of the monocytes to the eGC. The collapsed eGC in turn facilitates the access of the monocytes to the plasma membrane and binding to adhesion molecules (VCAM). Conclusions: The eGC is involved in the adhesion process of monocytes and serves as an important protective barrier. High Na⁺/aldosterone enhance the process of vascular inflammation. Since aldosterone receptor antagonism has protective effects on the eGC and attenuates the adhesion of monocytes this could be a useful strategy in the prevention of Na⁺/aldosterone-mediated inflammatory processes of the vasculature.

P073
Src-family tyrosine kinases are crucial for leukocyte recruitment in vivo

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Members of the Src tyrosine kinase family (SFK) are involved in tumor development, but also in various functions of leukocytes, including rolling, adhesion and extravasation. Targeting tyrosine kinases with small-molecule inhibitors became a
Nuclear factor kappa B (NFkB) in anucleated erythrocytes – just a molecular remnant?

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A single mature (anucleated) human erythrocyte with its nearly 270 million hemoglobin molecules is able to transport more than 1.1 billion oxygen molecules. For more than a century, erythrocytes were thus considered mainly as oxygen-transporters, thereby degrading them to the state of “monofunctional cells”. This misconception has contributed to the fact that the functional interactions of erythrocytes with other cells, i.e. immune cells and the physiological importance of such interactions have only been recognized recently. Human erythrocytes partly control the function of immune cells (e.g. of lymphocytes and immature dendritic cells (slanDCs) while residing in the peripheral blood (Fonseca et al. 2001; Melder et al. 2000; Profumo et al. 2011; Schäkel et al. 2006). The physical interaction between mature human erythrocytes and slanDCs leads to the inhibition of slanDCs-mediated production of the inflammatory cytokine interleukin-12 (IL-12), a condition of major clinical importance in psoriasis. Mature human erythrocytes also possess molecules that have been attributed to nucleated cells, namely transcription factors, e.g. all members of the redox-sensitive NFκB signalling pathway (Ghashghaeinia et al. 2011; 2012; 2013 and 2014). Our recent experimental data revealed an inverse correlation between NFkB abundance and eryptosis, the suicidal erythrocyte death (Ghashghaeinia et al. 2013). Our current unpublished studies now reveal a species-independent anti-eryptotic function of NFkB in anucleated murine erythrocytes. Furthermore, we currently investigate how NFkB influences this important biological pathway. Thus our investigations on the biological function of NFkB in anucleated erythrocytes and their clinical relevance is opening a new field of immunological research on erythrocytes-immune cell interaction, a challenge we want to take up by using a multi-disciplinary approach.

References:

Effect of acute exposure of blood platelets to the uremic toxin acrolein

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Background/aims: The polyamine degradation product acrolein accumulates in chronic kidney disease (CKD) and is thus considered an uremic toxin. Prior studies addressing the effect of acrolein on blood platelets yielded conflicting results suggesting that acrolein either fosters or inhibits thrombin induced platelet activation. Platelets are activated by increase of cytosolic Ca2+-activity ([Ca2+]i), which in turn leads to degradation (apparent from P-selectin exposure),

References:
integran activation, cell shrinkage, and aggregation. The Ca\(^{2+}\) entry is in part accomplished by the Ca\(^{2+}\) channel protein Orai1. The present study thus explored whether acrolein pretreatment modifies the thrombin induced platelet activation.

**Methods:** P-selectin, \(\alpha IIb\beta 3\) integrin and Orai1 protein abundance at the platelet surface was estimated utilizing CFT\(^{TM}\)488A conjugated antibodies, [Ca\(^{2+}\)]\(_i\) utilizing Fluoro3-fluorescence, cell volume utilizing forward scatter and platelet aggregation utilizing dual staining and subsequent measurement in flow cytometry.

**Results:** In the absence of thrombin, a 30 min exposure to 50 \(\mu\)M acrolein did not significantly modify P-selectin, \(\alpha IIb\beta 3\) integrin and Orai1 protein abundance at the platelet surface, [Ca\(^{2+}\)]\(_i\), forward scatter and platelet aggregation. Thrombin (0.02 U/ml) within 5 min decreased forward scatter and increased P-selectin, \(\alpha IIb\beta 3\) integrin, [Ca\(^{2+}\)]\(_i\) and platelet aggregation. The effects of thrombin on [Ca\(^{2+}\)]\(_i\), P-selectin abundance, \(\alpha IIb\beta 3\) integrin abundance, and platelet aggregation were significantly blunted by prior treatment with acrolein (50 \(\mu\)M).

**Conclusions:** A short term acrolein treatment blunts the activation of blood platelets by thrombin.

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**P076**

**Signaling of the A\(_{2a}\) and A\(_{2b}\) adenosine receptor on T helper cells**

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**Question:** Adenosine receptors (A\(_1\), A\(_{2A}\), A\(_{2B}\) and A\(_3\)) are G protein-coupled receptors that play an important role in purinergic signaling. It is known that the A\(_{2A}\) receptor (A\(_{2AR}\)) and the A\(_{2B}\) receptors are crucial modulators of the healing process after myocardial infarction (MI). While the A\(_{2AR}\) confers an anti-inflammatory action, there is controversy in the literature as to the role of the A\(_{2B}\)R. Therefore we investigated the role of the A\(_{2AR}\) and the A\(_{2B}R\) on T helper cells known to be important in the healing process after MI.

**Methods:** T helper cells were isolated from murine lymph nodes by cell sorting (FACS). Cells were cultured under stimulation conditions (3 \(\mu\)g/ml CD3 and 6 \(\mu\)g/ml CD28 antibodies) to simulate cell activation after MI. Expression of the adenosine receptors was measured by qPCR. To investigate the function of the adenosine receptors, cells were treated with a specific A\(_{2AR}\) agonist (CGS 21680) and A\(_{2B}R\) agonist (BAY 60–6583) and the production of 23 cytokines was analyzed using magnetic bead detection (Bio-Plex System).

**Results:** We found that the A\(_{2b}R\) was upregulated after antigenic stimulation, while no expression was detected on unstimulated T helper cells. Changes in cytokine profile after A\(_{2b}R\) and the A\(_{2a}R\) activation can be grouped into four patterns. I) Activation of both receptors inhibited cytokine production by T helper cells (IL-1\(\beta\), IL-3, IL-9, IL-10, IL-12(p70), IL-13, IL-17, G-CSF, GM-CSF, IFN-\(\gamma\), KC, MCP-1, MIP-1\(\beta\) and TNF-\(\alpha\)). In the majority of cases the A\(_{2b}R\) agonist was more potent than the A\(_{2a}R\) agonist. II) The A\(_{2a}R\) agonist significantly stimulated the release of cytokines (IL-12(p40) and RANTES), whereas the A\(_{2b}R\) agonist caused a slight, but significant inhibition (RANTES) or had no effect (IL-12(p40)). III) Activation of the A\(_{2a}R\) inhibited the formation of cytokines (IL-1a and IL-6), while the A\(_{2b}R\) had either no effect (IL-1\(\alpha\)) or showed an enhanced release (IL-6). IV) The A\(_{2b}R\) agonist inhibited the formation of cytokines (IL-4, IL-5 and Eotaxin), whereas the A\(_{2a}R\) agonist had no effect.

**Conclusion:** This study gives a first comprehensive overview on the effect of A\(_{2a}R\) and A\(_{2b}R\) on the release of various cytokines and chemokines from stimulated T helper cells. Compared to the A\(_{2a}R\), the A\(_{2b}R\) stimulation causes a pronounced reduction of Th2 cytokines (IL-4, IL-5, IL-10 and IL-13). Since an imbalance of Th1 and Th2 cytokines is associated with heart failure and may influence M1/M2 macrophage polarisation, our results provide evidence on the role of the A\(_{2b}R\) on activated T helper cells.
Hydrogen sulfide activates human CFTR which is heterologously expressed in Xenopus oocytes

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**Question:** Similar to the known gasotransmitters carbon monoxide and nitric oxide, hydrogen sulfide (H2S) has recently been recognized as a cellular signaling molecule which controls the activity of various ion channels. This study questioned whether or not the important human chloride channel CFTR (cystic fibrosis transmembrane conductance regulator) is influenced by H2S.

**Methods:** cRNA encoding human CFTR was injected into Xenopus laevis oocytes. The activity of such heterologously expressed CFTR was estimated by using the two-electrode voltage-clamp technique.

**Results:** The application of the H2S-forming salt Na2S dose-dependently (5-300 µM) increased the transmembrane current of CFTR-expressing oocytes. This effect was blocked by the CFTR-inhibitor CFTRinh-172. Furthermore, the organic slow H2S-releasing molecule GYY4137 also induced an increase of transmembrane current of CFTR-expressing oocytes, however, to a smaller extent compared to Na2S. There was no effect of Na2S or GYY4137 on native oocytes which did not express CFTR. The adenylate cyclase inhibitor MDL-12330A decreased the Na2S-induced activation of transmembrane current of CFTR-expressing oocytes.

**Conclusion:** These data indicate that H2S which is released by Na2S or GYY4137 activates heterologously expressed CFTR in Xenopus oocytes by the cAMP/adenylate cyclase/PKA signalling pathway.
Effect of CFTR expression on pH sensitivity of the human epithelial sodium channel (ENaC) in its δβγ- and δγγ-configuration

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Question: In cystic fibrosis (CF), dysfunction of the CF transmembrane conductance regulator (CFTR) chloride channel reduces chloride secretion in respiratory epithelia which impairs mucociliary clearance and favors recurrent pulmonary infections in CF patients. Enhanced sodium absorption via the epithelial sodium channel (ENaC) contributes to this effect. However, the mechanism how ENaC hyperfunction develops is not quite clear. ENaC function is sensitive to extracellular pH and pH is typically reduced in inflammatory tissue. The aim of the present study was to investigate whether CFTR affects pH sensitivity of human ENaC.

Methods: Human δβγ- or δγγENaC was expressed alone or together with human CFTR in Xenopus laevis (X. laevis) oocytes. Oocytes were superfused with ND96 adjusted to pH 7.4 or 8.4 (HEPES buffer), or 6.4, 5.9, 5.4, or 4.9 (MES buffer). Amiloride (100 µM)-sensitive whole-cell currents (ΔIami) were measured using the two-electrode voltage-clamp technique. CFTR was activated by pretreating oocytes for 20 minutes with IBMX (1 mM) and forskolin (1 µM).

Results: In δβγENaC expressing oocytes, step changes of extracellular pH from pH 7.4 to acidic values increased ΔIami. Stimulation was highest at pH 6.4 (~26%) and declined with decreasing pH (~5% at pH 4.9). In contrast, alkalization to pH 8.4 inhibited ΔIami by ~10%. This inhibition of ΔIami at pH 4.9 was increased (~21%) by co-expression with CFTR, whereas no effect on pH sensitivity was observed at acidic pH values. Active CFTR did not alter pH sensitivity in the range of pH 6.4 and 5.4, whereas there was a slightly lower stimulation of δβγENaC at pH 4.9 in the presence of CFTR. In δβγENaC expressing oocytes, ΔIami was stimulated at acidic pH with the highest effect at pH 5.9 (~72%) and the lowest at pH 4.9 (~44%). At pH 8.4, ΔIami was inhibited by ~20%. Overall, co-expression with CFTR did not alter pH sensitivity of δβγENaC. Only at pH 6.4, stimulation was higher in the presence of CFTR (~57% vs. ~65%). This effect was further enhanced after pretreating the oocytes with IBMX/forskolin (~58% vs. ~79%), whereas no other effect on pH sensitivity was observed.

Conclusion: Overall, we could confirm that δβγENaC is more sensitive to extracellular pH than δγγENaC. In the acidic pH range, inactive and active CFTR had no major effect on the pH sensitivity of δβγENaC. Moreover, the stimulatory effect of pH 6.4 on δβγENaC was slightly smaller in the absence than in the presence of CFTR. Thus, it is unlikely that modulatory effects of CFTR on the pH sensitivity of ENaC contribute to sodium hyperabsorption in CF.

Activation of the CFTR Cl- channel by n-alcohols requires the activity of adenylate cyclase

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Objective: Primary alcohols (n-alcohols) like 1-heptanol (HEP) and 1-octanol (OCT) are known as general anesthetics as well as a therapeutic option for certain diseases. Most investigations concerning the actions of n-alcohols have been done in excitable cells, less is known about their impact on ion transport processes in non-excitable cells like epithelia cells. The objective of this study was to investigate the effect of HEP and OCT on the activity of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl- channel in vitro as well as in native pulmonary epithelium.

Methods: Human CFTR (hCFTR) was heterologously expressed in Xenopus laevis (X. laevis) oocytes and modulation of CFTR activity by HEP and OCT was characterized by two-electrode-voltage-clamp measurements. Moreover, the impact of HEP and OCT on CFTR activity was investigated in native X. laevis lung tissue by Ussing chamber recordings.

Results: The hCFTR expression was confirmed by simultaneous application of IBMX (100 µM) and forskolin (5 µM). HEP and OCT (1 mM each) activated heterologously expressed hCFTR to a similar extent as IBMX/forskolin does. The effect of HEP/OCT on the transmembrane current (IM) was inhibited by CFTRinh-172 (5 µM). Moreover, the OCT induced effect was prevented by the adenylate cyclase (AC) inhibitor MDL 12330A (MDL, 50 µM). In Ussing chamber experiments using native X. laevis lung preparations both HEP and OCT induced an increase of the short-circuit current (HEP: 9.6 ± 1.1%, n=8, P<0.01; OCT: 11.7 ± 2.9%, n=9, P<0.01). This stimulation was abolished with known CFTR inhibitors (CFTRinh-172, 20 µM; glibenclamide, 750 µM; NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid), 100 µM). In contrast, the OCT effect was not sensitive to DIDS (4,4’diisothiocyanato-2,2'-stilbene disulphonic acid, 500 µM) an inhibitor of high-conductance anion channels and outwardly rectifying Cl- channels without affecting CFTR. The apical incubation of the pulmonary epithelium with MDL (50 µM) inhibited the effect of HEP and OCT.

Conclusion: Taken together, we conclude that HEP and OCT activate CFTR in vitro as well as in native pulmonary epithelia. Further, our approach provides strong evidence that AC is the main target in the HEP and OCT induced CFTR activation mechanism.
**P082**

**The role of HCN channels in urinary bladder contraction**

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The motility of the urinary bladder smooth muscle is controlled by the autonomous nerve system as well as by non-adrenergic-non-cholinergic transmission. In addition, pacemaker cells are capable to produce alternating membrane potential changes and thereby contribute to rhythmic motility of detrusor smooth muscle. Since hyperpolarization-activated cyclic nucleotide-gated non-selective (HCN) channels play a major role in other pacemaker cells such as the sinoatrial node, we have tested the effect of the HCN channel blocker ZD7288 on spontaneous motility of murine and human detrusor specimens. While ZD7288 did not change the frequency of spontaneous phasic contractions, the tonic contraction was significantly enhanced by this compound. Moreover, the gap junction blocker glycyrrhetinic acid prevented the ZD7288 effect indicating the involvement of gap junctions. On the other hand, the HCN channel activator lamotrigine caused a dose-dependent relaxation of detrusor smooth muscle. All HCN channel isoforms were detected in human detrusor samples by real-time PCR. Our results demonstrate that HCN channels are present in human urinary bladder, and the modulatory role of HCN channels in detrusor motility is mediated via gap junctions. In contrast to the well-known chronotropic effects in the sinoatrial node, HCN channels in the urinary bladder influence the basal tone rather than the frequency of spontaneous contractions.

**Conclusions:** Except for 2-APB, the TRPM7-modulating substances used in this study inhibited spontaneous activity only in dose ranges were they were very likely to exhibit non-specific inhibition also to other ion channels and proteins that are essential for contractile activity (e.g. connexins, L-type calcium channels). This conclusion is supported by the fact that agonist-induced contractions were inhibited to a similar extent as spontaneous activity. 2-APB itself has various cellular effects that are unrelated to TRPM7 but have been shown to affect pacemaking (e.g. IP3-receptor signaling). Furthermore, pacemaking in jejunum, bladder and portal vein were largely unaltered by 2-APB. The results of this study therefore suggest that TRPM7 is not a universal and irreplaceable element of the pacemaking process in smooth muscle.

**P083**

**The impact of TRPM7-channel modulators on the pacemaking activity of smooth muscle**

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**Question:** The ionic conductances underlying the pacemaker current in spontaneously active smooth muscle have yet been only incompletely characterized. From studies on cultured interstitial cells of Cajal (ICC) it has been suggested that TRPM7 may carry an important part of this current in intestinal smooth muscles from mice. The expression and the functional impact of TRPM7 in extraintestinal tissues is yet unknown. Furthermore, no studies have addressed the functional role of TRPM7 in native tissue preparations.

**Methods:** Intestinal (gastric antrum, jejunum) and extraintestinal (portal vein, uterus, bladder) smooth muscle tissues from rats were studied using the organ bath technique. Western Blot analysis was performed to confirm the expression of TRPM7 in these tissues. Isometric contractions were measured over time and the effects of TRPM7-modulators on the temporal pattern and on the amplitude of spontaneous and agonist / potassium induced contractions were studied.

**Results:** TRPM7 - protein could be detected in all tissues. The relative signal intensity was jejunum>portal vein>antrum>uterus. Compared to control, the TRPM7-modulator 10µM 2-APB reduced the frequency of spontaneous activity to 23% in gastric antrum (n=8, SD=17%, p<0.05), to 98% in jejunum (n=7, SD=12%, n.s.), to 81% in portal vein (n=5, SD=9%, n.s.), to 8% in uterus (n=7, SD=9%, p<0.05) and to 99% in urinary bladder (n=7, SD=10%, n.s.). Further reductions were seen in all preparations when 2-APB-concentration was increased to 20µM (e.g.: portal vein 51%, SD=23%; n.s.). Global contractility as measured by comparing responses to high potassium (60mM) in the presence and absence of 10µM 2-APB was changed to 97% in gastric antrum (n=8, SD=7%, n.s.), to 102% in jejunum (n=7, SD=8%, n.s.), to 106% in portal vein (n=5, SD=8%, n.s.) and to 81% in uterus (n=7, SD=6%, p<0.05). Carvacrol (up to 5*10^-4M), lanthanum (up to 5*10^-4M) and flufenamic acid (5*10^-5) significantly diminished the frequency of contractions in all preparations, but this effect was accompanied by a strong inhibition of global contractility.

**Conclusions:** Isometric contractions in all tissues were largely unaltered by 2-APB. The results of this study therefore suggest that TRPM7 is not a universal and irreplaceable element of the pacemaking process in smooth muscle.

**P084**

**Possible physiological role of the bile acid-sensitive ion channel (BASIC) in rat cholangiocytes**

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The bile acid-sensitive ion channel (BASIC) is an independent member of the DEG/ENaC family of ion channels which is not closely related to other members. On the mRNA level BASIC is mainly expressed in brain, liver and intestinal tract. In the liver immunohistochemical data suggest that it is mainly found in cholangiocytes, the epithelial cells lining the bile ducts. The physiological role of BASIC in these cells remains completely unknown. As the name BASIC implies, bile acids, especially the combination of hyodeoxycholic acid (HDCA) and chenodeoxycholic acid (CDCA) potently activate the rat ortholog of the channel. We employed the Ussing chamber technique to elucidate the physiological role of BASIC in normal rat cholangiocytes (NRC), a cell line derived from rat bile ducts. We show that BASIC is expressed in NRCs and that the apical application of bile acids (HDCA/CDCA (1/0.5mM)) induces reversible, Na+-carried, transepithelial short-circuit currents (Isc), which last several minutes. These currents were partly inhibited by
the diarylamine diminazene (Dimi), a specific inhibitor of BASIC. Pretreatment of the cells over night with the secretory peptide hormone somatostatin abolishes the bile acid induced currents. Taken together these data suggest that BASIC may contribute to absorptive processes in bile ducts.

P085
The chocolate test: a practical course to teach gastrointestinal functions
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We developed a simple approach to teach the regulatory function of the gastrointestinal system (GI) using carbohydrate digestion and resorption in our practical classes. Time requirement is just 60 minutes independent of the number of participants. The course was split up into two groups in which each student had to consume 55g sugar in different forms: Group 1: 0.5L Coca Cola (55g saccharose); Group 2: which each student had to consume 55g sugar in different forms by means of school grades (1-5). Blood glucose concentration was measured to the mean value of the [SCFA] measured = 80 ± 2 mmol/l-1 obtained via gas chromatography ([SCFA] measured = 80 ± 2 mmol/l-1 (r2 = 0.60)). Values for α and pK showed no correlation to the SCFA concentration of the solution. Conclusion: The isohydric principle holds for ruminal fluid and buffering follows equation (1) with α = 0.23 mmol/l-1·kPa-1 and pKαp = 6.1. The importance of establishing a generally accepted protocol with fixed levels of pCO2 for measuring the acid-base status of ruminal samples is discussed.

P086
A simple method for measuring the solubility α of CO2 and the pK of H2CO3 in a multi-buffer system
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Introduction: Using a clinically relevant example (ruminal fluid), we present a simple method allowing an estimate of Henry's constant α and the pK of H2CO3 in a multi-buffer system.

Methods: The total buffer value βbuffer(pH) of ruminal samples was determined by titration with HCl and NaOH at two levels of pCO2 (5% and 100%), yielding two different buffer curves. Since the buffer values of a multi-buffer system are additive (1), the difference Δβbuffer(pH) between the two buffer curves is given by: Δβbuffer(pH) = 2.302·α·ΔpCO2·10[SCFA]/[2+10(pH-pKSCFA)] (eq. 1) yielding values for the concentration of SCFA [SCFA] that were compared to those obtained using the Astrup technique with direct measurement of CO2 concentration. In a further step, the buffer curves were fitted to βbuffer(pH) = 2.302·α·pCO2·10[SCFA]/[2+10(pH-pKSCFA)] (eq. 2) yielding values for the concentration of SCFA [SCFA] that were compared to those obtained via gas chromatography.

Results: Using the classical Astrup technique, the solubility of CO2 (α) in ruminal fluid was found to be 0.244 ± 0.01 mmol/l-1·kPa-1, while pKα was 6.21 ± 0.03 (n/N=31/10). Curve fitting after titration yielded α = 0.223 ± 0.003 mmol/l-1·kPa-1 and pKα was 6.05 ± 0.01 (n/N=12/2). Fits also yielded an estimate for the [SCFA] of [SCFA] measured = 81 ± 4 mmol/l-1, close to the mean value of [SCFA] measured = 80 ± 2 mmol/l-1 obtained via gas chromatography ([SCFA] measured = 80 ± 2 mmol/l-1 (r2 = 0.60)). Values for α and pK showed no correlation to the SCFA concentration of the solution.

Conclusions: The isohydric principle holds for ruminal fluid and buffering follows equation (1) with α = 0.23 mmol/l-1·kPa-1 and pKα = 6.1. The importance of establishing a generally accepted protocol with fixed levels of pCO2 for measuring the acid-base status of ruminal samples is discussed.

P087
Cell specific modulation of gastrointestinal NO-induced relaxation by phosphodiesterases
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In the gastrointestinal (GI) tract NO is produced by nNOS and released from nerve varicosities to relax GI smooth muscle. NO-sensitive guanylyl cyclase (NO-GC), the receptor for NO, is expressed in at least three cell types (smooth muscle cells (SMC), interstitial cells of Cajal (ICC), fibroblast-like cells (FLC)) but where exactly nerve-released NO acts is still unclear. As ICC and FLC express much higher amounts of NO-GC than SMC and may therefore scavenge NO released from enteric varicosities, spillover and thus direct action of NO to SMC has been discussed for a long time. In these cells, not only the amount of NO-GC but also the type(s) of phosphodiesterase(s) (PDE) present will influence strength and duration of the NO signal. Furthermore, the role of cGMP/cAMP crosstalk in these cells remains unknown. To investigate the spillover and to clarify the individual impact of cGMP and cAMP for relaxation we used our mouse lines lacking NO-GC in SMC and ICC. Isometric force studies were performed to monitor the responsiveness to exogenous and endogenous NO in absence and presence of blockers of PDE3 (milrinone) and PDE5 (sildenafil). Immunohistochemistry was employed to identify PDE3/5 expression. These studies will help to define the impact of cGMP/cAMP crosstalk in GI relaxation and motility.

P088
Long-term live-imaging of adipose tissue inflammation
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Obesity is frequently associated with a chronic low-grade inflammation within adipose tissue (AT) [1]. Although classical signs of inflammation are missing in AT inflammation, there is a significant increase in macrophages and, to lesser extent, other immune cells, such as T-cells, B-cells, mast cells and neutrophils. The spatial and temporal activation of these cells as well as their accumulation in the AT seems to be tightly linked to so-called crown-like structures (CLS) [2]. CLS are accumulations of adipose tissue macrophages (ATMs) around dead adipocytes and are thought to reflect a scavenger response. At present, data on the life cycle of CLS with an unprecedented spatio-temporal resolution. Hence, applying long-term imaging allows studying cellular function within live AT explants and provides new insights into the role of ATMs and AT inflammation.

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P089
Reduced abundance of mineralocorticoid receptor in cirrhosis: a mechanism that is linked to hypoxia and proinflammatory cytokines
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Question: Aldosterone induces fibrosis in liver through the mineralocorticoid receptor (MR). During development of cirrhosis the activation of renin-angiotensin-aldosterone-system results in higher levels of aldosterone. The aim of this study was to investigate the role of MR during the development of cirrhosis.

Methods: Male wistar rats were treated 5, 8 or 12 weeks (w) with CCl4. Livers where excised and either conserved or treated for isolation of hepatocytes (pRH), hepatic stellate cell (pHSC) or endothelial cells (pLSEC). Gene expression was analyzed by realtime qRT-PCR. To gain more insight into the importance of MR in liver cirrhosis, HepG2 cells were treated with a combination of hypoxia and cytokines, mimicking conditions during development of cirrhosis, either with or without epirenone (MR antagonist) or aldosterone (10 nM).

Results: In hepatocytes MR-mRNA was decreased after 12w CCl4 treatment (-ΔΔCt: -1.46±0.39 compared to control; p<0.05) compared to pHSC and pSEC in cirrhotic livers. To investigate if the alteration in MR expression leads to a change in MR dependent gene expression, serum and glycocorticoid mimicking conditions during development of cirrhosis, either with or without epirenone (MR antagonist) or aldosterone (10 nM).

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P090 Molecular and functional characterization of the epithelial barrier of rat small intestinal Peyer’s patches
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Peyer’s patches are sensors of the small intestinal immune system. Although immune surveillance of the intestinal lumen by these aggregated lymphoid nodules has been analyzed in detail, information on specific barrier properties is scarce. Aim of our study was to analyze the barrier of Peyer’s patches follicle-associated epithelium compared to neighboring villous epithelium on molecular and functional level.

Methods: Experiments were performed on small intestinal tissue specimens of male Wistar rats. Conventional Ussing chambers or subjected to two-path impedance spectroscopy. For comparison, in vitro experiments were carried out on monolayers of the jejunal porcine epithelial cell line, IPEC-J2/PS. Zinc cytotoxicity was tested enzymatically in cultured cells.

Results: In IPEC-J2/PS, basolateral zinc was cytotoxic: application caused LDH release and an irreversible break-down of the epithelial barrier. Meanwhile, apical zinc application caused an immediate increase in trans- and paracellular resistances and a decrease in permeability to the paracellular marker fluorescein, reflecting overall barrier strengthening in vitro. All apical effects were fully reversible upon wash-out. In contrast, feeding high zinc doses exerted no significant electrophysiologically ascertainable short- and long-term adverse or protective effects on jejunal barrier function ex vivo, indicating that zinc was completely washed out during tissue preparation.

Conclusions: 1) Extracellular zinc acts acutely and reversibly from the apical side via tightening trans- and paracellular routes. 2) There is no evidence for long-term barrier effects: neither protective, e.g. through intracellular zinc loading of enterocytes nor detrimental through basolateral zinc accumulation.

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P091 Trans- and paracellular intestinal barrier effects of zinc
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Background: During the post-weaning period, piglets are prone to gastrointestinal infections. The resulting impairment of intestinal barrier function may cause diarrhea associated with growth retardation or even death of piglets. Oral zinc oxide is commonly used to prevent and treat diarrhea but its mode of action is still needs to be elucidated.

Aim: Functional analysis of zinc effects on porcine intestinal barrier function ex vivo and in vitro.

Methods: 26±1 days post partum, 48 piglets were weaned, allocated equally into three groups, and fed diets containing 57, 164, and 2425 ppm zinc oxide, respectively. On days 33±1 and 54±1, mid jejunal segments were obtained. Barrier properties of stripped jejunal explants were studied in conventional Ussing chambers or subjected to two-path impedance spectroscopy. For comparison, in vitro experiments were carried out on monolayers of the jejunal porcine epithelial cell line, IPEC-J2/PS. Zinc cytotoxicity was tested enzymatically in cultured cells.

Results: In IPEC-J2/PS, basolateral zinc was cytotoxic: application caused LDH release and an irreversible break-down of the epithelial barrier. Meanwhile, apical zinc application caused an immediate increase in trans- and paracellular resistances and a decrease in permeability to the paracellular marker fluorescein, reflecting overall barrier strengthening in vitro. All apical effects were fully reversible upon wash-out. In contrast, feeding high zinc doses exerted no significant electrophysiologically ascertainable short- and long-term adverse or protective effects on jejunal barrier function ex vivo, indicating that zinc was completely washed out during tissue preparation.

Conclusions: 1) Extracellular zinc acts acutely and reversibly from the apical side via tightening trans- and paracellular routes. 2) There is no evidence for long-term barrier effects: neither protective, e.g. through intracellular zinc loading of enterocytes nor detrimental through basolateral zinc accumulation.
Brain extracellular matrix retains functional connectivity in neuronal networks

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The processing and storage of information is the primary purpose of neuronal networks. It is achieved through precise interaction of interconnected neurons that form functional neuronal ensembles. Therefore, in order to understand how information is processed in healthy and diseased brain, it is important to reveal the underlying mechanisms of formation and maintenance of connectivity in neuronal networks. The hyaluronan-based brain extracellular matrix (ECM) is formed during postnatal development and surrounds most neurons in the adult mammalian brain. Importantly, the brain ECM has been shown to restrict plasticity and post-traumatic recovery in neuronal networks, but little is known about the mechanisms. In the present study, we pursued the idea that basic principles of spontaneous formation and maintenance of connectivity are preserved in cultured neuronal networks. In dissociated rat hippocampal cultures grown on micro-electrode arrays, we investigated the spontaneous development of functional connectivity in neuronal microcircuits, as well as the role of the brain extracellular matrix in regulation of functional interaction between spatially distributed neuronal clusters. Spontaneous formation of functional network connectivity correlated with increase of the ECM density in developing cultures, and was complete upon maturation by the end of fourth week *in vitro*. Further, we found that acute enzymatic degradation of the ECM by hyaluronidase in mature cultures led to a transient enhancement of neuronal activity. Intriguingly, blockade of fast inhibition by bicuculline in cultures with degraded ECM was ineffective to induce hyperexcitability and hypersynchronization of neuronal activity that were evident in inhibited cultures with intact ECM. Moreover, disinhibition in cultures pre-treated with hyaluronidase affected spatiotemporal properties of the network interaction, resulting in emergence of network burst patterns strongly resembling those seen in naïve developing cultures. Co-application of bicuculline with kynurenic acid partially rescued the effects of disinhibition on both the amount of activity and the properties of functional network interaction, suggesting that removal of the ECM promotes the spillover of glutamate and desensitization of glutamate receptors in extrasynaptic locations. Taken together, the effect of controlled degradation of the ECM on the functional network interaction revealed novel aspects of ECM-mediated regulation of glutamatergic neurotransmission in mature neuronal networks. Our results demonstrated that the ECM plays important role in retention of established connectivity in mature neuronal networks that can be exerted through synaptic confinement of released glutamate.

How synchronous is neuronal activity in human brain slices during spontaneous and elicited epileptic discharges?

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Using membrane potential imaging by voltage sensitive dyes in brain tissue together with conventional field potential recordings (local EEG), the simultaneous behaviour of neurons in a population during epileptic discharges were studied (Canepari and Zecevic, Eds., Membrane potential imaging in the nervous system, Springer 2010).

Tissue slices (400 μm thick) were prepared from human neocortex (Gyrus temporalis medius) and human amygdala obtained during epilepsy surgery (Bethel/Bielefeld, Germany). Epileptic activity occurred spontaneously, i.e. without experimental manipulations, in the human preparations. Epileptic discharges are characterized and were determined in this study by spikes, sharp waves and various combinations of both in the field potential recording (local EEG); for detection of membrane potentials fluorescence dyes were used. Comparing the changes of the local field potential (i.e. of the local EEG) with the fluorescence changes (i.e. with changes of membrane potentials of neurons) in the corresponding areas, it becomes apparent:

1) During spontaneously occurring epileptic discharges in the local EEG only a part of neurons of the populations is active simultaneously; a total synchronisation is missing. In all preparations a total synchronisation becomes apparent only if epileptic discharges are triggered by electrical stimulation of afferent fibres.

2) Spontaneous epileptic discharges in the local EEG which are similar in shape and time course are associated with different extensions of neuronal depolarisations. On the other hand, neuronal depolarisations similar in shape and time course are associated with epileptic discharges in the local EEG different in amplitude and duration (Speckmann, Das Gehirn meiner Kunst, 2nd Ed., Daedalus 2012).

Frequency dispersion in the cerebellar cortex


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To control timing of motor function, the cerebellar cortex receives broad-bandwidth neuronal signals with up to kilohertz frequencies from mossy fibers. However, it is unclear if the postsynaptic granule cells (GCs) are tuned to specific frequencies and whether such frequency dispersion is topographically organized. Here, we combined structural and functional analyses to investigate frequency tuning of GCs. We found that GCs near white matter (inner-zone GCs) are functionally different from GCs near Purkinje cells (outer-zone...
Epilepsy is a neurological disorder that is characterized by involuntary spontaneous seizures. Temporal lobe epilepsy (TLE) is a complex partial epilepsy in which patients experience recurrent epileptic seizures emerging mainly from the hippocampus and amygdala of the unilateral or bilateral temporal lobes of the brain. The pilocarpine animal model mimics both the underlying aetiology and symptoms of TLE. This includes: i) seizure foci in the hippocampus; ii) a latent period as a seizure-free time before phenotype manifestation; iii) hippocampal sclerosis. The model is very common in adult rats and induces chronic epileptic seizures in an already consolidated brain.

Our study goal is to electrophysiologically and pharmacologically shed light on why epileptic children have a high tolerance to bromide and why adults generally have severe side effects. Therefore, we developed a juvenile rat model which reconstructs epilepsy in children.

Epilepsy was induced in rat pups at different ages, with different pilocarpine doses, variable durations of treatment and diazepam doses. Based on survival rates and seizures, a protocol was selected for further validation. EEG was recorded at 30, 60 and 120 days after treatment in the hippocampal CA1 region and motor cortex for 3-7 days and seizures were analyzed with a Seizure Detection Module (NeuroScore, DSM).

Furthermore, literature suggests that voltage activated Ca2+ channels are likely to be predominant candidates for calcium elevation during most epileptiform activity. Therefore, we studied molecular changes in the juvenile epileptic rat hippocampal system using quantitative Real Time PCR analysis. In conclusion, our model is easy to use and yields the desired outcome. Mortality rates are as low as 10-20% while 70-90% of the survival animals present an epileptic phenotype. It presents seizures in the hippocampus and motor cortex on a reproducible manner and proofs the distinct role of voltage-gated calcium channels in epileptogenesis.

The model can be used to mimic epilepsy in children and for testing antiepileptic drugs such as bromide.

P095
A young pilocarpine model for epilepsy
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Epilepsy is a neurological disorder that is characterized by involuntary spontaneous seizures. Temporal lobe epilepsy (TLE) is a complex partial epilepsy in which patients experience recurrent epileptic seizures emerging mainly from the hippocampus and amygdala of the unilateral or bilateral temporal lobes of the brain. The pilocarpine animal model mimics both the underlying aetiology and symptoms of TLE. This includes: i) seizure foci in the hippocampus; ii) a latent period as a seizure-free time before phenotype manifestation; iii) hippocampal sclerosis. The model is very common in adult rats and induces chronic epileptic seizures in an already consolidated brain.

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The model can be used to mimic epilepsy in children and for testing antiepileptic drugs such as bromide.

P096
Long-term, long-range recordings of living dorsal root ganglion neurons by scanning ion conductance microscopy
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Scanning ion conductance microscopy (SICM, Hansma et al., 1989) is a method for contactless investigations of textures of non-conducting surfaces. It is especially suited to investigate shape changes of the encircling plasma membrane of living cells, which can be used for a determination of the cell volume. SICM is based on the principle that the conductance sensed by the tip of an electrolyte-filled glass capillary decreases when the distance between the pipette opening and an insulator surface approaches the tip diameter. The advantage of this technique is that it does not require a denaturing preparation of the cells and no mechanical force on the membrane is exerted in the recording process. Here we show, that the method is applicable to perform up to fifty consecutive recordings of a living neuron, isolated from an embryonic mouse dorsal root ganglion. The observed neuron migrated in a saltatory manner with a mean velocity of approximately 20 μm/h. A detailed quantitative investigation of the series of recordings shows phases of nucleokinesis, in which the movement of the nucleus precedes the movement of the cell base. Especially when cells changed from a stationary to a migratory state, frontal cell volume changes preceded the movement of the nucleus as already seen in oligodendrocyte precursor cells (Happel et al., 2013).

P097
Plasticity of intrinsic excitability in mature granule cells of the dentate gyrus
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The dentate gyrus is the main entry gate for cortical input to the hippocampus and one of the few brain areas where adult neurogenesis occurs. Surprisingly, it is difficult to induce synaptic plasticity in mature but not newborn granule cells. This has prompted some authors to speculate that mature granule cells are functionally "retired" and that plastic properties of the dentate are mainly associated with newborn granule cells. We found, using a combination of electrophysiological and two-photon imaging techniques, that physiologically relevant conditioning protocols, such as theta burst stimulation or spike-timing-dependent plasticity (STDP), consistently modified the probability to generate action potentials in response to a given synaptic input in mature granule cells; in the case of the STDP protocol even without any modification of synaptic strength. We present evidence suggesting that dendritic A-type potassium channels are likely candidates for mediating these changes. Our
Calcium-activated K+ channels (KCa) play a major role in coupling the membrane potential to the intracellular Ca2+ concentration. Following a series of action potentials (AP), hippocampal CA1 neurons typically show an afterhyperpolarizing potential (AHP) involving KCa2.2 channels. In hippocampal slices showing epileptic activity acutely induced by bath application with gabazine, we have previously found a reduction of the AHP following an AP series. Here, we have performed Western blot analyses detecting KCa2.2 in the CA1 area in gabazine-treated hippocampal slices. We found a significant reduction of KCa2.2 protein in slices bathed in gabazine (5 µM) for 60 min compared to control levels. This reduction could not be prevented by co-application of gabazine together with the protein kinase A (PKA) blocker H-89 indicating that KCa2.2 reduction was independent of PKA phosphorylation. However, co-application of gabazine with MG-132 and chloroquine to block both proteasomal and lysosomal degradation, respectively, significantly raised the KCa2.2 content to control levels. Thus, gabazine-induced epileptic activity is associated with a reduction of KCa2.2 protein in the CA1 area due to proteasomal and lysosomal degradation.

**P099**
Comparison of spike parameters from optically identified GABAergic and glutamatergic neurons in sparse cortical cultures

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Combining recordings of electrical activity and fluorescent microscopy can be powerful tools to study both active and passive cell physiology. Here, we use multi-electrode array (MEA) recordings from spontaneously active cultures of wildtype and GAD67-GFP transgenic mice to evaluate which spike parameters differ between GABAergic interneurons and principal, putatively glutamatergic neurons. Simultaneous calcium imaging confirmed the proper assignment of spikes to individual, active neurons and immunohistochemistry confirmed that excitatory and inhibitory neurons are present in our culture system at a comparable ratio as described in vivo. Spike waveforms and firing patterns at different developmental stages were analyzed and correlated with the spatial orientation of the recorded neuron within the recording field. The results suggest that in our primary cortical culture systems spike waveform characteristics and firing patterns of neurons were mostly developed after two weeks in culture. Spike amplitude, but not other spike waveform parameters, correlated with the distance between recording electrode and location of the assigned neuron’s soma. Cluster analysis of spike waveform properties recorded from wildtype cultures revealed no particular cell population that may be assigned to putative inhibitory or excitatory neurons. Moreover, experiments in sparse primary cultures from transgenic GAD67-GFP mice, which allow optical identification of GABAergic interneurons and thus unambiguous assignment of extracellular signals, did not reveal any significant difference in spike timing and spike waveform parameters between inhibitory and excitatory neurons. Our detailed characterization of spike waveforms and spike timing properties of cortical neurons in vitro emphasizes the need for direct confirmation of electrophysiological classifications on single cell level before applying on a population-based level and demonstrates the strength of combined electrophysiological and optical recordings.

**P100**
Studying molecular mechanisms of synaptic modulation with an optogenetic approach

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Synaptic signalling modulates a variety of physiological processes in postsynaptic neurons. One important mechanism is the inhibition of potassium (K+) channels by activation of Gq protein-coupled receptors (GqPCRs), which has depolarizing effects on the postsynaptic neuron. It is assumed that Phospholipase C (PLC)-mediated depletion of Phosphatidylinositol-4,5-bisphosphate (PIP2) is involved in GqPCR-mediated inhibition of at least some types of K+ channels. We have previously found that pharmacological activation of GqPCRs can lead to strong depletion of PIP2 in pyramidal neurons of the hippocampal CA1 region. However, such PIP2 dynamics have not been investigated in detail in response to physiological synaptic activity.

In order to address this issue, we aimed at establishing a versatile model for modulatory synaptic transmission. We study the effects of cholinergic signalling on CA1 pyramidal neurons in acute septohippocampal mouse brain slices. CA1 pyramidal neurons receive cholinergic inputs from the medial septum (MS) and diagonal band of broca (DBB) via the fimbria/fornix pathway, acting via Gq-coupled M1/3 receptors. We trigger ACh release with an optogenetic approach using transgenic mice that express channelrhodopsin (ChR2), a blue light-gated cation channel, in cholinergic neurons. Upon blue light irradiation, neurons in the MS/DBB cell body layer depolarize, resulting in ACh release in the vicinity of CA1 pyramidal neuronal dendrites. In patch-clamp measurements on acute septohippocampal slices from ChR2 transgenic mice, depolarization of CA1 pyramidal neurons could be recorded in response to single or repeated blue light irradiation for 5 s. Some cells responded
with a brief, but strong increase in action potential (AP) frequency or AP bursts 10–25 s after the onset of the stimulus. Others showed a transient increase in membrane potential with latency and duration in the range of minutes. Occasionally, both effects occurred combined. In most cases, these reaction were reversible.

Based on these findings we conclude that this approach may provide a useful model for studying GqPCR mediated signalling, including PIP2 dynamics, in central neurons under near physiological stimulation, that is, synaptic activity.

**Question:** The canonical form of Spike timing-dependent plasticity (STDP) is commonly induced by high repeat numbers of single pre- and postsynaptic spike pairings synchronized within a precise temporal window (~10ms). Positive time intervals (pre-post) induce long-term potentiation (LTP) and negative time intervals (post-pre) long-term depression (LTD). Traditional protocols use among 60 to 100 spike pairings for STDP induction. However the physiological impact of the number of spike pairings has not been evaluated systematically so far. Thus, we are interested whether the mechanisms for STDP induction might be influenced by the number of spike pairings.

**Methods:** The current study aimed at elucidating the influence of the number of spike pairing on the mechanisms for STDP induction at Schaffer collateral (Sc)-CA1 synapses. Briefly, STDP was induced with different numbers of spike pairings (i.e., 3, 6, 12, 25, 50 and 70 repeats) of one presynaptic action potential (AP), leading to an excitatory postsynaptic potential (EPSP) in combination with one postsynaptic AP (1EPSP/1AP) with a Δt= +10ms at low frequency (0.5Hz) for Sc-CA1 synapses (see Edelmann *et al*, at this meeting).

**Results:** Here, we show a novel 1EPSP/1AP protocol, which induced robust t-LTP with only six spike pairings (1EPSP/1AP_6X). We also observed, that this STDP paradigm provokes significant changes in short term plasticity at Sc-CA1 synapses, suggesting that this kind of plasticity is not dominated by presynaptic changes. Furthermore, our data show that different numbers of spike pairings may have a strong influence on the influence of different neuromodulators on successful STDP. It is known, that dopamine D1-like receptors play a role in dopaminergic signaling in CA1 region of the hippocampus. We previously demonstrated that STDP induced with 1EPSP/1AP_70X at Sc-CA1 synapses can be completely blocked using a specific antagonist for this D1 receptor (SCH23390, 10µM). However, when using only 6 repetitions of the 1EPSP/1AP_6X STDP protocol D1 receptor block was only partial. Moreover, recently in our laboratory, we have found that different numbers and patterns of spikes pairings cause changes in the locus (i.e. pre- vs. postsynaptic) and the molecular mechanisms leading to t-LTP expression at Sc-CA1 synapses (see Edelmann *et al*, at this meeting).

**Conclusion:** STDP can be induced with a wide range of spike pairings that differentially activate molecular pathways leading synaptic modification, which means that the number of spike pairings might orchestrate the activation of essential neuromodulatory pathways for integration and processing of memories in the hippocampus.

This work was supported by the Center of Behavioral Brain Sciences (CBBS) and the Deutsche Forschungsgemeinschaft (DFG, SFB 779/B6).

**P101**

Influence of number of spike pairings on induction mechanisms in Spike timing-dependent plasticity

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**Question:** The canonical form of Spike timing-dependent plasticity (STDP) is commonly induced by high repeat numbers of single pre- and postsynaptic spike pairings synchronized within a precise temporal window (~10ms). Positive time intervals (pre-post) induce long-term potentiation (LTP) and negative time intervals (post-pre) long-term depression (LTD). Traditional protocols use among 60 to 100 spike pairings for STDP induction. However the physiological impact of the number of spike pairings has not been evaluated systematically so far. Thus, we are interested whether the mechanisms for STDP induction might be influenced by the number of spike pairings.

**Methods:** The current study aimed at elucidating the influence of the number of spike pairing on the mechanisms for STDP induction at Schaffer collateral (Sc)-CA1 synapses. Briefly, STDP was induced with different numbers of spike pairings (i.e., 3, 6, 12, 25, 50 and 70 repeats) of one presynaptic action potential (AP), leading to an excitatory postsynaptic potential (EPSP) in combination with one postsynaptic AP (1EPSP/1AP) with a Δt= +10ms at low frequency (0.5Hz) in acute hippocampal slices.

**Results:** Here, we show a novel 1EPSP/1AP protocol, which induced robust t-LTP with only six spike pairings (1EPSP/1AP_6X). We also observed, that this STDP paradigm provokes significant changes in short term plasticity at Sc-CA1 synapses, suggesting that this kind of plasticity is not dominated by presynaptic changes. Furthermore, our data show that different numbers of spike pairings may have a strong influence on the influence of different neuromodulators on successful STDP. It is known, that dopamine D1-like receptors play a role in dopaminergic signaling in CA1 region of the hippocampus. We previously demonstrated that STDP induced with 1EPSP/1AP_70X at Sc-CA1 synapses can be completely blocked using a specific antagonist for this D1 receptor (SCH23390, 10µM). However, when using only 6 repetitions of the 1EPSP/1AP_6X STDP protocol D1 receptor block was only partial. Moreover, recently in our laboratory, we have found that different numbers and patterns of spikes pairings cause changes in the locus (i.e. pre- vs. postsynaptic) and the molecular mechanisms leading to t-LTP expression at Sc-CA1 synapses (see Edelmann *et al*, at this meeting).

**Conclusion:** STDP can be induced with a wide range of spike pairings that differentially activate molecular pathways leading synaptic modification, which means that the number of spike pairings might orchestrate the activation of essential neuromodulatory pathways for integration and processing of memories in the hippocampus.

This work was supported by the Center of Behavioral Brain Sciences (CBBS) and the Deutsche Forschungsgemeinschaft (DFG, SFB 779/B6).

**P102**

Age-dependent role of nitric oxide in synaptic plasticity at medial perforant path-granule cell synapses

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In several regions of the brain neurons produce nitric oxide (NO) acting as an intercellular messenger. During synaptic transmission, NO synthesis can be triggered by postsynaptic NMDA receptor activation. Thus, NO may play a role as a retrograde messenger linking postsynaptic induction of synaptic plasticity to presynaptic changes in transmitter release. Here, we compared the influence of NO synthesis on long-term potentiation (LTP) and long-term depression (LTD) at the medial perforant path-granule cell synapse (MPP) in early postnatal (P9-15) and adult (P30-60) rats. In slices from early postnatal rats, delta-burst stimulation (dBS, 2 trains of 10 bursts at 1.25 Hz with 8 stimuli at 80 Hz) resulted in a stable LTP (147 ± 6%). Application of the NO synthase blocker L-NAME (100 µM) reduced LTP (128 ± 15%) suggesting that NO participates in the generation of LTP in early postnatal rats. We further asked whether NO might also affect LTD. In P9-15 slices, low-frequency stimulation (LFS, 900 pulses at 1 Hz) reliably induced LTD (80 ± 4%). In contrast to LTP, LTD was significantly enhanced by application of L-NAME prior to LFS (66 ± 6%). In slices from adult (P30-60) rats, however, LFS-induced LTD (81 ± 3%) was not affected by L-NAME (85 ± 6%). These results indicate that NO differentially modulates LTP and LTD of the medial perforant path-granule cell synapse. Moreover, NO-mediated modulation of synaptic plasticity appears to be age-dependent.
**P103**

Individual hippocampal mossy fiber synapses show differential plasticity depending on their history

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The hippocampal mossy fiber synapse displays a wide range of different forms of synaptic plasticity, which are expressed on the presynaptic as well as on the postsynaptic side at different time scales and stimulation intensities. These different types of plasticity require a variety of differential properties of the synapse. It has been suggested that there are different states of the mossy fiber synapse that may be established sequentially by different types of activation (“metaplastic switch”, Rebola et al. 2011, Nat. Neurosci.). If this is the case, one would expect that individual mossy fiber synapses at the same cell are encountered in such different states. In order to analyze synaptic transmission and synaptic plasticity at individual mossy fiber synapses, we combined single-bouton stimulation (Vyleta and Jonas 2014, Science) and 2-photon calcium imaging in individual spines postsynaptic to mossy fiber boutons. We labelled hilar mossy cells in organotypic entorhino-hippocampal slice cultures in the whole-cell patch clamp configuration using dye-filled pipettes. Alexa 594 was used to visualize the morphology of the spines whereas Fluo-4ff served to report calcium transients in single spines. A second pipette filled with Alexa 488 in extracellular solution was advanced under 2-photon excitation to the already labelled spines. Mossy fiber boutons presynaptic to these spines were visualized as shadows covering the spines, contrasting against the stained extracellular space (Kitamura et al. 2008, Nat. Methods). Stimulation of the attached mossy fiber bouton elicited EPSPs in the postsynaptic cell as well as calcium transients in the spine. We found a striking variability in the EPSP amplitudes as well as in the calcium responses, pointing to a varying AMPAR and NMDAR contribution, respectively. Furthermore, stimulation of the attached bouton paired with delayed back-propagating APs elicited in the postsynaptic cell resulted in strong potentiation of some synapses but failed to result in any changes in other spines with respect to EPSP amplitudes and amplitudes of calcium transients. The degree of potentiation was related to the primary state of the synapse before application of the paired induction protocol. These results suggest that individual hippocampal mossy fiber synapses reside in different states that reflect previous activity at these synapses.

M.F. is Senior Research Professor for Neuroscience of the Hertie Foundation.

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**P104**

The reduction of extrasynaptic AMPA receptors limits synaptic plasticity in a model of hepatic encephalopathy

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Hepatic encephalopathy (HE) is a frequent neuropsychiatric complication of both acute and chronic liver disease and can lead to cognitive dysfunction including learning and memory deficits. Ammonia is one of the key mediators in HE. Here, we have investigated glutamatergic neurotransmission under high ammonia conditions in a novel in vitro model of HE. Dissociated hippocampal neurons were co-cultured with astrocytes in a sandwich culture system. At DIV14, both neurons and astrocytes were stressed with clinically relevant concentrations of ammonia (1-5 mM) for 36 hrs. Molecular and functional aspects of glutamatergic neurotransmission were then selectively analyzed in neurons. Quantitative real time PCR and immunoblot analysis revealed a dose-dependent decrease in the expression of AMPA-type glutamate receptors (AMPARs), concerning both their pore-lining GluA and their auxiliary TARP and CNII subunits. Consistent with the expression data, we found a dose-dependent reduction of AMPAR currents in outside-out patches reflecting a decrease in the functional surface expression of extrasynaptic AMPARs. By contrast, basal synaptic transmission evaluated by AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) remained completely unaffected. There was neither a change in amplitude nor in frequency of AMPAR-mediated mEPSCs even under high ammonia conditions of 5 mM. However, when such neurons were challenged in a model of synaptic plasticity, functional deficits in glutamatergic neurotransmission were unveiled. In high ammonia, neurons did no longer show successful induction of chemical long-term potentiation (cLTP) anymore. We hypothesize that in HE, ammonia limits synaptic plasticity by reducing the extrasynaptic reserve pool of AMPARs required for fueling synaptic potentiation. (The first two authors contributed equally to this work).

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**P105**

GluN2B is not involved in depotentiation in chronically epileptic tissue

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In chronically epileptic tissue, transcriptional down-regulation has been reported for a number of ion channels. Almost all available data so far have focused on the dysfunction of putatively anti-convulsive ionic currents such as potassium channels. However, we have previously found that the NMDA receptor subunit GluN2B was up-regulated in CA1 neurons from pilocarpine-treated chronically epileptic rats. As a result, long-term potentiation (LTP) was enhanced in
excitatory to inhibitory evoked synaptic currents increased. This effect was evident only for rats aged about 29 to 36 days. Thereafter, mainly the amplitude of sEPSCs increased after iTBS.

**Conclusions:** First, we can confirm that a reduction in PV expression, as achieved by Orduz et al. [3] with PV-/− mice, increases the excitability and induced spike-train frequency of FS interneurons. Although reduced PV favours paired-pulse facilitation at the GABAergic synapses of FS neurons, our findings on spontaneous EPSCs and postsynaptic currents evoked by single-pulse stimulation of nearby neurons also indicate generally increased excitatory but reduced inhibitory cortical activity after iTBS. Second, we found an age-dependent effect of iTBS, indicating that cortical PV-type interneurons are most sensitive to iTBS during a time window coinciding with the maturation of these interneurons with respect to growth of the perineuronal nets and use-dependent refinement of excitatory inputs.

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**P106**

**Excitability changes of rat parvalbumin-positive cortical interneurons induced by transcranial magnetic theta-burst stimulation are age-dependent**

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**Question:** We could recently show that the strong reduction of parvalbumin (PV) expression in cortical fast-spiking (FS) interneurons by transcranial magnetic theta-burst stimulation of the intermittent kind (iTBS) is age-dependent, evolving first around postnatal day 32 when the perineuronal nets surrounding these neurons started to grow [1]. We now investigated whether this goes along with changes in the electrophysiological properties of FS interneurons.

**Methods:** Rats aged 26 to 62 days received either sham- or verum-iTBS (3 blocks of 600 pulses each) 60 minutes before killed for whole-cell patch-clamp recordings from frontal brain slices through somatosensory cortex. To specifically target the PV+/FS interneurons, we labelled their perineuronal nets with Cy3-conjugated Wisteria floribunda agglutinin (WFA) [2]. Passive and active electric cell membrane properties, spontaneous excitatory postsynaptic currents (sEPSCs) and postsynaptic currents (EPSCs/IPSCs) evoked by electrical stimulation within the same layer (2/3) were analysed.

**Results:** FS interneurons of verum-iTBS treated rats showed a higher excitability, expressed as a higher spike rate response to low current injection (100 pA) and a more depolarized resting membrane potential without changes in input resistance. Both, the frequency of sEPSCs and the ratio of these animals. It has been reported that GluN2B is required for depotentiation following the expression of LTP. To address this question, we studied depotentiation at the Schaffer collateral-CA1 synapse after theta-burst stimulation (TBS)-induced LTP. First, we confirmed that TBS-induced LTP was enhanced in epileptic tissue (161±8%, 60 min after TBS) compared to controls (134±5%, P<0.05). Then LTP was significantly reversed in epileptic tissue (122±9%, P<0.05), but less so in controls (124±8%). Bath application of the NMDA receptor antagonist D-AP5 (50 μM) prior to LFS abolished depotentiation in both control (138±10%) and epileptic tissue (171±8%) demonstrating NMDA receptor-dependency. Since GluN2B was up-regulated in epileptic tissue, we next tested the effect of the GluN2B subunit-specific blocker Ro 25-6981 (1 μM) on LFS-induced depotentiation. Under these conditions, depotentiation could be still induced in epileptic tissue (126±10%, P<0.05), but not in controls (136±15%). These results indicate that Schaffer collateral-CA1 synapses from epileptic animals expressed enhanced LTP as well as enhanced NMDA receptor-dependent depotentiation. However, while depotentiation is GluN2B-dependent in control tissue, GluN2B is not required for depotentiation in epileptic tissue.

**P107**

**Aromatase inhibition influences cellular correlates of hippocampus-dependent learning**

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Hippocampal neurons express aromatase, the final enzyme of estradiol synthesis, and actually synthesize and secrete estradiol. Inhibitors of aromatase are suspected of inducing memory deficits in women. Our recent data [1, 2] showed that inhibition of local estradiol synthesis by phosphorylation of aromatase in these neurons resulted in loss of spine synapses in female but not in male mice. Along these lines we focused on the effects of letrozole on long-term potentiation (LTP), which is a cellular correlate of memory and known to induce spines [3]. Our studies revealed a clear-cut sexual dimorphism in estrogen-induced synaptic plasticity. Inhibition of neuronal estradiol synthesis caused a dramatic LTP impairment in acute slices and also in hippocampal slice cultures and, as a likely consequence, induced spine synapse loss in slices of female animals but not of male animals [2]. Since inhibition of aromatase results in elevated levels of testosterone the question arises whether testosterone may be necessary for the maintenance of spine synapses and synaptic potentiation in the male hippocampus. In final steroidogenesis testosterone is either converted to estradiol by activity of aromatase or is irreversibly metabolized to 5alpha-dihydrotestosterone (DHT) by activity of 5alpha-reductase. Here we show that both enzymes are expressed in hippocampal neurons of both genders and as a consequence hippocampal neurons of male animals as well as of female
animals, are capable of synthesizing sexual neurosteroids, such as DHT and 17beta-estradiol. Consistently, stereological counts of spine synapses revealed a significant increase in spine synapse number in “male” hippocampal slice cultures in response to treatment with DHT, a non-aromatizable androgen, as compared to “female” hippocampal slice cultures. Application of finasteride, an inhibitor of 5alpha-reductase, results in a significant spine synapse loss in slice-cultures of males but not of females. Consistently, application of finasteride to acute slices reduced LTP predominantly in male animals. Similar effects were seen after blockade of androgen receptors with flutamide. Taken together, our data indicate sex-specific roles of sexual neurosteroids in the maintenance of hippocampal synapses.

Reference list

Stem cells and cardiac functions

P108
RBM20 deficiency prevents expression of the mature N2B isoform of titin in cardiomyocytes derived from human induced pluripotent stem cells
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Rationale: Although cardiomyocytes derived from human induced pluripotent stem cells (iPSC) have great potential for basic and applied research, they still stop short of full maturation. Titin, the giant elastic muscle protein, exhibits dramatic changes in the isoform-expression pattern during perinatal heart development. Initially a long and compliant, fetal, N2BA isoform (3.7 MDa) is expressed, which is then replaced around birth by shorter N2BA variants (3.3-3.6 MDa) and the stiff N2B isoform (3.0 MDa), which predominates in the adult human heart. The titin-isoform switch is due to alternative splicing within the elastic titin segment and is regulated by splicing factors such as RNA-binding motif protein 20 (RBM20), which contains a conserved serine/arginine (S/R)-rich domain mediating the mRNA binding. Mutated RBM20 causes dilated cardiomyopathy (DCM) in humans and animal models. We hypothesized that mutations within the S/R-rich domain of RBM20 affect the titin-isoform pattern in iPSC-derived cardiomyocytes by preventing the expression of adult N2B titin.

Materials and Methods: iPSC-derived cardiomyocytes were generated from healthy donors (control) and from two patients with a severe and a mild form of DCM, respectively, due to mutations in RBM20. In the patient with severe DCM, RBM20 carries a mutation within the S/R-rich domain; in the patient with mild DCM, the mutation in RBM20 is outside the S/R-rich domain. Titin isoforms were electrophoretically separated on loose SDS-polyacrylamide gels and detected by immunoblot using antibodies to N- and C-terminal titin sites.

Results: All cardiomyocytes derived from human iPSCs expressed the long, fetal N2BA isoform of titin. Control cardiomyocytes and cells with the mild DCM mutation outside the S/R-rich domain of RBM20 also expressed the adult N2B isoform in 71% and 25% of cases, respectively. Cardiomyocytes with the severe DCM mutation inside the S/R-rich domain of RBM20 never expressed the adult N2B-titin isoform.

Conclusions: The S/R-rich RNA-binding domain of RBM20 is crucial for alternative splicing of titin during cardiomyocyte maturation. Mutations in this domain prevent the expression of adult N2B titin, which may contribute to the severe DCM phenotype in affected patients.

P109
Purification of cardiomyocytes from iPSC cells with a non-clonal strategy enables automated planar patch clamp analysis of long QT-syndrome 3
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Induced pluripotent stem (iPS) cells can be generated from patients with cardiac disease and differentiated into functional cardiomyocytes. The aim of this study was to establish a straight-forward selection strategy to obtain pure cardiomyocytes for characterization of a cardiac disease using automated electrophysiological analysis. We therefore designed a novel non-clonal purification approach using a lentivirus to express a puromycin resistance gene under the control of a cardiac-specific promoter. With this virus we infected our previous reported wild-type and LQT3-specific mouse iPS cells. Because the lentivirus construct also expressed a neomycin resistance under the stem cell promoter Rex1, iPS cells were selected without time-consuming picking of individual clones and could be stably propagated in bulk culture without virus silencing. Application of puromycin during differentiation gave rise to a nearly pure cardiomyocyte population (wild-type iPS cell line: 92.8±6.2 %, n=5 and LQT3-specific iPS cell line: 87.7±9.7 %, n=4).

Using conventional patch clamp experiments we found that purified cardiomyocytes from the non-clonal LQT3-specific iPS cell line preserved the phenotype indicated by prolonged action potential duration (ADP) at slow pacing rate and early afterdepolarizations which both were not seen in wild-type controls. The slope of ADP restitution...
Cardiotrophin-1 (CT-1) regulated signaling pathways. Cardiomyocytes differentiated form early (P12-P18) and late (P19-P35) passages of non-clonal IPS cells showed identical action potential parameters (resting potential, APD, Vmax, APD restitution slope) proving the stability of the non-clonal IPS cell lines. For automated electrophysiological analysis we used planar patch clamp recordings (Patchliner, Nanion) of purified cardiomyocytes. Using this method the LQT3 phenotype with positive APD restitution slope and early afterdepolarizations could be automated and reliably detected. Also we found no significant differences in action potential parameters compared with the manual patch clamp data. Hence, LQT3-specific cardiomyocytes can be purified from IPS cells with a non-clonal lentiviral strategy, maintain the hallmarks of the cardiac disease and can be used for automated electrophysiological analysis.

P110
Nuclear translocation of cardiotrophin-1 and its effects on cardiomyogenesis of embryonic stem cells

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Cardiotrophin-1 (CT-1) controls cardiomyogenesis of mouse embryonic stem (ES) cells. CT-1 enhanced cardiomyogenesis, increased the cardiac transcription factors MEF2c, Nkx-2.5, TEAD3 and GATA4, the cardiac proteins α-actinin, MLC2a, MYH7, MLC1a, MLC2v and HCN4 as well as vascular endothelial growth factor (VEGF), platelet-derived growth factor-BB (PDGF-BB), fibroblast growth factor-2 (FGF-2) and atrial natriuretic peptide (ANP). CT-1 downregulation by small interfering RNA (siRNA) inhibited cardiomyogenesis and decreased VEGF, PDGF-BB, FGF-2 and ANP expression. CT-1 raised intracellular calcium which was abolished by the intracellular calcium chelator BAPTA, AM and thapsigargin. Moreover, CT-1 treatment increased reactive oxygen species (ROS), followed by nitric oxide (NO) generation and NOS3 activation. During ES cell differentiation CT-1 was translocated to the cell nucleus. Exogenous CT-1 induced nuclear translocation of endogenous CT-1 which was inhibited by BAPTA, the NOS inhibitor L-Nω-Nitroarginine methyl ester (L-NAME), the radical scavenger N-(2-mercaptopyrrolyl)glycine (NMPG) as well as the janus kinase 2 (JAK2) inhibitor AG490 and the PI3 kinase (PI3K) inhibitor LY294002. In conclusion, nuclear translocation of CT-1 may be central to cardiomyogenesis and involves calcium, NO, ROS as well as CT-1 regulated signaling pathways.

P111
Structural and functional maturation of stem cell-derived cardiomyocytes

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Cardiomyocytes derived from induced pluripotent stem cells (iPSC-CMs) represent promising tools for cell-based therapy of human myocardial diseases. Despite their cardiogenic properties, these cells maintain immature features in culture, especially regarding the structural organization of their microarchitecture, myofilament orientation and spontaneous beating behavior. However, successful application for myocardial repair requires a similar physiological complexity of iPSC-CMs like in mature cardiomyocytes. Therefore, better insight into the mechanisms driving cardiac maturation are needed in order to develop novel strategies to enhance the cardiac phenotype in iPSC-CMs. Here, we tested the hypothesis that structural and functional features of iPSC-CMs may be strongly influenced by their immediate environment and cardiac maturation may be enhanced by optimization of the respective culture conditions. We investigated the effect of different predefined geometries on cell and myofilament orientation and the resulting functional changes at the level of Ca2+ handling during excitation-contraction (EC)-coupling in single iPSC-CMs. Using the new technology of direct laser writing, we developed flexible 3D scaffolds from polyethylene glycol-based photosensitive polymers in defined rectangular and polygonal shapes. Murine iPSC-CMs were seeded onto these fibronectin-coated scaffolds and cultured in forced geometry for one week. Changes in the structural arrangement of myofilaments and the expression pattern of EC-coupling relevant proteins were assessed by immunocytochemistry in dependence of the different shapes of cells and scaffolds. Functional analysis of Ca2+ transients was performed by live-imaging of changes in fluorescence intensity of Ca2+-sensitive fluo-4 using confocal laser-scanning microscopy. In contrast to cells grown without any peripheral constraints, rectangular shapes, which mimic the characteristic form of an adult cardiomyocyte, improved the orientation of myofilaments in iPSC-CMs and synchronized Ca2+ transients, leading to enhanced temporal characteristics of the cytosolic Ca2+ signals. These results indicate functional maturation of Ca2+ handling during EC-coupling. In addition, spontaneous contractions revealed improved rhythmicity in rectangular-shaped cardiomyocytes. In conclusion, we show that structural restrictions critically influence the morphological and functional development of iPSC-CMs at the level of Ca2+ signaling during EC-coupling. These environmental requirements of iPSC-CMs have to be taken into account for the design of novel cardiac constructs, which need to present functional features that are compatible with adult cardiac tissue.
P112
Induction of cardiac gene expression in cultivated epicardium-derived cells (EPDC) by coculture with microvesicles / exosomes from apoptotic cardiomyocytes

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EPDC have been reported to transdifferentiate into functional cardiomyocytes; however, this is a rather rare event. In the present study, we therefore aimed to identify mechanisms that may promote the conversion of EPDC into the cardiomyocyte lineage.

EPDC formed in the rat heart by ischemia (60 min) with subsequent reperfusion were enzymatically isolated 5d post myocardial infarction (MI). We found that EPDC can be well cultivated for up to 20 passages with a doubling time of 35.3 +/- 8.5 hrs. They exhibit an MSC-like morphology and retain the expression of Wt1, the gold marker for EPDC, throughout in vitro expansion. EPDC express distinct cardiac marker genes such as Gata4, Nkx2.5, Tbx5, tropinin T and Mhc-beta. They also display endocytic properties and avidly take up nanoparticles. To identify the biological relevance of this process, we have prepared microvesicles/exosomes derived from apoptotic cardiomyocytes by sequential centrifugation steps. Microvesicles/exosomes were well incorporated by EPDC and expression analysis revealed that this uptake significantly increased gene expression of cardiac and endothelial marker genes such as Tbx5, tropinin T and Mhc-beta as well as cadherin 5 and Pecam-1.

In a translational attempt, we have successfully established a protocol for the isolation of EPDCs from human atrial biopsies (bypass surgery after MI). Human EPDCs display a similar phenotype, expression profile and similar functional properties as their murine counterparts. In conclusion, we have characterized cultured EPDC from rat and men and demonstrate that they avidly take up microvesicles/exosomes which most likely contain instructive signals to promote the conversion of EPDC into cardiomyocytes.

P114
Expression of fast vs. slow cardiac myosin heavy chain in human embryonic stem-cell derived cardiomyocytes: effects on force generation, tension cost and time course of twitches

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**Aim:** Human embryonic or induced pluripotent stem cell-derived cardiomyocytes (hESC-CMs or hiPSC-CMs), after typical differentiation protocols lasting ~14 days still express high levels of the fast cardiac myosin heavy chain (α-MyHC). In human ventricular cardiomyocytes, however, the slow β-MyHC predominates. To assess effects of α-MyHC in hESC-CMs used as cellular models for human diseases we investigated the functional significance of α- vs. β-MyHC expression for contractile properties of hESC-CMs at the cellular and sarcomere level.

**Methods:** To examine cellular contractility, hESC-CMs differentiated and propagated as “cardiac bodies” in suspension culture were dissociated and plated on laminin-coated coverslips. Cells were electro-stimulated to record twitch kinetics by edge detection and intracellular calcium-transients after loading with fura-2 AM. Time to peak (ttp) and half relaxation time (hrt) of twitch and calcium transients were analyzed. After this functional analysis, MyHC-isof orm expression of each individual CM was identified using α-MyHC and or ATP increased the number of cardiac clusters and the frequency of contractions. Molecular analysis of cardiogen-specific genes showed an enhanced expression of α-MHC, MLC2v, HCN4, α-actinin and connexin43 (Cx43) on both gene and protein levels upon ADP/ATP treatment, indicating increased cardiomyogenesis and pacemaker cell differentiation. Quantitative RT-PCR analysis of purinergic receptor expression demonstrated presence of P2Y1, P2Y2 and P2Y4 on differentiating ES cells. ATP and ADP but not UTP or UDP transiently increased the intracellular calcium concentration ([Ca2+]i) as evaluated by the calcium indicator Fluo-4, whereas no changes in membrane potential were observed. Cytosolic [Ca2+], transients induced by ADP/ATP were abolished by the phospholipase C (PLC) inhibitor U-73122, suggesting involvement of metabotropic P2Y receptors. Furthermore, partial inhibition of [Ca2+], transients was achieved in presence of MRS2179, a selective P2Y1 receptor antagonist, whereas PPADS, a non-selective P2YRs inhibitor, completely abolished the [Ca2+], response. Consequently, cardiomyocyte differentiation was decreased upon long term co-incubation of cells with ADP and P2 receptor antagonists. In summary it is demonstrated that the activation of purinoceptors and the subsequent intracellular [Ca2+]i transients enhance the differentiation of ES cells towards cardiomyocytes. Purinergic receptor stimulation may be a promising strategy to drive the fate of pluripotent ES cells into a particular population of cardiomyocytes.
β-MyHC specific antibodies and a cell mapping technique. For analysis of sarcomere contraction, chemically demembranated hESC-CMs were attached to a force probe and a motor. For activation/relaxation of these cells, solutions with different calcium-concentrations were rapidly exchanged. To boost CMs towards pure α-MyHC-expression, some CMs were treated for 7 or 14 days with T3 or T4, respectively.

Results: At a stimulation frequency of 0.5 Hz, ttp (170-205ms) and hrt (104ms) of twitches on average were not different for hESC-CMs expressing either α-MyHC or β-MyHC. Also, ttp of calcium transients was very similar for CMs expressing either of the two MyHC isoforms. Likewise, twitch kinetics at normal beating frequencies of human cardiomyocytes of 1 and 2 Hz were independent of the myosin isoform. To dissect the role of calcium handling vs. speed of cross-bridge cycling for the hESC-CM-twitch kinetics, we also measured contraction parameters of demembranated CMs. Maximum isometric force was similar (30-40 kPa) for cells with β- or α-MyHC (T3-treated). Two key parameters for the speed of cross-bridge cycling, force redevelopment after a quick release (kTR) and the first phase of force decay upon rapid calcium removal (kLIN) were 0.7 s⁻¹ and 2.4 s⁻¹ for kTR of β-MyHC and α-MyHC-expressing CMs, respectively, while klin was 0.37 s⁻¹ for β-MyHC cells and 1.47 s⁻¹ for α-MyHC. 

Conclusion: Twitch-kinetics of hESC-CMs at 0.5-2 Hz beat-frequency appear dominated by kinetics of calcium transients while cross-bridge kinetics are not limiting. Importantly, however, the faster kLIN for α-MyHC-expressing CMs indicates higher tension cost compared to hESC-CMs with the ventricular β-MyHC. This should be considered when using CMs expressing mainly α-MyHC for cellular disease models, tissue engineering or for testing effects of new drugs.

P115 Electrophysiological characterization of cardiomyocytes from the LQT3 and control human iPS cell lines was performed using classical whole-cell patch clamp recordings. Peak Na⁺ current density was not different, but recovery from inactivation of Na⁺ current was significantly faster in LQT3 (3.6 ± 0.6 ms, n=5) compared to control cells (10.6 ± 2.3 ms, n=13). Action potential duration (APD) was recorded at various pacing frequencies and showed a prolongation at slow heart rates in LQT3, but not in control cells. Although APD at 1 Hz was not significant different overall, however, analyzing only cardiomyocytes with ventricular-like action potentials showed significant longer APD in cells from LQT3 iPS cells (234.9±35.2 ms, n=8) compared with controls (137.3±15.2 ms, n=5). To quantify the frequency-dependent APD prolongation, the slope of APD restitution (APD versus pacing period) was determined for each individual cell by linear regression analysis. This yielded a negative slope (-3.3±3.5 ms/s, n=5) for control cells, whereas a clearly positive slope (19.3±5.4 ms/s, n=8) was observed in LQTS 3 iPS-derived cardiomyocytes. Application of the Na⁺ channel blocker mexiletine (100 µM) reduced APD prolongation only in LQT3 cardiomyocytes (-25.8±6.7%, n=5), but not in control cells (+3.2±4.8%, n=8). Thus, iPS cell-derived cardiomyocytes from a LQT3 patient showed known biophysical features of LQT3 and allow patient-specific pharmacological screening in the future.

P116 AdipoR1 and AMPK are involved in CTRP7 and CTRP9 induced glucose uptake in cardiomyocytes

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Background: The adipose tissue-derived cytokine adiponectin plays a major role in glucose metabolism. A family of adiponectin paralogs, designated as C1q/tumor necrosis factor-alpha-related proteins (CTPRs), has recently been discovered. However, no data exists on their effects on glucose metabolism in cardiomyocytes.

Aims: The aim of this study was to determine whether CTPRs affect glucose uptake via increased GLUT1 and GLUT4 translocation and to investigate possible signaling pathways in H9C2 cardiomyoblasts and isolated adult rat cardiomyocytes treated with CTPRs. A further objective was to examine the role of adiponectin receptor 1 (adipoR1).

Materials and Methods: H9C2 cardiomyoblasts or adult rat cardiomyocytes were treated with different recombinant CTPRs (4 µg/ml) produced in E. coli. Glucose uptake was monitored with radiolabeled glucose ([3H] 3-O-methyl-D-glucose), while translocation of glucose transporters (GLUT) was assessed by Western blotting and confocal fluorescence microscopy. We determined protein phosphorylation

The long QT syndrome type 3 (LQT3) is an inherited heart disease caused by gain of function mutations of Na⁺ channels. It is characterized by long QT intervals in the ECG because of prolonged action potentials in cardiomyocytes especially at slow heart rates. Affected patients have a high risk of torsades de pointes ventricular tachycardia and sudden cardiac death during rest or sleep.

To investigate the phenotype of patient-specific human cardiomyocytes with LQT3, induced pluripotent stem cell lines were generated from a LQT3 patient with the R1644H mutation in the α-subunit of the Na⁺ channel and from a not affected control person. To this aim fibroblasts were obtained by skin biopsies and transduced with retroviruses to obtain fibroblast derived iPS cells with long QT syndrome type 3

Electrophysiological characterization of cardiomyocytes from the LQT3 and control human iPS cell lines was performed using classical whole-cell patch clamp recordings. Peak Na⁺ current density was not different, but recovery from inactivation of Na⁺ current was significantly faster in LQT3 (3.6 ± 0.6 ms, n=5) compared to control cells (10.6 ± 2.3 ms, n=13). Action potential duration (APD) was recorded at various pacing frequencies and showed a prolongation at slow heart rates in LQT3, but not in control cells. Although APD at 1 Hz was not significant different overall, however, analyzing only cardiomyocytes with ventricular-like action potentials showed significant longer APD in cells from LQT3 iPS cells (234.9±35.2 ms, n=8) compared with controls (137.3±15.2 ms, n=5). To quantify the frequency-dependent APD prolongation, the slope of APD restitution (APD versus pacing period) was determined for each individual cell by linear regression analysis. This yielded a negative slope (-3.3±3.5 ms/s, n=5) for control cells, whereas a clearly positive slope (19.3±5.4 ms/s, n=8) was observed in LQTS 3 iPS-derived cardiomyocytes. Application of the Na⁺ channel blocker mexiletine (100 µM) reduced APD prolongation only in LQT3 cardiomyocytes (-25.8±6.7%, n=5), but not in control cells (+3.2±4.8%, n=8). Thus, iPS cell-derived cardiomyocytes from a LQT3 patient showed known biophysical features of LQT3 and allow patient-specific pharmacological screening in the future.
by immunoblotting and used PCR to analyze mRNA level of genes involved in glucose metabolism and expression level of CTRPs. The role of adiponectin receptor 1 (adipoR1) was determined via siRNA-mediated knockdown in the H9C2 cell line.

**Results:** PCR revealed that CTRPs are ubiquitously expressed in rat organs and each of the CTRPs has a unique expression profile. Furthermore, most CTRPs altered the expression of genes related to glycolysis and gluconeogenesis. Furthermore, CTRP7 and CTRP9 induced translocation of GLUT4 and GLUT1 from cytosol to cell membrane in H9C2 cardiomyoblasts, while only CTRP 9 induced GLUT1 translocation in cardiomyocytes. After AMPK inhibition, the effect of CTRPs on glucose uptake and glucose transporter translocation disappeared. In adult rat cardiomyocytes, only CTRP 9 increased glucose uptake and translocation of GLUT1 and GLUT4 whereas CTRP 7 had no effect. The CTRP 7 and CTRP 9-induced phosphorylation of AMPK and ACC is mediated by adipoR1 as seen in knockdown experiments.

**Conclusions:** The adiponectin paralogs CTRP7 and CTRP9 enhance glucose uptake and induce GLUT1 and GLUT4 translocation in cardiomyocytes through an AdipoR1 and AMPK-dependent signaling pathway. CTRPs may provide a therapeutic target in metabolic diseases such as diabetes and obesity.

**P117**

*RhoA controls myofibroblast characteristics*

**Purpose:** During the remodeling of the heart cardiac fibroblasts (CF) undergo a transition towards a myofibroblastic phenotype showing an increased proliferation and migration rate. This process and the contributing signaling cascades are still poorly understood. Nevertheless, it was observed that a significant remodeling of the fibroblast cytoskeleton is involved. Since RhoA is known to be a major regulator of the cytoskeleton we aim to investigate its role in cardiac fibroblasts and its effect on myofibroblast characteristics.

**Methods:** To investigate the role of RhoA in neonatal rat fibroblasts we used a lentivirus induced knockdown (kd-CF). Wildtype fibroblasts were treated with 10 µM Fasudil cardiac fibroblasts we used a lentivirus induced knockdown of RhoA. To investigate the role of RhoA in neonatal rat fibroblasts we used a lentivirus induced knockdown (kd-CF). Wildtype fibroblasts were treated with 10 µM Fasudil.

**Results:** RhoA kd-CF develop an epithelial-like morphology lacking stress fibers and higher order actin structures like geodesic domes. The orientation of focal adhesions sites along the cell stress axis was also impaired. This phenotype could be mimicked by the treatment of CF with Fasudil. Furthermore, in knockdown cells cytoskeletal proteins were found unchanged except for a decrease in the myofibroblast marker smooth muscle actin by 43% and an increase in acetylated tubulin by 57% without a shift in the expression of HDAC6. This phenotype could be induced by TubA. In order to analyze both changes in actin and tubulin cytoskeleton we investigated the migration and proliferation rate of CF. The reduction of RhoA caused a significantly faster adhesion but migration velocity was inhibited (shControl 4043±316 nm/h versus shRhoA 3021±153 nm/h). The serum-driven proliferation rate of kd-CF was reduced by 50%. Fasudil significantly decreased migration velocity by 62% but had no effect on the proliferation rate. TubA treated CF showed just a 30% decrease in migration velocity but a 60% reduced proliferation rate. EHM complemented with kd-CF showed no change in resting tension but a reduction in contractile force and the cardiomyocyte marker calsequestrin by 30%.

**Conclusion:** RhoA influences the two main myofibroblast characteristics, migration and proliferation, by interfering with the actin and tubulin cytoskeleton. This can be mimicked by the use of Fasudil and Tubastatin A, respectively. The downregulation of RhoA leads to loss of cardiac contractile function and influences cardiomyocyte survival.

**P118**

*Analysis of the cardiac mitochondrial phosphoproteome*

**Purpose:** To analyze the AKT1 and AKT2 isofrom-specific signaling in the heart we have generated conditional KO mice allowing inducible deletion of AKT1 and AKT2 isoforms in cardiomyocytes of the adult heart (iCMATKTKO). Using tandem-affinity purification and proximity ligation assay we identified the α1-subunit of the ATP synthase as a novel interaction partner of AKT1 in HEK293T cells and adult murine cardiomyocytes, demonstrating that AKT1 appears to be imported into mitochondria and regulates ATP synthesis.

**Methods:** To investigate the role of RhoA in neonatal rat fibroblasts we used a lentivirus induced knockdown (kd-CF). Wildtype fibroblasts were treated with 10 µM Fasudil for ROCK inhibition or 5 µg/mL of Tubastatin A (TubA) for blocking the tubulin-specific deacetylase HDAC6. Cytoskeletal proteins were analyzed by immunoblot and immunofluorescence. Adhesion velocity and migration was determined by life cell imaging and the serum-driven proliferation rate by nuclei counting over a time course of four days. The contribution of kd-CF to cardiac function was investigated by generating engineered heart muscle (EHM).

**Results:** RhoA kd-CF develop an epithelial-like morphology lacking stress fibers and higher order actin structures like geodesic domes. The orientation of focal adhesions sites along the cell stress axis was also impaired. This phenotype could be mimicked by the treatment of CF with Fasudil. Furthermore, in knockdown cells cytoskeletal proteins were found unchanged except for a decrease in the myofibroblast marker smooth muscle actin by 43% and an increase in acetylated tubulin by 57% without a shift in the expression of HDAC6. This phenotype could be induced by TubA. In order to analyze both changes in actin and tubulin cytoskeleton we investigated the migration and proliferation rate of CF. The reduction of RhoA caused a significantly faster adhesion but migration velocity was inhibited (shControl 4043±316 nm/h versus shRhoA 3021±153 nm/h). The serum-driven proliferation rate of kd-CF was reduced by 50%. Fasudil significantly decreased migration velocity by 62% but had no effect on the proliferation rate. TubA treated CF showed just a 30% decrease in migration velocity but a 60% reduced proliferation rate. EHM complemented with kd-CF showed no change in resting tension but a reduction in contractile force and the cardiomyocyte marker calsequestrin by 30%.

**Conclusion:** RhoA influences the two main myofibroblast characteristics, migration and proliferation, by interfering with the actin and tubulin cytoskeleton. This can be mimicked by the use of Fasudil and Tubastatin A, respectively. The downregulation of RhoA leads to loss of cardiac contractile function and influences cardiomyocyte survival.
Analysis of the mitochondrial phosphoproteomes led to the identification of 190 phosphopeptides corresponding to 106 mitochondrial proteins. Among these, 64 phosphosites were newly discovered. After insulin stimulation, 165 mitochondrial phosphopeptides corresponding to 92 phosphoproteins and 47 new phosphosites were found. Phosphoproteins were located in all submitochondrial compartments and were associated with all mitochondrial functions including β-oxidation, apoptosis, respiratory chain, etc. Several mitochondrial proteins appeared to be differentially phosphorylated in AKT-KO mice. The α-subunit of the ATP-synthase was a prominent target for phosphorylation. Of six identified phosphosites, Ser52 was regulated in an AKT-dependent manner.

Taken together, mitochondrial proteins are modified substantially by phosphorylation. Direct interaction and regulated phosphorylation of the α-subunit of the ATP synthase suggest a regulatory role of AKT in ATP synthesis.

P119
Role of Protein kinase C (iota) for cardiac function: electrophysiological insights from cardiac specific gene transfer


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The protein kinase family C (PKC) is well known to regulate signalling pathways involved in cell development, proliferation, differentiation, metabolism and cell death. While we readily understand the roles of conventional and novel PKCs in cardiac function close to nothing is known about the putative importance of the atypical PKCs (aPKCs), PKC(iota) and PKC(zeta), for the heart. aPKCs are not activated by diacylglycerol (DAG) or Ca²⁺ but are believed to gain activity through direct protein-protein interactions. Here we set out to investigate the role of PKC(iota) for cardiac function by cardiac myocyte specific expression of PKC(iota) mutants, a constitutively active (CA) and dominant negative (DN) isoform delivered by serotype 9 adenovirus (AAV) both fused to the red fluorescent protein TagRFPT, whose sole expression also served as a control.

We investigated both in vivo and in vitro parameters by employing echocardiography to study cardiac function on the systemic level and investigation of cellular calcium handling and contractility as well as cellular electrophysiological properties to reveal cellular changes.

We found significant hypertrophy for PKC(iota)-DN hearts and ventricular relaxation times were largely altered in this group. On the level of individual myocytes, aberrant calcium handling as well as an altered calcium-contraction relationship was revealed indicating a changed calcium handling in both CA and DN isoforms. Using the patch clamp technique in its whole cell configuration we found that the time course of action potentials was significantly prolonged (APD80) for cells expressing PKC(iota)-DN while surprisingly the cellular capacitance as a parameter for cell size was significantly reduced for both PKC(iota) isoforms, DN and CA, despite hypertrophy in DN injected mice (see above). Furthermore calcium current peak sizes differ significantly between CA and DN, emphasizing our results in calcium imaging experiments.

From our data we conclude that employing dominant negative and constitutively active isoforms of PKCs, for which no specific inhibitors are available, is a valuable tool to specifically investigate their cardiac function. Our study clearly emphasizes the involvement of PKC(iota) in cardiac function such as hypertrophy as well as cardiac myocyte function such as their electrophysiology properties, calcium handling and contractility. These data highlight aPKCs as possible novel targets for modulating cardiac function and malfunction such as hypertrophy.

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P120
Structural interplay of KCNE1 and K\textsubscript{7.1} is crucial for both, SQT2 and LQT1

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Tetrameric K\textsubscript{7.1} channels dynamically coassemble with preferentially two β-subunits KCNE1 to conduct the cardiac delayed rectifier current I\textsubscript{Ks}. Dysfunction has been associated with both, ventricular and atrial cardiac repolarization disorders, resulting in increased risk of cardiac arrhythmia and sudden death. K\textsubscript{7.1} loss-of-function mutations often lead to long QT syndrome 1 (LQT1) whereas gain-of-function mutations may cause short QT syndrome (SQT) or atrial fibrillation (AF).

Here, we describe a vulnerable amino acid position that, if mutated, leads to both, AF and LQT1, depending on the specific mutation. Employing mutagenesis and molecular modeling we found that the wild-type-residue is crucial for KCNE1 effects on ion selectivity, gating and conductance rate. Hence, the amino acid position is central in the molecular interplay between the outer domain of KCNE1 and K\textsubscript{7.1}. Mutations can cause both, gain-of-function or loss-of-function depending on the K\textsubscript{7.1}/KCNE1 stoichiometry. Using RT-qPCR, we investigated the subunit stoichiometry in human atria and ventricles and found that the relative expression of K\textsubscript{7.1} and KCNE1 transcripts was in the range from about 1:4 up to 1:24.

Summarizing, we identified a clinically relevant key amino acid in K\textsubscript{7.1} crucial for the interplay between K\textsubscript{7.1} and KCNE1. Its functional effects may vary among different heart regions and possibly among patients determined by differences in the ratio of expressed K\textsubscript{7.1} versus KCNE1 potentially leading to large clinical variations of SQT.
P121
Heart rate reduction and longevity in mice
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**Background:** Heart rate correlates inversely with life span across all species, including humans. In patients with cardiovascular disease, higher heart rate is associated with increased mortality, and such patients benefit from pharmacological heart rate reduction. However, cause-and-effect relationships between heart rate and longevity, notably in healthy individuals, are not established. We therefore prospectively studied the effects of a life-long pharmacological heart rate reduction on longevity in mice. We hypothesized, that the total number of cardiac cycles is constant, and that a 15% heart rate reduction might translate into a 15% increase in life span.

**Methods:** C57BL6/J mice received either placebo or ivabradine at a dose of 50 mg/kg/day in drinking water from 12 weeks to death. Heart rate and body weight were monitored. Autopsy was performed on all non-autolytic cadavers, and parenchymal organs were evaluated macroscopically. Results: Ivabradine reduced heart rate by 14 (median, interquartile range 12 to 15)% throughout life, and median life span was increased by 6.2 % (p=0.01). An inverse relationship existed between individual heart rate and life span in all tested mice (r=0.86; p=0.046). Body weight and macroscopic findings were not different between placebo and ivabradine. Conclusion: Life span was not increased by the same extent as heart rate was reduced, but nevertheless significantly prolonged by 6.2 %, which in humans translates to an additional 5 years for a person of 80 years.

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P122
Gq signaling in the heart impacts primary pacemaking and conduction propagation
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In the mammalian heart, a plethora of processes are controlled or modulated via G-protein coupled receptors (GPCRs) that evoke intracellular signal transduction. While the role of Gq-, Go- and Gs-proteins for excitation generation, that is pacemaking, impulse propagation, i.e. cardiac conduction system, and the myocardial function, e.g. contractility, are well understood, the detailed contribution of Gq-proteins especially to pacemaking and excitation conduction remains largely elusive. Here, we employed a novel mouse model expressing a design receptor exclusively activated by design drugs (DREADD) specifically coupling to Gq-proteins, the r/hM3Dq-receptor, to gain detailed insight into Gq-mediated signal transduction in the heart. This DREADD has no physiological agonist but can be activated by the synthetic ligand CNO. Striated muscle specific expression was established with a muscle creatine kinase promoter. We demonstrate cardiac myocyte expression with immunofluorescence directed against an incorporated HA-tag. Initially, we studied survival of the mice following injection of various doses of CNO and found a dose-dependent lethality. At highest concentration, transgenic but not wt mice ceased between 0.5 and 4 hours after i.p. injection of CNO. In-vivo effects of DREADD activation were investigated in more details in mice with implanted blood pressure and ECG sensors. While in wt mice, CNO had no effect on blood pressure and ECG parameters, transgenic mice showed severe arrhythmic events, including first to third degree atrioventricular blocks and tachycardiac episodes (>850 bpm) shortly after CNO injection. Isolated, intact hearts displayed concordant behavior demonstrating the cardiac specificity of our findings. While administration of CNO induced an increase of the amplitude of electrically-induced global calcium transients in isolated ventricular and atrial myocytes around 40% of the atrial myocytes became electrically not-excitable after 10 minutes. We therefore conclude that the r/hM3Dq-receptor represents a novel and important tool to specifically study the role of Gq-coupled signaling revealing novel functions of Gq-signal transduction for pacemaking and impulse propagation going well beyond mere production of inositol-trisphosphate. In light of our findings, the precise role of Gq signaling in the heart has to be reconsidered and revisited in future studies aiming to unravel the signaling cascades leading to Gq-dependent alterations of the electrophysiological properties of the heart.

P123
Increased gene expression of the cardiac endothelin system in obese mice
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**Question:** Obesity is a well-known risk factor of atherosclerosis and heart failure. In the human heart, a local endothelin system containing prepro-endothelin-1,
P124

Analysis of effective connectivity in sensory-isolated neuronal networks reveals intrinsic synchronization of spontaneous bursting activity

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Spontaneous activity is a remarkable feature of living neuronal networks: certain level of neuronal activity is present even in the absence of apparent external stimuli. In early development, spontaneous activity plays crucial role in circuitry formation in neuronal networks both in vivo and in vitro. Dissociated neuronal cultures represent neuronal networks that consist of a relatively small number of interconnected neuronal elements. In contrast to intact brain, cultured neuronal networks are chronically isolated from physiologically relevant sensory inputs. Therefore, spontaneous activity observed in neuronal cultures is intrinsic and represents the sole mechanism that drives the formation of connectivity. Furthermore, due to sensory isolation, cultured neuronal networks are void of external input-driven modification of connectivity characteristic for intact brain.

Notably, repetitive neuronal firing in series (bursts) of action potentials (spikes) followed by periods of quiescence is the predominant pattern of activity in neuronal cultures, which becomes particularly prevalent upon maturation. The bursting was shown to reflect the intrinsic mode of activity of neurons in the hippocampus, and was proposed to represent more reliable carrier of information or a distinct neural code.

We hypothesized that spontaneous bursting in neuronal networks is involved not only in development, but also in the maintenance of the network connectivity. To address this question, we monitored the spontaneous neuronal activity in rat hippocampal cultures over several consecutive weeks (DIV14-DIV35) corresponding to different developmental stages associated with formation and maturation of synaptic connectivity. Throughout the analyzed period, spontaneous spike trains in neuronal cultures grown on microelectrode arrays were characterized by high variability in the temporal domain reflecting the presence of series of spikes and relatively long inter-burst intervals. However, the analysis of distribution of inter-burst intervals demonstrated that the initiation of spontaneous bursts can be considered as Poisson process, and remains stochastic at all developmental stages. Next, we evaluated the development of the effective connectivity represented by synchronization of bursting onset between remote network locations. We found that individual pairwise connections underwent developmental optimization associated with significant reduction of the burst onset lag, which was stabilized after DIV28 within gamma temporal window (8-30 ms). Taken together, our results suggest that synchronization of spontaneous bursts across the network can serve as a mechanism of formation of connectivity and might represent a default mode of the network activity necessary for the maintenance of existing network architecture.

P125

SPRED2 – a crucial regulator of synaptic efficacy and of behavior

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Question: SPRED proteins are inhibitors of Ras/ERK-MAPK signaling and thus of gene transcription and cell proliferation. Recently, we demonstrated that SPRED2 is indispensable for appropriate regulation of hormonal homeostasis. We detected a complete up-regulation of stress hormone secretion from all levels of the HPA axis in our SPRED2 KO mouse model. This elevated release of CRH, ACTH, corticosterone, and aldosterone was provoked by an up-regulated hypothalamic Ras/ERK/MAPK signaling and associated with an...
Pavlov’s scientific heritage

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In 2014 memorable dates are connected with the name of Ivan Petrovich Pavlov. First of all, it is the 165th anniversary since the scientist’s birthday (1849) and the 110th anniversary since Pavlov was awarded with the Nobel Prize for his fundamental contribution to science. Moreover, it is the 140th anniversary since the beginning of his research work, e.g. Pavlov’s publication of his first works on the physiology of digestion and blood circulation (1874–1875).

Three main periods in Pavlov’s scientific carrier can be differentiated: researches in the field of blood circulation, digestion and physiology of the higher nervous system. The so called “synthetic physiology of digestion” represents the theory proposed by Pavlov. The concept of synthetic physiology attempts to cover the digestive system as a whole. The difficulty of creating a whole concept of physiology was based on lack of new research methods. I.P. Pavlov created some new experimental methods, e.g. the method of imaginary feeding. Another experimental method, the so called “small stomach”, was created by Pavlov to analyze two phases of the gastric juice secretion.

While studying the dynamics of the secretory process in different parts of digestive tract, I.P. Pavlov described the leading role of the nervous system in the regulation of digestive process. In other words, one can imagine the nervous system as a conductor of an orchestra. Moreover, Pavlov studied the coordination of secretory and motor activities of digestive organs. He discovered the close functional connection of the whole digestive tract, e.g. the fine-tuned correlation between stomach’s secretion and duodenal reaction.

In 1897 I.P. Pavlov published the collection of his scientific work “Lectures on the Main Digestive Glands Activity”, in which he summarized results of his research on the physiology of digestion. On 20th October 1904 I.P. Pavlov was awarded with the Nobel Prize for his works on the physiology of digestion.

The higher nervous activity was the next milestone of Pavlov and his numerous collaborators and students. The focus of his scientific research was mostly dedicated to cerebral cortex. Pavlov analyzed the dynamics of cortical processes. He focused on sleep and hypnotic phenomenon. Further studies showed that the cortex activity played the dominant role in coordinated functioning of the organism.

Studying qualitative differences of human and animal higher nervous systems Pavlov put forward the theory of two signaling systems.

He always kept in mind the main biological principle of the unity of an organism and the environment. For him an organism was a syncytium composed of single units. The functional unity of an organism was controlled by the activity of the higher nervous system.

I.P. Pavlov considered the environment to be the main trigger of all changes that take place in the dynamic vital processes of an organism. Due to Pavlov speech represented another trigger which was tightly connected to human consciousness.
P127
Neuronal correlates of sustained fear in the anterolateral part of the bed nucleus of the stria terminalis

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Much of our understanding for mechanisms of fear and anxiety is based on animal studies in which Pavlovian paradigms have been used to evaluate short-term fear responses to a conditioned threat (“phasic fear”). Recently, experimental studies have begun to focus on more long-lasting states of fear that are elicited by less predictable threats (“sustained fear”). The bed nucleus of the stria terminalis (BNST), a region within the extended amygdala, has been identified as a critical element for phasic as well as sustained fear responses, but its neuronal activity pattern in relation to these different fear states is unclear so far. Therefore, we developed a novel fear conditioning paradigm in freely behaving mice, which allows us to differentiate between phasic and sustained fear states. This paradigm is based on unpredictable CS-US presentations during fear acquisition followed by repetitive brief auditory white noise burst presentations during fear memory retrieval 24 hours later. Freezing and/or fear-potentiated startle were evaluated as measures of fear. Behavioral data revealed that freezing and startle responses indicate phasic and sustained components of fear, characterized by an initially high phasic freezing and startle response followed by a prolonged, sustained but less elevated fear response throughout the entire CS presentation (6 minutes) during retrieval of fear. Next, we combined the novel fear conditioning paradigm with extracellular local field potential and single unit recordings in the anterolateral part of the BNST. Cluster analysis of neuron specific factors (preselected by principal factor analysis) like (a) neuronal activity throughout the phasic/sustained fear retrieval, (b) neuronal response to startle inducing bursts, (c) waveform characteristics and (d) freezing correlated neuronal activity revealed 3 distinct types of neuronal subpopulations in the anterolateral part of the BNST: (1) High phasic fear off neurons (approximately 30% of identified units), decreasing their activity at initially high phasic fear states, (2) phasic and sustained fear off neurons (app. 35%), reducing their activity at phasic and sustained fear states, and (3) low sustained fear on neurons (app. 25%), showing a significantly increased activity upon the sustained fear state. Particularly emphasized in this context are the high phasic fear off and low sustained fear on neurons, which activities closely resemble the predicted shift of phasic to sustained fear states of our behavioral data. Taken together, our results show that activity patterns of neuronal subpopulations of the BNST indeed correlate with the behavioral expression of phasic and sustained fear states.

P128
Functional innervation of the nasal mucosa by meningeal afferents – link between nasal and meningeal nociception

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Besides intranasal pain, noxious stimuli applied to the nasal cavity have been reported to cause headaches, which are believed to be functionally linked to meningeal nociception. The nasal mucosa is innervated by nociceptive trigeminal fibers of the nasociliary nerve, which passes part-way intracranially through the dura mater of the anterior cranial fossa. We asked if the activation of these nasal trigeminal afferents causes nociceptive effects in the dura mater. In vivo, single fiber recordings were made from filaments of the nasociliary nerve. Skulls of mice and rats were sagitally hemisected, the brain was removed and the nasociliary nerve was sucked into a glass recording electrode filled with physiological solution. Receptive fields were identified by von Frey filaments and electrical stimuli. In vitro, stimulated calcitonin gene-related peptide (CGRP) release from the dura mater of the anterior cranial fossa was studied in coronally divided rodent skulls which served as incubation chambers. The nose was flushed with physiological solution or capsaicin, the superfusion fluid of the anterior cranial fossa was collected every 10 min and CGRP levels were analyzed with a commercial ELISA.

In vivo, meningeal blood flow in the dura mater covering the olfactory bulb was measured by laser Doppler flowmetry in the rat open cranial window preparation. The ganglion blocker hexamethonium was injected i.v. prior to nasal application of physiological solution or capsaicin. Receptive fields of single Aδ and C-fibers responding to mechanical and electrical stimuli were identified in the dura mater as well as in the nasal mucosa. Intracranial receptive fields were localized in the anterior cranial fossa and extracranial receptive fields were found along the dorsal half of the nasal cavity up to the rostral cartilaginous part. Stimulation with capsaicin but not vehicle increased meningeal blood flow for some minutes. The flow increase was not abolished by systemic application of hexamethonium. We conclude that activation of nociceptive trigeminal afferents innervating the nasal mucosa induces intracranial nociceptive events such as CGRP release and increase in meningeal blood flow. These effects are independent of the autonomic nervous system. We assume that meningeal collateral fibers of nasociliary nerve fibers cause these functions. Intranasal application of substances might influence meningeal nociception and headaches via such collaterals.
P129  
Effects of the cytokine Interferon-γ on nociceptive nerve fibers from rat knee joint in vivo  
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**Question:** Whether the cytokine Interferon-γ (IFN-γ) has pro-inflammatory or anti-inflammatory effects is still disputed. In this study we investigated whether IFN-γ has the potency to induce mechanical hyperalgesia in normal or in acutely inflamed knee joints and whether such effects are specific to an action on the IFN-γ receptor.

**Methods:** Healthy adult Wistar rats were anesthetized with sodium thiopentone (100 mg/kg, i.p.) and the medial articular nerve of the right knee was exposed. The knee joint was mechanically stimulated by innocuous (20 mM) or noxious (40 mM) rotations of the lower leg against the fastened femoral bone for 15 sec each. Action potentials were recorded from nerve fibers that were classified as C- or as Aδ-fibers by their conduction velocity (<1.4 m/s or <10 m/s, respectively). Compounds were injected into the joint cleft at a volume of 0.1 ml each.

**Results:** In normal knee joints a single intraarticular injection of IFN-γ dose-dependently decreased the net response rate of C-fibers to noxious stimulation within three hours (0.1 ng by 21±51, 1 ng by 155±32, 10 ng by 171±56 APs/15 s; mean±SEM, respectively), but had no influence on the net response rate to innocuous stimulation. The response rates in Aδ-fibers were only insignificantly changed by IFN-γ. After 7 hours of acute knee joint inflammation induced by kaolin and carrageenan in C-fibers a similar dose-dependent decrease in response rate was seen: A dose of 10 ng of IFN-γ caused a decrease by 211±61 APs/15 s after three hours of application of the cytokine, a dose of 1 ng of IFN-γ caused a decrease by 176±52 APs/15 s, and a dose of 0.1 ng of IFN-γ had no effect. Interestingly, in Aδ-fibers recorded from inflamed knees, a dose of 0.1 ng of IFN-γ had no effect on the net response rate, but both 1 ng and 10 ng of IFN-γ caused rather an increase in the net response rate (1 ng by 135±40, 10 ng by 195±64 APs/15 s, respectively). Simultaneous injection of 10 μg of a blocking IFN-γ receptor antibody together with 10 ng of IFN-γ in normal knee joints prevented the decreasing effect of the cytokine in C-fibers, and responses in Aδ-fibers did not change.

**Conclusion:** Thus IFN-γ in slowly conducting nerve fibers both in normal and in inflamed knee joints acts as an anti-nociceptive cytokine, whereas its effect on fast conducting nociceptive fibers depends on the situation in the knee joint.

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P130  
Sensitization of diode-laser heat pain in glabrous skin through topical capsaicin application in humans  
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**Question:** The capsaicin-receptor TRPV1 is activated – beyond several others – by topical capsaicin application and by noxious heat stimuli. We now investigated whether sensitization and/or desensitization of radiant heat pain induced by a diode-laser can be achieved in glabrous skin by application of an 8% capsaicin patch (Qutenza®, Astellas Pharma GmbH) for 1h as clinically used to deactivate peripheral nociceptors in course of neuropathic pain therapy.

**Method:** Capsaicin patches (diameter 4cm²) were applied for 1h to glabrous skin of hand in 25 healthy volunteers. A vehicle patch (Qutenza® Demo Patch) was applied to the corresponding contralateral areal. Near-infrared diode laser stimuli (1475nm wavelength, stimulated area about 18μm²) of variable intensities (by varying energy up to 100mW and/ or stimulus duration up to 390ms) were used to characterize radiant heat pain before as well as 1h and 24h after removal using a numeric rating scale of pain intensity (0-100; no to most intense pain imaginable). In 8 volunteers laser-evoked brain potentials (LEPs) were recorded using 7 channel EEG. Mechanosensitivity was determined using von-Frey filaments and pinprick stimulators (MRC Systems GmbH) according to the Quantitative sensory testing (QST) protocol of the DFNS.

**Results:** When applied for 1h capsaicin significantly increased laser-heater pain ratings after 24h (e.g., 17.7 ± 2.2 versus 11.4 ± 1.6; p<0.01, n=16, student’s paired t-test; Capsaicin vs. vehicle) and markedly decreased contact-heat pain threshold (31.0 ± 0.4°C vs. 41.3 ± 1.1°C, p=0.001; n=16) indicative of a prominent heat hyperalgesia. Furthermore, a significant decrease of the warm detection threshold was seen after 24h (31.1 ± 0.3°C vs. 32.8 ± 0.3°C; p<0.001; n=16; baseline temperature 30°C). The main N2P2 component of the LEPs paralleled those subjective measures displaying a significant increase (e.g.,14.2 ± 2.1μV vs. 9.4 ± 2.0μV at 33mW for 390ms; p<0.05, n=8). Furthermore, after 24h there also was a trend to hyperalgesia to pinprick stimuli. In contrast, no systematic change in laser heat- and mechanical pain sensitivity was observed 1h after removal of the patch (n=16). Application of capsaicin for 24h to the glabrous skin completely abolished diode laser pain as measured 24h after removal.

**Conclusion:** These data indicate that topical application of 8% capsaicin for one hour sufficiently sensitizes human heat pain whereas longer application of the substance is necessary to desensitize pain perception within 24h after the treatment. Thus, this procedure may be used as a pain model to investigate primary hyperalgesia.
P131
System-wide protein profiling of chronic pain
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The treatment of chronic pain represents a major medical challenge largely owing to our limited understanding how protein networks change during these conditions. In the last years different -omics approaches have been used to decipher the broad molecular changes underlying chronic pain. Among them RNA deep sequencing is powerful to quantify mRNA frequency, however its predictability of protein-coding is limited. On the other hand, classical shotgun proteomics are semi-stochastic and suffer from under-sampling. New targeted proteomics approaches can shortcut these problems, being able to theoretically achieve proteome completeness and quantitative accuracy of an unlimited number of target proteins in a given sample. Their applicability in pain research needs to be tested.

**Question:** We set up a workflow based on targeted proteomics to investigate system-wide protein changes underlying inflammatory and neuropathic pain in mice.

**Methods:** We employed the complete Freund's adjuvant (CFA) and the spared nerve injury (SNI) models of inflammatory and neuropathic pain, respectively. Following behavioral tests, we isolated lumbar DRG and performed state-of-the-art targeted proteomics profiling, network analysis and several proteomic-independent orthogonal validations.

**Results:**
We reproducibly quantified more than 2500 proteins of which 143 were significantly regulated. Only 11 proteins were commonly altered in both pain models. Interestingly, some of these candidates have been linked to pain in other animal models and in humans serving as an in vivo support of our unbiased approach. Network analysis revealed the regulation of certain cellular processes. After having validated the regulation of a number of candidates in vitro, we are currently testing the role of specific candidates in vivo using pharmacological inhibition prior to behavioral tests in mice.

**Conclusions:** Our results provide new insights into nociceptor biology and identify potential new players in chronic pain.

In the present study, we analyzed different aspects of fear learning and fear extinction in differently aged APP/PS1 mice. This AD mouse model combines the Swedish APP (K670/671NL) mutation with the PS1L166P mutation under control of the Thy1 promoter (Radde et al., 2006, EMBO), resulting in a rather mild but constant post-developmental expression of Aβ and subsequent plaque formation. By testing amygdala-dependent cued fear learning, we observed only slight impairments in 12 months old but not in younger APP/PS1 mice. In the adjacent fear extinction training, we observed no impairments in the extinction of these cued fear memories, neither in short nor in long-term extinction memory. In contrast to the cued fear learning, we observed deficits in contextual fear learning in six months old APP/PS1 animals. Here, animals could not discriminate between the conditioned and a neutral context. However, the subsequent extinction of these contextual fear memories seemed to be unimpaired. Currently, we are analyzing the protein level of Aβ42 in the hippocampus, amygdala and medio-prefrontal cortex of the tested animals in order to correlate the local occurrence of these toxic Aβ-species with the behavioral performance of the animals. In addition, we started to analyze long-term potentiation (LTP) in acute hippocampal slices from APP/PS1 mice. Here, first results indicate an impaired LTP in the CA1 region of six but not three months old APP/PS1 mice.

In conclusion, we could demonstrate selective impairments in contextual fear learning in middle-aged APP/PS1 mice. Experiments trying to further analyze the underlying mechanisms for this deficit by analyzing expression levels of solubilizable forms of Aβ protein and altered hippocampal synaptic plasticity are in progress.

This work was supported by the Center for Behavioral Brain Sciences (CBBS) and the Deutsche Forschungsgemeinschaft (DFG, SFB 779/B6).

P132
Fear and fear extinction learning in APP/PS1 mice
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One of the most challenging topics in neuroscience research is the identification of novel treatment approaches for Alzheimer’s disease (AD). It has been shown by several studies, that in AD patients emotional processing, e.g. recognition of fearful faces or the learning of fear, is impaired. Interestingly, these impairments occur already at early stages of the AD etiopathology. Thus, an altered emotional processing might be regarded as an early symptom in the development of AD.

In the present study, we analyzed different aspects of fear learning and fear extinction in differently aged APP/PS1 mice. This AD mouse model combines the Swedish APP (K670/671NL) mutation with the PS1L166P mutation under control of the Thy1 promoter (Radde et al., 2006, EMBO), resulting in a rather mild but constant post-developmental expression of Aβ and subsequent plaque formation. By testing amygdala-dependent cued fear learning, we observed only slight impairments in 12 months old but not in younger APP/PS1 mice. In the adjacent fear extinction training, we observed no impairments in the extinction of these cued fear memories, neither in short nor in long-term extinction memory. In contrast to the cued fear learning, we observed deficits in contextual fear learning in six months old APP/PS1 animals. Here, animals could not discriminate between the conditioned and a neutral context. However, the subsequent extinction of these contextual fear memories seemed to be unimpaired. Currently, we are analyzing the protein level of Aβ42 in the hippocampus, amygdala and medio-prefrontal cortex of the tested animals in order to correlate the local occurrence of these toxic Aβ-species with the behavioral performance of the animals. In addition, we started to analyze long-term potentiation (LTP) in acute hippocampal slices from APP/PS1 mice. Here, first results indicate an impaired LTP in the CA1 region of six but not three months old APP/PS1 mice.

In conclusion, we could demonstrate selective impairments in contextual fear learning in middle-aged APP/PS1 mice. Experiments trying to further analyze the underlying mechanisms for this deficit by analyzing expression levels of solubilizable forms of Aβ protein and altered hippocampal synaptic plasticity are in progress.

This work was supported by the Center for Behavioral Brain Sciences (CBBS) and the Deutsche Forschungsgemeinschaft (DFG, SFB 779/B6).

P133
Hearing impairment causes psychical stress and enhances cognitive demands in an acoustic discrimination task
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**Question:** Hearing impaired people complain of psychical stress that is enhanced if they are asked to listen carefully or if they should solve auditory recognition tasks. In contrast, an increased level of psychical stress is assumed to contribute to the extra-aural effects of noise, i.e. increased heart rate, hypertension or disturbances in the endocrine system. In the present study we wanted to test whether psycho-vegetative parameters and central processing of auditory signals are changed in hearing impaired people and whether these changes are aggravated if auditory signals are masked by noise.
POSTER PRESENTATIONS

**P134**

**In vivo recording and identification of projection-specific subtypes of midbrain dopamine neurons**

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Based on their axonal targets, midbrain dopamine (DA) neurons might be segregated into three major populations – mesostriatal, mesolimbic and mesocortical DA neurons. These distinct DA neurons are distributed across the substantia nigra (SN) and ventral tegmental area (VTA) in a loose topographical order (Lammel et al. 2008). However, given their substantial overlap, in particular in the medial SN, mesostriatal and mesolimbic DA neurons cannot be identified in vivo solely by juxtacellular labeling to define their respective anatomical positions (Subramaniam et al. 2014). Thus, we aimed to combine projection-specific retrograde labeling with fluorogold (FG) with single-unit in vivo extracellular recording and juxtacellular labeling of DA SN neurons in anaesthetized adult C57Bl6N mice. In contrast to previous retrograde labeling studies of the nigrostriatal pathway in mice (Liu et al. 2003; FG 4%, 0.5 µl), a dramatic reduction of the FG concentration to 0.002% (0.5 µl) was necessary to prevent the degeneration or the occurrence of morphological changes (e.g. dendritic beading) in targeted DA SN neurons. We are currently carrying out in vivo extracellular single unit recordings combined with juxtacellular labeling to compare the physiological properties of FG-labeled and FG-unlabeled DA SN neurons one week after ipsilateral intrastriatal fluorogold (0.002%; 0.5 µl) infusion (50 nl/min). The current study aims to define a retrograde labeling procedure that does not affect the morphology or in vivo function of retrogradely labeled DA neurons to enable the identification of genuine differences between mesostriatal and mesolimbic DA SN neurons.

**References:**


**P135**

**A computational study on the diversity of neural activity pattern and responsiveness**

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In experimental recordings a diversity of neuronal activity pattern and response characteristics can be observed. No neuron reacts in exactly the same way as another one what even can be seen in recordings from the same type of neurons or peripheral sensory receptors that may have identical sets of ion conductances, only differing quantitatively in the ion channel densities. Even responses of the same neuron on exactly identical stimuli exhibit certain variability. For the elucidation of the crucial membrane properties of neuronal diversity we have used a Hodgkin-Huxley type model that has originally been developed to simulate the temperature dependencies of peripheral cold receptors [1] but, with the same set of ion conductances and only minor modifications, can also account for major response characteristics of warm receptors and electroreceptors as well as for the activity pattern and stimulus dependencies in several brain areas like the entorhinal cortex, thalamus or hypothalamus [2]. We demonstrate that the neurons’ responses, e.g. firing threshold and bifurcations between different activity patterns, critically depends on the neurons’ dynamic state, determined by the nonlinearities and time delays of current activation. The neurons dynamic, can dramatically change with only slight alterations of the activation range of ionic conductances (slope, strength, half-activation values) neurons’ operating point. However, also so-called passive membrane properties like the leak conductances or membrane capacity can be important factors determining the neurons’ responsiveness in changing its operating point. Such effects can also be examined in model neurons with a minimal set of ioic conductances as, for example, realized in one of the Virtual Physiology teaching tools: “SimNeuron” (free downloads fromwww.virtual-physiology.com).

**References:**

The dentate gyrus (DG) is thought to enable efficient hippocampal memory acquisition via pattern separation. With patterns defined as spatiotemporally distributed action potential sequences, the principal DG output neurons (granule cells, GCs), presumably sparsen and separate similar input patterns from the perforant path (PP). In electrophysiological experiments, we have demonstrated that during temporal lobe epilepsy (TLE), GCs downscale their excitability by transcriptional upregulation of “leak” channels. Here we studied whether this cell type-specific intrinsic plasticity is in a position to homeostatically adjust DG network function. We modified an established conductance-based computer model of the DG network such that it realizes a spatiotemporal pattern separation task, and quantified its performance with and without the experimentally constrained leaky GC phenotype. Two proposed TLE seizure mechanisms were implemented in various degrees and combinations: recurrent GC excitation via mossy fiber sprouting and increased PP input. While increasing PP strength degraded pattern separation only gradually, already the slight elevation of sprouting drastically (non-linearly) impaired pattern separation. In most tested hyperexcitable networks, leaky GCs ameliorated pattern separation. However, in some sprouting situations with all-or-none seizure behavior, pattern separation was disabled with and without leaky GCs. In the mild sprouting (and PP increase) region of non-linear impairment, leaky GCs were particularly effective in restoring pattern separation performance. These results are compatible with the hypothesis that the experimentally observed intrinsic rescaling of GCs serves to maintain the physiological function of the DG network.
The neurotrophin BDNF (brain-derived neurotrophic factor) has been shown to be an important mediator of synaptic strength and to be crucially involved in learning and memory processes. Several recent studies have demonstrated an important role of BDNF also in amygdala-dependent cued fear learning. Recently, we could demonstrate that heterozygous BDNF knockout (BDNF+/-) mice exhibit a learning deficit in response to a weak fear conditioning protocol when animals became older than 3 months of age (Endres & Lessmann (2012) Learning Mem.). In order to analyze this learning deficit at the cellular level, we analyzed synaptic plasticity in amygdala slice preparations.

Since we previously showed impaired long-term potentiation (LTP) at the thalamic input to the lateral amygdala (LA) already in one month old BDNF+/- mice (Meis et al. (2012) J. Physiol.), we hypothesized that age-dependent changes of plasticity at cortico-LA afferents might cause the observed learning deficit. Therefore, we tested LTP at this input structure of the amygdala in 3-4 months old BDNF+/- mice and their wild type (WT) littermates. However, we observed unpaired LTP in BDNF+/- mice at this synapse as well as at other intra-amygdala synapses, i.e. lateral-basal and basal-central amygdala synapses.

In order to better understand how fear learning alters synaptic plasticity in the LA, we performed ex vivo occlusion experiments. In fear conditioned WT mice, in contrast to pseudo-conditioned animals, LTP at cortico-LA synapses was occluded 24 h after fear conditioning training, stressing the fear learning relevance of our applied LTP paradigm. Interestingly, LTP at the same synapses was not occluded in fear conditioned BDNF+/- mice. This lack of occlusion 24 h after fear conditioning parallels the fear learning deficit in adult BDNF+/- mice. Currently we are testing occlusion of LTP at cortico-LA synapses 4 h after fear conditioning. First results show that LTP at this time point is also not occluded in adult BDNF+/- mice. This observation is paralleled by impaired fear memory in BDNF+/- mice at this time point. Interestingly, fear memory was still unimpaired 30 min after fear conditioning, indicating that the acquisition of fear memory is still intact in adult BDNF+/- mice.

In conclusion, our results suggest that in adult BDNF+/- mice the acquisition of fear is still intact, as indicated by intact short-term fear memory 30 min after fear conditioning. As we observed neither occlusion of LTP nor intact fear memory 4 or 24 h after fear conditioning, the observed learning deficit seems to result from an unsuccessful early consolidation process.

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Posters

**P140**
**LRRK2 sensitive Na⁺/Ca²⁺ exchanger activity in dendritic cells**


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**Background:** Gene variants of the leucine-rich repeat kinase 2 (LRRK2) are associated with susceptibility to Parkinson’s disease. Besides the brain and peripheral organs, the kinase is expressed in various immune cells including dendritic cells (DCs), antigen-presenting cells linking innate and adaptive immunity. However, the function of LRRK2 in the immune system is still incompletely understood.

**Methods:** To investigate the role of LRRK2 in DCs, Ca²⁺ signaling was analyzed in DCs isolated from Lrrk2 gene targeted mice lacking functional Lrrk2 (Lrrk2⁻/⁻) and their wild-type littermates (Lrrk2⁺/⁺). Cytosolic Ca²⁺ levels ([Ca²⁺]ᵢ) were determined utilizing Fura-2 fluorescence and whole cell currents to decipher electrogenic transport.

**Results:** The increase of [Ca²⁺]ᵢ following inhibition of sarcoplasmatic Ca²⁺-ATPase in the absence of extracellular Ca²⁺ was significantly larger in Lrrk2⁻/⁻ than in Lrrk2⁺/⁺ DCs. It is believed that the increase of [Ca²⁺]ᵢ could be augmented by impaired Ca²⁺ extrusion due to decreased Na⁺/Ca²⁺ exchanger activity. To quantify the activity of the K⁻/Ca²⁺ exchanger, the increase of [Ca²⁺]ᵢ ([Δ[Ca²⁺]ᵢ]) and Na⁺/Ca²⁺ exchanger induced current (Ica) were determined during abrupt replacement of Na⁺ containing (130 mM) and Ca²⁺ free (0 mM) extracellular perfusate. As a result, both slope and peak of Δ[Ca²⁺]ᵢ as well as Ica were significantly lower in Lrrk2⁻/⁻ than in Lrrk2⁺/⁺ DCs.

**Conclusions:** LRRK2 deficiency leads to downregulation of Na⁺/Ca²⁺ exchanger activity and as a result to an augmentation of Ca²⁺ signals in DCs. The present observations thus disclose a completely novel functional significance of LRRK2, i.e. regulating of Ca²⁺ signaling by modulation of Na⁺/Ca²⁺ exchanger activity in DCs.

**P142**
**Y39 in the unique N-terminal region of NBCe1-B plays an inhibitory role in NBCe1-B expression**

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The sodium bicarbonate cotransporter NBCe1 is crucial for the reabsorption of HCO₃⁻ in the kidney and for the secretion of HCO₃⁻ in the pancreatic ducts. The two major isoforms kidney NBCe1-A and pancreatic NBCe1-B exhibit differential electrogenicity. It is hypothesized that there is an inhibitory mechanism in NBCe1-B. This study was to identify these inhibitory component(s). Various NBCe1 mutants were generated via deletion and site-directed mutagenesis and were expressed in either frog oocytes or HEK 293 cells. The activities of cells expressing the wild-type NBCe1 protein and its mutants were analyzed and compared in the presence of HCO₃⁻. Deletions of amino acids 31-41, 41-51 and substitution mutants Y35A, P36A and Y39A of NBCe1-B resulted in the disinhibition of its activities. Our results suggest that there are multiple specific subdomains and individual amino acid residues that are responsible for inhibiting the expression and physiological activity of NBCe1-B located in its unique N-terminal end. Specifically, amino acid residue Y39 of the unique N-terminal domain of NBCe1-B was shown to play such an inhibitory role of expression in both oocytes and HEK 293 cells. This research is significant for understanding the reabsorption and secretion mechanisms of HCO₃⁻ in physiology and pathophysiology.
Acute hypoxia decreases Na⁺ absorption by H441 distal lung epithelial cells
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**Question:** Na⁺ transport across distal lung epithelia involves the influx of Na⁺ ions into epithelial cells via apically located epithelial Na⁺ channels (ENaC) as well as the extrusion of these ions at the basolateral side by the Na⁺/K⁺-ATPase. This Na⁺ transport generates the osmotic driving force for lung liquid clearance. It is well established that Na⁺ transport is impaired under long-term hypoxic conditions. However, effects of acute hypoxia are less investigated. Therefore we questioned whether or not acute hypoxia influences Na⁺ transport across distal lung epithelial cells.

**Methods:** The human H441 cell line was employed as a model for distal lung epithelia. Cells were cultured to epithelial monolayers on permeable supports at air/liquid interface and in the presence of dexamethasone in order to enhance Na⁺ transport capacity. Na⁺ absorption across these monolayers was measured as ion current signals in Ussing chambers. Chambers were filled with a bicarbonate Ringer’s solution and fumigated with a gas mixture containing 10% oxygen and 5% CO₂ (balanced N₂) and tempered to 37°C.

**Results:** Acute hypoxia inhibited ion currents across H441 monolayers by 30.12%. This effect was reversible. Half-maximal inhibition of ion current occurred within 5.55 ± 0.73 min. There was no change in transepithelial resistance, which indicates that there was no damage to the monolayers induced by hypoxia. There was no difference in intracellular ATP concentrations between hypoxic and control monolayers, indicating that the inhibition of ion transport was not a result of metabolic depletion. Comparison of ion currents which were sensitive to the ENaC-inhibitor amiloride revealed that acute hypoxia decreased ENaC-mediated Na⁺ absorption. Furthermore, pre-treatment of H441 monolayers with amiloride abolished the inhibition of ion current by acute hypoxia.

**Conclusion:** Acute hypoxia rapidly and reversibly decreases ENaC-mediated Na⁺ absorption by H441 lung epithelial cells. The fast kinetics of hypoxic inhibition may suggest signalling mechanisms which act independently of altered ENaC expression and rather control the activity or membrane abundance of this ion channel.

P2X receptor-mediated inhibition of NaCl absorption in the thick ascending limb occur independent of nitric oxide
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**Background:** Our group established that basolateral (bl.) P2X receptors acutely and markedly reduce NaCl absorption in mouse medullary thick ascending limb (mTAL). Others propose a mechanism of P2X receptor-mediated NO synthesis leading to NKCC2 inhibition. P2X receptor stimulation causes an increase in cytosolic Ca²⁺ and therefore this could be proximal to the generation of NO.

**Objective:** Here we tested if blocking NO synthesis or removal of extracellular Ca²⁺ inhibits the ATP-mediated (P2X) transport inhibition.

**Methods:** We used isolated, perfused mTALs from mice to electrically measure NaCl absorption. By microelectrodes we determined the transepithelial voltage (Vₜₑ) and the transepithelial resistance (Rₑ) and via these the transepithelial Na+ absorption (equivalent short circuit current, $I_{SC}$).

**Results:** We confirm that bl. ATP (100 µM) induced a marked, acute and reversible inhibition of Na⁺ absorption (28% ± 6%, n=6). In the presence of the NO synthase blocker L-NAME (100 µM, with 3 min preincubation) the ATP effect remained unaffected (23% ± 9%, n=6). Also higher concentrations of L-NAME (1 mM) were without effect. Furthermore, the removal of extracellular Ca²⁺ (100 nM, 2 min preincubation) had no effect on the ATP-induced transport inhibition ([Ca²⁺] =100 nM: 21% ± 6%, n=5, [Ca²⁺] =1 µM: 24% ± 4%, n=6).

**Conclusion:** We find no evidence for nitric oxide (NO) being involved in the signaling pathway for P2X receptor-dependent transport inhibition. Similarly, Ca²⁺ signaling appears not involved in ATP-mediated inhibition of NaCl absorption.
The phenotype of these Rictor fl/fl*KspCre mice was characterized and patch-clamp experiments were performed on split open tubular segments from the transition zone of the late connecting tubule and early cortical collecting duct (CNT/CCD).

Results: Rictor fl/fl*KspCre mice were viable and did not show any obvious phenotype under control conditions except for a 2.5 fold increase in serum aldosterone. However, when given a high K+ diet, Rictor fl/fl*KspCre mice rapidly developed hyperkalemia. Patch-clamp experiments demonstrated that in outside-out patches Ba^2+ sensitive apical K+ currents were barely detectable in the majority of Rictor fl/fl*KspCre mice. In contrast, whole-cell current measurements demonstrated that the activity of the epithelial sodium channel (ENaC) was largely preserved in Rictor fl/fl*KspCre mice.

Conclusion: These findings indicate that the reduced ability of Rictor fl/fl*KspCre mice to maintain K+ homeostasis is mainly due to an impaired apical K+ conductance but not due to a reduced electrical driving force for K+ secretion by ENaC inhibition. Thus, these data unravel a vital and non-redundant role of mTORC2 for distal tubular K+ handling.

P146 Hydrogen sulfide inhibits β-adrenergic agonist induced stimulation of pulmonary transepithelial sodium absorption

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Question: The absorption of water across pulmonary epithelia is essential for lung liquid homeostasis. The underlying osmotic driving force is a transepithelial absorption of sodium ions through epithelial sodium channels (ENaC). In the lung, β-adrenergic agonists stimulate ENaC activity and hence lung liquid clearance. We have previously shown that the neurotransmitter hydrogen sulfide (H2S) reduces basal sodium fluxes across pulmonary epithelia (Althaus et al. 2012, Br J Pharmacol). This study investigated on the possible interference of H2S with the β-adrenergic regulation of ENaC activity.

Methods: ENaC-mediated transepithelial sodium absorption was measured as ion current signals in Ussing chambers. Experiments were performed with freshly-dissected lung epithelia from Xenopus laevis or human H441 epithelial cells which were cultured at air/liquid interface in the presence of dexamethasone. Intracellular ATP and cAMP concentrations were measured with an ATP luminescence assay and ELISA, respectively. Activation of protein kinase A (PKA) was indirectly assessed by immunoblotting against its target protein CREB. Lung liquid clearance was measured in rat lungs in situ.

Results: The application of the β-adrenergic agonist terbutaline increased transepithelial ion currents in Xenopus lungs. This stimulatory effect was sensitive to the ENaC inhibitor amiloride, and was completely lost in the presence of the H2S-forming molecule Na2S (50 µM). In principle, β-adrenergic signalling involves cAMP formation, subsequent activation of PKA and exocytosis of ENaC containing vesicles which increases ENaC membrane abundance. Stimulation of cAMP formation by forskolin and 3-isobutyl-1-methylxanthine increased amiloride-sensitive currents in H441 cells. This effect was inhibited by Na2S in a dose-dependent manner (5-50 µM). Na2S did not interfere with cAMP formation or PKA activation in these cells. However, Na2S prevented a cAMP-induced increase in apical sodium conductance in basolaterally permeabilized H441 monolayers. Application of Na2S to the alveolar compartment of rat lungs decreased baseline liquid absorption and abrogated the stimulation of liquid absorption by terbutaline.

Conclusions: H2S prevents the β-adrenergic activation of ENaC in pulmonary epithelial cells and, consequently, lung liquid clearance. H2S acts downstream of cAMP formation and PKA activation and prevents the delivery of ENaC to the apical membrane.

P147 Reduced renal K+ excretion with compensatory hyperaldosteronism in Kcn1.1 channel β2-subunit KO mice

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The kidneys are the primary organs responsible for excreting K+, ensuring whole body K+ homeostasis by precisely matching K+ excretion to dietary K+ intake. K+ is secreted into the urine in the collecting ducts, and two distinct mechanisms for K+ secretion exist: a constitutive mechanism mediated by ROMK (Kir1.1) in principal cells and a flow-induced mechanism mediated by BK channels (Kcn1.1) in intercalated cells. Both mechanisms are up-regulated by aldosterone. Here we studied renal K+ excretion in knock out mice for the β2-subunit of the BK channel (β2 KO). The β2 KO mice have increased plasma aldosterone, low renin expression and normal plasma [K+] when kept on a control diet. The low renin in β2 KO mice indicates that hyperaldosteronism was triggered by a K+ handling deficiency, rather than hypovolemia and activation of the renin-angiotensin-aldosterone system. We hypothesize that β2 KO mice have decreased BK channel-mediated renal K+ secretion, which is compensated by hyperaldosteronism and up-regulation of ROMK-mediated K+ secretion, allowing β2 KO mice to maintain normal plasma [K+]. In fact, when treated with eplerenone (mineralocorticoid receptor antagonist) for 4 days, β2 KO mice develop slight hyperkalemia (4.15 mM ± 0.13 in WT vs. 4.60 mM ± 0.10 in KO, P = 0.013).

Urinary K+ excretion following oral K+ load (20% of normal daily intake) was not different between WT and KO mice under control conditions. However, when treated with eplerenone, β2 KO mice had a significantly lower urinary K+ excretion rate (P=0.044) and significantly higher plasma [K+] 3 hours after
oral K+ load (10.0 mM ± 0.4 in WT vs. 11.4 ± mM ± 0.5 in KO, P = 0.044). Our data support that hyperaldosteronism in β, KO mice is part of a chronic compensation to a decreased BK channel-mediated renal K+ secretion.

**P148**
The role of the chloride transport protein ClC-7 in renin expressing cells

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**Question:** The protease renin is stored, processed and secreted from acidic, lysosome related organelles in juxtaglomerular cells of the kidney. Since the mechanism of acidification of renin containing vesicles is yet unknown, we have characterized the potential role of the lysosomal Cl-/H+ exchanger ClC-7 for the function of JG cells, in particular for the control of renin synthesis and secretion.

**Methods:** We have analyzed the expression of ClC-7 in JG cells of normal kidneys and we have characterized renin expression and secretion in mice lacking ClC-7 in the renin cell lineage (Ren1d Cre/+ ClC-7 fl/fl).

**Results:** We found ClC-7 and also LAMP-1 expressed in renin-containing cells partially overlapping with renin immuno-reactivity. In mice lacking ClC-7 in the renin cell lineage, renin expression and plasma renin concentrations were reduced to about 30% of wild type controls. This reduction of renal renin expression was accompanied by a decrease of the number of renin expressing cells and paralleled by morphological changes of the cells. In contrast to wild type mice, the remaining renin cells of ClC-7 deficient mice were smaller and contained much less electron dense renin storage vesicles. Experiments with isolated perfused kidneys revealed that the normal control of renin secretion by catecholamines, by angiotensin II and by the renal perfusion pressure was almost blunted in ClC-7 deficient mice. In addition, the typical recruitment of renin expressing cells induced by inhibition of the renin angiotensin system was strongly attenuated in ClC-7 deficient mice.

**Conclusion:** These findings suggest that ClC-7 exists in renin expressing cells and is highly relevant for the function of renin producing JG cells. Defect of ClC-7 impairs renin expression, storage and secretion. It remains to be clarified if the reduction of renin synthesis is the cause or the consequence of reduced renin storage induced by defects of ClC-7.

**P149**
Carbonic anhydrase isoform II physically interacts with monocarboxylate transporter isoform 4 to form a "proton-collecting antenna"

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Carbonic anhydrase isoform II (CAII), the fastest of the α-CAs, catalyzes the reversible hydration of CO₂ to HCO₃⁻ and H⁺. We have previously shown that CAII enhances transport activity of MCT1 and MCT4 by a non-catalytic mechanism, when the proteins were heterologously expressed in Xenopus oocytes (1, 2). This CAII-induced augmentation of MCT1/4 transport activity is mediated by the enzyme’s intramolecular H⁺-shuttle His64 (3). Since efficient H⁺ transfer between enzyme and transporter seems to require close proximity of the two molecules, we analyzed the direct interaction between CAII and MCTs by measuring changes in intracellular H⁺ concentration with H⁺-sensitive microelectrodes in Xenopus oocytes and pull-down experiments with different mutants of CAII and MCTs. Our results suggest that functional interaction between MCT1/4 and CAII requires direct binding of the enzyme to a glutamic acid cluster in the C-terminal of the transporter. Binding of CAII to the C-terminal of MCT1/4 is mediated by CAII-His64, which is also involved in H⁺-shuttling. Transport activity of MCT2, which lacks a putative CAII-binding site, is not augmented by CAII (4). However, introduction of a CAII-binding moiety into the C-terminal of MCT2 resulted in CAII-induced augmentation of MCT2 transport activity. To further investigate the augmentation of MCT activity by a putative H⁺-shuttle, we inserted a cluster of histidine residues into the C-terminal of MCT4. Indeed, introduction of this “proton-collecting antenna” led to a significant increase in MCT4 transport activity in the absence of CAII, which was comparable to the CAII-induced augmentation. These results suggest that CAII, when directly bound to MCTs, can act as a “proton-collecting antenna”, which removes or donates H⁺ from or to the transporter pore, depending on transport direction, and distribute H⁺ along the membrane to enhance H⁺-driven lactate flux.

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**References**
**P150**
**Na+/HCO₃⁻ cotransporter SLC4A7 mediates the increased basolateral bicarbonate uptake in renal thick ascending limbs during metabolic acidosis**

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**Objectives:** We will determine if NBCn1 contributes to basolateral net acid extrusion in TAL and if it is functionally up-regulated during MAC.

**Methods:** We measured intracellular pH(pHi) in isolated perfused TAL from WT and NBCn1 KO mice. To induce MAC, mice were loaded with 0.196 M NH₄Cl in the drinking water for 4 days.

**Results:** RT-PCR and immunoblotting demonstrated expression of NBCn1 in TAL of WT but not NBCn1 KO mice. RT-PCR also revealed expression of other SLC4 HCO₃⁻ transporters in TAL. These include NBCe1, NDCBE, AE4 and BTR4. Following an acute intracellular acid load, all mice showed pHi recovery dependent on Na⁺,HCO₃⁻ cotransport. In untreated mice, pHi recovery rate was not different between WT and NBCn1 KO mice. NH₄⁺ loaded WT mice showed a 3-fold increase in pHi recovery rate compared to untreated animals, while the NBCn1 KO mice showed only a small increase.

**Conclusion:** In control mice, NBCn1 apparently does not contribute to net acid extrusion in TAL. However, under MAC, NBCn1 is responsible for the 3-fold increase in pHi recovery rate. Other SLC4 transporters are expressed in TAL and likely mediate the Na⁺,HCO₃⁻ cotransport under basal conditions. We propose that NBCn1 is important for facilitating net acid extrusion under MAC.

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**P151**
**Expression and function of the non-gastric H⁺/K⁺ ATPase in INS-1E rat beta-cells**

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**Question:** Beta-cell apoptosis is involved in the pathogenesis of both type-1 and type-2 DM. Intracellular acidification, disturbed ion homeostasis and cell shrinkage under isotonicity (apoptotic volume decrease, AVD) are early events during apoptosis and precede caspase activation, DNA degradation and loss of cell membrane integrity. In pancreatic beta-cells intracellular pH (pHi) is regulated by Na⁺/H⁺ exchangers (NHES), HCO₃⁻ dependent mechanisms and H⁺-ATPases. This study was performed to disclose a possible contribution of H⁺/K⁺ ATPases to pH/volume homeostasis in beta-cells. H⁺/K⁺ ATPase alpha-subunits comprise the gastric (Hkalpha1) and the non-gastric (Hkalpha2; encoded by gene Atp12a) isoforms, which are involved in gastric acid secretion, H⁺/K⁺ transport in the distal nerton and colon, acidification of prostate fluids and secretion of HCO₃⁻ rich fluids in the exocrine pancreas.

**Methods:** Experiments were performed on INS-1E rat insulinoma cells using RT-PCR, qRT-PCR, Western blot and flow cytometry. Results: On the mRNA level we found expression of Hkalpha2 as well as NHE isoforms 1, 2 and 7, but not Hkalpha1. Western blotting revealed the expression of Hkalpha2 on the protein level. The abundance of Atp12a mRNA increased 4-fold by incubating cells under high medium glucose (25 mM) for 48 hours, indicating regulated gene expression. Using combined Coulter-volume measurements and flow cytometry we found that in absence of other pro-apoptotic stimuli the H⁺/K⁺ ATPase blocker SCH-28090 applied for 24 hours caused a significant reduction of the mean cellular volume (MCV), an increase in annexin-V and caspase-positive cells. Pump inhibition exerted an additive effect on the pro-apoptotic action of the short-chain fatty acid butyrate (10 mM). Cell surface staining with an anti-Hkalpha2 antibody gave a positive signal in 8% of cells under control conditions. The percentage significantly increased to 23% upon treatment with butyrate for 24 hours. Cells expressing Atp12a displayed a significantly reduced MCV compared to Hkalpha2-negative cells. Conclusions: Our data show regulated and functional expression of the non-gastric H⁺/K⁺ ATPase Atp12a in beta-cells, which might be associated with apoptosis. The pump might counteract intracellular acidification, K⁺ loss and cell shrinkage, and delay the progression of apoptotic cell death.

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**P152**
**Loss of Slc26a9 anion transporter results in reduced pancreatic fluid and bicarbonate secretion electrolyte in female but not in male mice**

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**Background:** Slc26a9 is a member of the Slc26 multifunctional anion transporter family with strong expression in the lung and the stomach. In the bronchial and duodenal epithelium it may function as a chloride conductance interacting with CFTR. Polymorphisms in Slc26a9 are associated with an increased incidence of meconium ileus and diabetes in cystic fibrosis patients.

**Aim:** We investigated the expression of Slc26a9 in the pancreas and elucidated its potential role in pancreatic ductal electrolyte and fluid secretion.

**Methods and Results:** The mRNA expression of Slc26a9 was low in pancreatic parenchyma but 20-fold higher in microdissected pancreatic ducts. No Slc26a9 mRNA expression was detected in the liver, while bile ducts displayed low Slc26a9 expression. The main pancreatic and the common bile duct were cannulated and pancreatic and biliary fluid and bicarbonate secretion assessed in anesthetized Slc26a9 rich fluids in the exocrine pancreas.
knockout mice and age- and sex-matched wild-type (WT) littermates in the basal state and after intravenous stimulation with secretin. Significantly reduced basal as well as secretin-stimulated pancreatic fluid secretory rates were observed in young adult (6-8 weeks) female Slc26a9 KO mice, with no difference in the bicarbonate secretory rates. In young male mice, as well as male and female old mice (>1 year), no significant difference in pancreatic ion secretion was observed. In addition, biliary fluid and bicarbonate secretion were not affected by loss of Slc26a9 expression.

Conclusions: Deletion of Slc26a9 is associated with a reduction in pancreatic fluid but not bicarbonate secretion in young female mice. The results are consistent with our earlier work in the airways, stomach and duodenum which underline the importance of Slc26a9 in these epithelia particularly at young age. Once the critical age is survived, adaptation allows proper functioning of many but not all organs. For example, in cystic fibrosis patients the incidence of diabetes in higher in females.

K+ channels

P153
Structural movement of the TM4 segment during pore gating in TREK-1 channels

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The two-pore domain (K2P) potassium channels are important regulators of excitability in various cell types and are regulated by a wide range of stimuli including temperature, voltage, mechanical stress, phosphorylation, various lipids, and extracellular and intracellular pH. For several K2P channels X-ray structural information is now available, but the structural basis of the pore gating is still poorly understood. We have previously shown in TREK-1 channels that pore gating likely occurs at the selectivity filter and that the intracellular pore entrance does not close or show large changes in opening diameter during gating. However, in TREK-1, the important stimuli sensing domain appears to be the cytoplasmic C-terminus that also harbours the intracellular pH sensor. Here we studied the coupling mechanism between the C-terminal domains and the selectivity filter by characterising the effect of chemical modification of cysteines, systematically introduced into the TM4 segment starting from its extracellular end up to the pH sensor in the C-terminus. Our results revealed an unusual pore accessibility profile with different clusters of modifiable cysteines, which differ in respect to the effect of chemical modification. Based on the structural information of TRAAK, the TM4 segment is kinked about in the middle and modification of residues above this kink resulted in channel activation, while modification of residues below this kind caused channel inhibition. Furthermore, residues in the kinking region displayed clear state dependence with modification in the closed state and its lack in the open state. These results represent functional evidence for a structural movement in the TM4 region that is likely to underline the coupling of the C-terminus to the selectivity filter causing its opening in response to changes in intracellular pH or the binding of lipids, such as PIP2.

P154
Engineered voltage sensitive phosphatases: what do they tell us about the activation mechanism?

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Voltage sensitive phosphatases (VSPs) contain a phosphoinositide-specific phosphatase domain (PD) whose activity is controlled by a voltage sensor domain (VSD). The underlying coupling mechanism between both domains is not fully understood. By a combinatorial approach of all-atom molecular dynamics simulations and experimental mutagenesis studies on Ciona intestinalis (Ci-) VSP, we identified an interaction between positively charged amino acids in the region that links the PD to the VSD and negatively charged residues in the TI loop of the PD, in particular with Asp400. Our results suggest that this interaction is crucial for stabilizing the substrate binding pocket of Ci-VSP’s PD. To clarify the relevance of this interaction for VSPs in more detail, we used the engineered phosphatases PTENCiV and hVSP1 CiV, which were previously described (Lacroix et al., JBC 2011, Halaszovich et al., J Lipid Res 2012). These chimeras consist of Ci-VSP’s VSD and the PD of PTEN or hVSP1, respectively. In both enzymes, Asp400 (Ci-VSP numbering) is not conserved. Nonetheless, the chimeras have robust voltage dependent phosphatase activity, analogously to Ci-VSP. Mutations in the linker of the chimeras show similar effects on protein function as previously reported for homolog mutations in Ci-VSP. These results suggest a common linker-mediated gating mechanism for the three VSPs. We further mutated Asp400 in Ci-VSP to Asn or Arg mimicking the Ti loop of hVSP1 and PTENCiV, which were previously described (Lacroix et al., JBC 2011, Halaszovich et al., J Lipid Res 2012). These chimeras consist of Ci-VSP’s VSD and the PD of PTEN or hVSP1, respectively. In both enzymes, Asp400 (Ci-VSP numbering) is not conserved. Nonetheless, the chimeras have robust voltage dependent phosphatase activity, analogously to Ci-VSP. Mutations in the linker of the chimeras show similar effects on protein function as previously reported for homolog mutations in Ci-VSP. These results suggest a common linker-mediated gating mechanism for the three VSPs. We further mutated Asp400 in Ci-VSP to Asn or Arg mimicking the Ti loop of hVSP1 and PTENCiV, respectively. Vice versa, we introduced Asp in both chimeras mimicking the Ti loop of Ci-VSP. The voltage dependent phosphatase activity of the mutants suggests that the interaction between the linker and Asp400 is not necessarily crucial for the dephosphorylation activity of VSPs, but it allows a more efficient VSD-PD coupling. Further work is required to clarify the interactions which are crucial for the coupling process in VSPs.
**P155**

*Porcine airway epithelium: action sites of luminal acetylcholine in intracellular calcium signaling*

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**Introduction:** The present study investigates the action sites of luminal ACh in intracellular calcium signaling, which is assumed to mediate Cl− secretion via Ca2+-dependent Cl− and K+ channels.

**Methods:** Porcine tracheal preparations were mounted in Ussing-chambers for transepithelial short-circuit-current (Isc) recordings.

**Results:** Luminal ACh induced a Isc with an immediate rise followed by a slow attenuation. The action of luminal ACh were simulated by the cholinergic receptor agonist carbachol (resistant to the action of cholinesterases) and the muscarinic acetylcholine receptor (mAChR) agonist muscarine, but no effect was observed in response to the nicotinic acetylcholine receptor (nAChR) agonist nicotine or the membrane-impermeable nAChR agonist DMPP. Furthermore, the luminal ACh-induced Isc was largely blocked by the nonselective mAChR antagonist atropine (M1-M5), the M1 preferring antagonist pirenzepine as well as by the M3 preferring antagonist 4-DAMP. An antagonist of phosphatidylinositol-phospholipase C (U73122) was without any effect on the luminal ACh-induced Isc, which was, in contrast, attenuated by a phosphatidylycholine-phospholipase C inhibitor (D609). An inhibitor of adenyly cyclase (MDL) showed no effect on the luminal ACh-induced Isc. Bilateral calcium free solution studies revealed, that the luminal ACh-induced Isc was independent from the presence of Ca2+ in the extracellular medium, but was inhibited by blockade of intracellular sarcoplasmic, endoplasmatic Ca 2+-ATPases (SERCA) with Thapsigargin. Against this the SERCA inhibitor cyclopiazonic acid showed no effect. Further the responses of luminal ACh were insensitive against inhibition of intracellular Ca2+ release channels on the sarcoplasmic reticulum. In the presence of the ryanodine receptor antagonist ruthenium red and the inositol-1,4,5-trisphosphate (IP3) receptor antagonist 2-APB no changes in the luminal ACh-induced Isc were observed. Basolateral depolarization by a high K+ concentration revealed a good correlation between the Isc and a basolateral K+ current rather than a luminal Cl− current under ACh-stimulated conditions.

**Conclusion:** In conclusion, luminal M1 and M3 mAChR couple with phosphatidylycholine-phospholipase C, leading to a Ca2+-activated K+ channel-mediated K+ conductance. This suggests the importance of K+ channels in airway mucociliary clearance.

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**P156**

*β-secretase BACE1 interacts with neuronal KCNQ channels and regulates hippocampal M-current*


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The β-secretase BACE1 (β-site APP-cleaving enzyme 1) plays a pivotal role in the amyloidogenic pathway of APP processing, leading to the generation of amyloid-β peptides and, eventually, plaque formation, which is a neuropathological hallmark of Alzheimer’s disease (AD). Pharmacological inhibition of BACE1 is therefore a prime target for drug therapy in AD. One should bear in mind, however, that, with an ever-increasing number of BACE1 substrates other than APP being identified, many of the physiological functions of BACE1 are still elusive. BACE1-deficient mice offer a suitable approach to address this issue. Among other neurological abnormalities, BACE1-knockout mice display an epileptic phenotype. Whereas a causal role of altered Na+ channel expression and function for the epileptic phenotype remains controversial, we focused here on the effects of BACE1 on M-current, a voltage-dependent, non-inactivating K+ current with slow kinetics. M-current can be mediated by different assemblies of neuronal KCNQ (Kv7) channels (KCNQ2-5), with a preponderance of KCNQ2/Q3 heteromers.

Compared to their wild type counterparts, hippocampal neurons from BACE1−/− mice exhibited enhanced intrinsic excitability, in particular impaired frequency adaptation, which we link to a dramatic decrease in their M-current. In HEK293T cells, BACE1 increased K+ currents through heteromeric KCNQ2/Q3 channels as well as through homomeric KCNQ2, KCNQ4 and KCNQ5 channels. Importantly, BACE1 altered channel gating of heterologously expressed KCNQ2/3 Q3 complexes in a proteolysis-independent fashion. BACE1 did not increase surface levels of these heteromers. Two independent lines of biochemical evidence strongly suggest that BACE1 physically interacts with KCNQ channels in a β-subunit-like manner. Our data indicate that BACE1 is required for regular M-current function, which reveals a new feature of how BACE1 regulates neuronal activity in the intact and diseased brain.

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P157
Down-regulation of inwardly rectifying Kir2.1 K⁺ channels by human parvovirus B19 capsid protein VP1

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Background: Parvovirus B19 (B19V) has previously been shown to cause endothelial dysfunction. The parvoviral B19V capsid protein VP1 harbours a lysophosphatidylcholine producing phospholipase A2 (PLA2). Lysophosphatidylcholine inhibits Na⁺/K⁺ ATPase, which in turn may impact on the activity of inwardly rectifying K⁺ channels. The present study explored whether VP1 modifies the activity of Kir2.1 K⁺ channels.

Materials and Methods: cRNA encoding Kir2.1 was injected into Xenopus oocytes without or with cRNA encoding VP1 isolated from a patient suffering from fatal B19V-induced inflammatory cardiomyopathy or the VP1 mutant H153AVP1 lacking a functional PLA2 activity. K⁺ channel activity was determined by dual electrode voltage clamp. In addition, Na⁺/K⁺-ATPase activity was estimated from K⁺ induced pump current (I_pump) and ouabain-inhibited current (I_ouabain).

Results: Injection of cRNA encoding Kir2.1 into Xenopus oocytes was followed by appearance of inwardly rectifying K⁺ channel activity (I_K), which was significantly decreased by additional injection of cRNA encoding VP1, but not by additional injection of cRNA encoding H153AVP1. The effect of VP1 on I_K was mimicked by lysophosphatidylcholine (1 μg/ml) and by inhibition of Na⁺/K⁺-ATPase with 0.1 mM ouabain. In the presence of lysophosphatidylcholine, I_K was not further decreased by additional treatment with ouabain.

Conclusion: The B19V capsid protein VP1 inhibits Kir2.1 channels, an effect at least partially due to phospholipase A2 (PLA2) dependent formation of lysophosphatidylcholine with subsequent inhibition of Na⁺/K⁺-ATPase activity.

P158
Kv1.5 blockers preferentially inhibit TASK-1 channels: TASK-1 as a target against atrial fibrillation and obstructive sleep apnea?

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Atrial fibrillation and obstructive sleep apnea are responsible for significant morbidity and mortality in the industrialized world. There is a high medical need for novel drugs against both diseases, and here, Kv1.5 blockers have emerged as promising drug targets. In humans, TASK-1 has an atrium-specific expression and TASK-1 is also abundantly expressed in the hypoglossal motor nucleus. We asked whether known Kv1.5 channel blockers, effective against atrial fibrillation and/or obstructive sleep apnea, modulate TASK-1 channels. Therefore, we tested Kv1.5 blockers with different chemical structures for their TASK-1 affinity, utilizing two-electrode voltage clamp (TEVC) recordings in Xenopus oocytes. Despite the low structural conservation of Kv1.5 and TASK-1 channels, we found all Kv1.5 blockers analyzed to be even more effective on TASK-1 than on Kv1.5. For instance, Q1 the half-maximal inhibitory concentration (IC50) values of AVE0118 and AVE1231 (A293) were 10- and 43-fold lower on TASK-1. Also for MSD-D, ICAGEN-4, S20951 (A1899), and S9947, the IC50 values were 1.4- to 70-fold lower than for Kv1.5. To describe this phenomenon on a molecular level, we used in silico models and identified unexpected structural similarities between the two drug binding sites. Kv1.5 blockers, like AVE0118 and AVE1231, which are promising drugs against atrial fibrillation or obstructive sleep apnea, are in fact potent TASK-1 blockers. Accordingly, block of TASK-1 channels by these compounds might contribute to the clinical effectiveness of these drugs. The higher affinity of these blockers for TASK-1 channels suggests that TASK-1 might be an unrecognized molecular target of Kv1.5 blockers effective in atrial fibrillation or obstructive sleep apnea.
The aspartyl protease beta-site APP-cleaving enzyme 1 (BACE1) is the rate-limiting enzyme for the generation of Abeta peptides. Its crucial role in the amyloidogenic pathway makes BACE1 a promising therapeutic target for Alzheimer’s disease. However, as the number of its known substrates is ever increasing, one should be aware that most physiological functions of BACE1 remain to be elucidated. Previous work from our group using BACE1-/- mice and a heterologous expression system revealed a complex interaction between BACE1 and voltage-gated potassium channels of the KCNQ (Kv7) family. Importantly, the majority of the effects of BACE1 on KCNQ channels were independent of its enzymatic activity. Rather, BACE1 emerged as a mandatory constituent of proper KCNQ function, which promotes channel gating in a beta-subunit-like fashion. As a consequence, lack of BACE1 weakens KCNQ-mediated hippocampal M-current, offering a plausible explanation for the epileptic phenotype of BACE1-deficient mice.

The above studies relied heavily on electrophysiological methods to explore the functional implications of the novel and unorthodox interaction between BACE1 and KCNQ channels. To gain insight into the mechanisms that govern the formation of KCNQ/BACE1 complexes, their stoichiometry, and compartments of interaction, we established optical and immunological methods. This tool box comprises advanced microscopy techniques including fluorescence recovery after photobleaching (FRAP), single particle tracking (SPT) in total internal reflection fluorescence (TIRF) microscopy, stimulated emission depletion (STED) microscopy, and proximity ligation assay (PLA), an antibody-based interaction assay. Preliminary findings show that BACE1/KCNQ complexes can be formed at the plasma membrane as well as in intracellular compartments. TIRF imaging allowed us to track and compare the diffusion pattern of unbound BACE1 with that of BACE1/KCNQ complexes, providing the first evidence that BACE1 is trapped by co-expressed KCNQ. Our data strongly suggest that the use of advanced optical techniques represents a promising approach to unravel the intricacies of the non-proteolytic interactions between BACE1 and KCNQ channels at the level of single protein complexes, thereby offering novel insights into BACE1-dependent mechanisms that regulate neuronal excitability in the normal and demented brain.
a physical occlusion of the permeation pathway. Although $K_{cp}$ channels are thought to be voltage-independent, we found that the local anesthetic drug bupivacaine blocks TASK-1 channels in a voltage-dependent manner. This effect was not observed for other TASK-1 blockers including the TASK-1 specific drug A1899. To identify the molecular basis for this unusual voltage-dependent block of a leak channel, we Ala-scanned the M2- and M4-segments and parts of the pore helix to identify the bupivacaine binding site in TASK-1. Although the screen identified some residues of the high affinity drug-binding site of A1899, the residues of the bupi- vacaine binding site are clearly distinct from the ‘classical drug’ binding site located in the central cavity. Molecular dy- namics simulations confirm that the majority of the identified residues face into the recently discovered side-fenestrations of $K_{cp}$ channels. In-silico docking experiments and molecular dynamics simulations also suggest a binding of the drug in these side-fenestrations, with bupivacaine bound to the back-side of the pore-helices. As TASK-1 channels have a voltage-dependence and as the selectivity filter forms the gate of $K_{cp}$ channels, we propose that the targeted binding to the pore-helix from the side-fenestrations provides the molecular basis for a voltage-dependent block of $K_{cp}$ leak channels.

P162
Novel insight into the regulation of TALK-2 (TASK-4) channels by membrane voltage, intracellular ions and pharmacological compounds
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TALK channels (TWIK-related alkaline pH-activated K+ channel) comprise a K2P channels subfamily including TALK-1, TALK-2 and TASK-2 that are activated by high extracellular pH. Beside the activation by extracellular pH little is known about the functional properties, pharmacology and regulation of these channels. Here we investigated TALK-2 channels that are mainly expressed in the pancreas, but at a lower level also in liver, placenta, heart and lung. TALK-2 channels were expressed in Xenopus oocytes and studied in inside-out patches. We report that TALK-2 channels are strongly activated by membrane depolarisation that appears to arise from an ion dependent gating mechanism in the selectivity filter. Replacing intracellular K+ by NH4+, Cs+ and Rb+ dramatically enhanced voltage activation in these channels. The activation by Rb+ was abolished by a mu- tation of a threonine (T116C) in the selectivity filter that is known to coordinate K+ at the S4 site and to alter the K+ occupancy in the pore. Furthermore we identified two novel pharmacological activators of TALK-2 channels that were 2-APB (2-aminoethoxydiphenyl borate) and the structural related antispasmodic agent drofenine. Both compounds strongly activated TALK-2 channels at higher micromolar concentrations and activation in contrast to e.g. Rb+ activation was voltage insensitive. TALK-2 channels activated by 2-APB were very sensitive to inhibition by intracellular Tetrapentylammonium (TPA+, IC50 ∼ 15µM) that is known to bind in the pore cavity of K2P channels and block ion permeation. Intriguingly, TALK-2 channels activated by Rb+ permeation were markedly less sensitive to TPA+ inhibition (IC50 = 12mM) suggesting differences in the pore structure (i.e. TPA+ binding site) for TALK-2 channels activated by 2-APB compared to the Rb+ activated pore. In summary we present novel information on the activation of TALK-2 channels by membrane voltage, intracellular ions and pharmacological compounds.

P163
TASK-1 and TASK-3 form heterodimers in human atrial cardiomyocytes
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TASK-1 channels have emerged as promising drug targets against atrial fibrillation, the most common arrhythmia in the elderly. While TASK-3, the closest relative of TASK-1, was previously not described in cardiac tissue, we found a very prominent expression of TASK-3 in right human auricles. Transcriptional profiling revealed that TASK-3 in the human heart is also expressed at similar levels in the atria and sinoatrial node, while in the ativoventricular node and the ventricles TASK-3 expression levels were even more pronounced. Immunocytochemistry experiments of human right auricular cardiomyocytes showed that TASK-3 is pri- marily localized at the plasma membrane. Single-channel record- ings of right human auricles in the cell-attached mode, using divalent-cation-free solutions, revealed a TASK-1-like channel with a single-channel conductance of about 30 pS. While homomeric TASK-3 channels were not found, we observed an intermediate single-channel conductance of about 55 pS, reflecting the heteromeric channel formed by TASK-1 and TASK-3. Subsequent experiments with TASK-1/TASK-3 tandem channels or with co-expressed TASK-1 and TASK-3 channels in HEK293 cells or Xenopus oocytes, supported that the 55 pS channels observed in right auricles have electrophysiological characteristics of TASK-1/TASK-3 heteromers. In addition, co-expression ex- periments and single-channel recordings suggest that hetero- meric TASK-1/TASK-3 channels have a predominant sur- face expression and a reduced affinity for TASK-1 blockers. In summary, the evidence for heteromeric TASK-1/TASK-3 channel complexes together with an altered pharmacologic response to TASK-1 blockers is likely to have further impact for studies isolating $I_{task}$, from cardiomyocytes and for the
development of drugs specifically targeting TASK-1 in atrial fibrillation treatment.

**P164**

**Voltage dependent activation of silent TWIK-1 channels by intracellular and extracellular Rb+ ions**

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The K2P potassium channels, TWIK-1 and TWIK-2 are expressed in various tissues, including heart, brain, pancreas, lung and kidney. Electrophysiological characterization and understanding of the functional significance of TWIK channels has been difficult by the low or absent functional expression in heterologous expression systems. Several explanations have been proposed to account for the low activity of TWIK channel in cell membranes including silencing of the channel by sumoylation, rapid internalization by a di-isoleucine endocytosis motif and recently the presence of a hydrophobic barrier inside the pore that prevent ion conductance due to pore dehydration. Here we studied TWIK-1 channels in inside out patches from Xenopus oocytes. We found that although removal of the di-leucine endocytosis motif somewhat increased channel activity the observed TWIK currents were still tiny. However, exchange of intracellular K+ by Rb+ generated large macroscopic currents (several nanoamperes) that displayed very strong outward rectification. This outward rectification appeared to result from a voltage dependent activation mechanism that proceeded with a time constant of about 20 ms. The time course of voltage activation was not voltage dependent but the fraction of channel activated steeply increased with any further depolarization without any sign of saturation. This outcome suggests that Rb+ ions induce a structural change in pore (likely at the selectivity filter) that promoted the pore opening while other intracellular ions such as Na+, K+, Tl+, Cs+ and NH4+ did not induce opening. This ion activation profile is different to other K2P channels including TREK, TRAAK, TRESK, TALK-2 and TASK-3 that we have previously shown to be activated by e.g. K+; NH4+ and Cs+ in addition to Rb+ suggesting the TWIK-1 pore has unique properties. In line with this we found that TWIK-1 Rb+ currents were strongly inhibited by intracellular K+ with IC50 of about 3 mM while in other K2P channel much higher K+ concentration are needed to block Rb+ activation. Furthermore, other K2P channels are not activated by extracellular Rb+ ions while in TWIK-1 extracellular Rb+ produced large inward current at negative voltages. Our results show that TWIK-1 channels display low activity in cell membranes primarily because of their unique pore properties that include the inability of K+ to promote voltage dependent activation.

**P165**

**BACE1 modulates gating of KCNQ1 (Kv7.1) and cardiac delayed rectifier KCNQ1/KCNE1**

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KCNQ1 (Kv7.1) is one of the five known members of the KCNQ family and forms a homotetrameric channel producing a voltage-dependent K+ current. Co-assembly of KCNQ1 with the auxiliary β-subunit KCNE1 strongly up-regulates this current. In cardiac myocytes, KCNQ1/KCNE1 complexes are thought to give rise to the delayed rectifier current IKs which contributes to action potential repolarization. Prompted by a previous study (Sachse et al., *FASEB J* 27:2458-24678, 2013), in which we identified KCNE1 has a novel substrate of the beta-secretase BACE1 (β-site APP-cleaving enzyme 1), a type I membrane protein which is best known for its detrimental role in Alzheimer’s disease, we explored here the functional consequences of the presumed interaction of BACE1 with KCNQ1/KCNE1. We performed whole-cell recordings from HEK 293T cells which were transiently transfected with KCNQ1, KCNE1 and BACE1 in various combinations. Our main findings were the following: (1) BACE1 slowed the inactivation of KCNQ1 current producing an apparently overshooting response to depolarizing voltage steps. (2) Activation and deactivation kinetics of KCNQ1/KCNE1 currents were significantly slowed in the presence of co-expressed BACE1. (3) BACE1 augmented reconstituted cardiac IKs when heart action potentials were used as voltage command waveforms. (4) The electrophysiological effects of BACE1 reported here were independent of its enzymatic activity, because they were preserved when the proteolytically inactive variant BACE1 D289N was co-transfected in lieu of BACE1.

(5) Co-immunoprecipitation of transfected HEK293T-cells supported our hypothesis that BACE1 modifies biophysical properties of KCNQ1 and KCNQ1/KCNE1 by physically interacting with the channel proteins in a β-subunit-like fashion. Our data suggest that BACE1, which is expressed in cardiac tissue, should have an impact on heart electrophysiology by enhancing a prominent repolarizing current.

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P166
SPAK and OSR1 sensitivity of voltage-gated K⁺ channel Kv1.5
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Question: SPAK (SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1) are potent regulators of several transporters and ion channels. The kinases are under regulation of WNK (with-no-K[Lys]) kinases. The present study explored whether SPAK and/or OSR1 modify the expression and/or activity of the voltage gated K⁺ channel Kv1.5, which participates in the regulation of diastolic cardiac action potential and tumor cell proliferation.

Methods: cRNA encoding Kv1.5 was injected into Xenopus laevis oocytes with or without additional injection of cRNA encoding wild-type SPAK, constitutively active T233E SPAK, WNK insensitive T233SSPAK, WNK insensitive T233ASPAK, WNK insensitive T233SSPAK, wild-type OSR1, constitutively active T185EOSR1, WNK insensitive T185OSR1 and catalytically inactive D164AOSR1. Voltage gated K⁺ channel activity was quantified utilizing dual electrode voltage clamp and Kv1.5 channel protein abundance in the cell membrane utilizing chemiluminescence of Kv1.5 containing an extracellular hemagglutinin epitope (Kv1.5-HA).

Results: Kv1.5 activity and Kv1.5-HA protein abundance were significantly decreased by wild-type SPAK and T233ESPASK, but not by T233SSPAK and D212ASPAK. Similarly, Kv1.5 activity and Kv1.5-HA protein abundance were significantly down-regulated by wild-type OSR1 and T185EOSR1, but not by T185OSR1 and D164AOSR1.

Conclusion: Both, SPAK and OSR1 decrease cell membrane Kv1.5 protein abundance and activity.

P167
In vitro biosynthesis of two-pore domain potassium channels for functional studies in giant unilamellar vesicles
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The Two-pore domain potassium channels (K2P) function as regulators of membrane resting potential and cellular electrical excitability. The family comprises of 15 homo- and heterodimeric members, most of which can be characterized by electrophysiological recordings of transfected cells and RNA-injected Xenopus oocytes. However, drawbacks of this heterologous expression arise for example from unregulable effects of cellular regulation events, unknown lipid membrane environment, or lack of measurable wildtype currents at the plasma membrane. Alternative methods to investigate such channels often demand recombinant protein production, which is still difficult for membrane proteins. We report the in vitro biosynthesis of several K2P channels as well as mutant and GFP-tagged fusion constructs in a lipid-containing wheat-germ cell-free system. We show that Giant Unilamellar Vesicles (GUV) of defined lipid composition can be included in the cell-free reaction to promote production of channel proteins without the addition of detergents. Channel insertion and orientation in the GUV lipid membrane was visualized by fluorescence microscopy of GFP-tagged fusion proteins and a proteolytic assay. Channel protein containing GUV were probed by standard patch-clamp methods, planar lipid bilayer experiments, as well as biochemical analysis. Our results indicate that K2P channels are formed in a functional folded state. The cell-free synthesis in GUV provide the opportunity of obtaining ion channels in defined, membrane-mimicking lipid environment to study lipid regulation effects or mechanosensitivity. Problems can be addressed exclusive of endogenous regulatory or accessory proteins and coexpression experiments could reveal interaction partners or heteromers. Furthermore, patch clamp technique can be extended from plasma membrane directed ion channels to channels of possibly intracellular location and could help to understand the role of K2P family members so far referred to as „silent“. As reaction mixture and GUV are accessible for reagent addition site-specific labeling by fluorophores, cysteine reactive agents or non-natural amino acids can readily be achieved and might prove beneficial in studying structural changes in gating or regulation events.

P168
The Ca²⁺-activated potassium channel (Kᵦ₃.1) blocker Senicapoc inhibits CXCL12-induced migration of highly metastatic A549 lung carcinoma cells
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Non-small cell lung cancer (NSCLC) is a leading cause of cancer-related death worldwide. Due to the early development of metastasis the survival rate is less than 20%. Unfortunately, chemotherapies are of limited benefit in NSCLC and thus, novel therapeutic targets are urgently needed. CXCL12, the ligand for the CXCR4 receptor, is known to increase the metastatic potential of non-small cell lung cancer cells. Furthermore, it is also known that the Ca²⁺-activated K⁺ channel Kᵦ₃.1 is involved in the migration of cancer cells. In former experiments, we could already show that high Kᵦ₃.1 channel expression correlates with tumor aggressiveness and poor patient survival. Here, we investigated whether a link exists between the Kᵦ₃.1 channel and the CXCL12/CXCR4 axis in the metastatic cascade of NSCLC. Migration of highly aggressive A549 cells, stimulated with the chemokine CXCL12 could be inhibited when treated with the Kᵦ₃.1 channel blocker Senicapoc. A correlation between CXCR4 expression and Kᵦ₃.1 channel expression was found on the protein level in highly metastatic A549 cells. High Kᵦ₃.1 channel expression correlates with high CXCR4 expression. Furthermore, CXCL12 as well as the growth factor EGF enhanced the extravasation and invasion of highly metastatic A549 cells. In this experiment, we cultured human microvascular endothelial cells (Hmec-1) on a collagen-based matrix until they formed a confluent endothelial layer. Afterwards, A549 cells together with CXCL12 or EGF were added to the endothelial layer. The extravasation and invasion capability were then
analyzed using time lapse video microscopy. Treatment with CXCL12 or EGF caused an 8-fold increase of transmigration and invasion compared to that of untreated control cells. Our results suggest that increased $K_{c,3.1}$ channel expression is accompanied with increased CXCR4 expression. Moreover, they lend further support to the idea that $K_{c,3.1}$ channels are involved in the metastatic process of NSCLC.

**POSTER PRESENTATIONS**

**P169**

Functional role of amino acids in the intracellular vestibule of human P2X7 receptors

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The human P2X7 receptor (hP2X7R) is an ATP-gated cation channel expressed in immune cells. According to the recently dissolved three-dimensional structure of a zebrafish P2X4 receptor (zP2X4R) the hP2X7R subunits have a large extracellular domain, two transmembrane domains and intracellularly located N- and C-termini. The second transmembrane domain (TM2) has been shown to be involved in the ion permeation and the pore opening of P2X receptor channels. At its C-terminal end, it forms an intracellular vestibule which is supposed to be involved in P2X receptor gating. We performed single cysteine scanning mutagenesis of the hP2X7 constructs and ATP-dependent whole cell and single channel currents were measured using the two microelectrode voltage clamp technique and the patch clamp technique, respectively.

hP2X7R constructs with cysteine substitutions between amino acids L346 and Y358 were found to be insensitive to the extracellularly applied sulfhydryl group modifying reagent MTSEA+. As a result of the cysteine exchange of the amino acids G345, A348 and D352, the ATP-dependent currents became smaller compared to the wt hP2X7R. These amino acids C-terminal to G345 seem therefore not to be involved in permeation and gating of the hP2X7R.

Application of MTSEA+ but not the larger MTSET+ to hP2X7G345C increased the P2X7R-dependent current if applied extracellularly to open P2X7R channels indicating the location of hP2X7R gate and selectivity filter N-terminal to G345. MTSET+ however increases hP2X7G345C-dependent currents if applied intracellularly to closed channels. Therefore we conclude that G345 marks the C-terminal border of the hP2X7R selectivity filter and gate.

**P170**

Na\(_{1.8}\) channels are prime targets of oxidative stress in dorsal root ganglia neurons

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Dorsal root ganglia (DRG) are pivotal elements for signal transmission between peripheral organs and the central nervous system. Oxidative stress and a decay of the anti-oxidant system result in aberration of electrical signaling, particularly in inflammatory conditions and aged organisms. We hypothesize that this process involves modification of voltage-gated ion channels and therefore studied the impact of oxidative stress on the electrical properties of acutely isolated murine DRG neurons.

Application of the mild oxidant chloramine-T (ChT) as well as blue-light illumination profoundly prolonged the duration of stimulated action potentials in wild-type animals. ChT (10 µM, 150 s) increased the time to peak from 6.8 ± 0.2 ms to 7.3 ± 0.3 ms (8.1%) and the decay time (80-20%) from 2.6 ± 0.3 ms to 3.0 ± 0.2 ms (15.1%, n = 13). Blue light directed to the cells via the epifluorescence channel of an inverted microscope (450–490 nm from a 100-W mercury source via a 20x objective) increased the time to peak from 6.4 ± 0.4 ms to 6.8 ± 0.4 ms (6.4%) and the decay time from 2.2 ± 0.2 ms to 2.4 ± 0.2 ms (6.4%, n = 12). This influence on the action potential shape was strongly diminished in DRG neurons from animals lacking the TTX-resistant Na+ channel Na\(_{1.8}\) (Na\(_{1.8^-}\)). ChT decreased action potential time to peak and decay time by 3.4% and 5.5% (n = 13), respectively, while the impact of blue light was practically absent (0.2%, -0.6%, n = 13).

Na+ currents were investigated under voltage-clamp control. ChT decreased peak currents at 10 mV of Na\(_{1.8}\) channels studied in DRG neurons of Na\(_{1.9^-}\) mice by 17.4 ± 2.6% but did not significantly change the inactivation (mean inactivation index (Ri) from time of peak to 20 ms: Ri = 0.07 ± 0.01 to 0.09 ± 0.01, n = 14). Blue light reduced the peak current by only 3.7 ± 1.4% but slowed down the inactivation from Ri = 0.15 ± 0.01 to 0.21 ± 0.02 (n = 14). Neither blue light nor ChT had a significant impact on TTX-sensitive Na+ channels, measured in DRG neurons of Na\(_{1.8^-}\) mice in the absence of TTX. ChT did not change the peak current (+1.4 ± 3.4%) and did not affect the mean inactivation from time of peak to 10 ms: Ri = 0.02 ± 0.01 to 0.02 ± 0.01 (n = 9). Blue light decreased the peak current by 2.9 ± 3.7% and minimally slowed-down the inactivation (Ri = 0.04 ± 0.01 to 0.05 ± 0.01; n = 9).
These results highlight Na\(_{v}1.8\) channels as prime targets of oxidative stress in DRG neurons directly affecting electrical signaling.

**P171**

**Differential expression of transmembrane channel-like genes 1 to 8 in skeletal and cardiac muscle of mice**

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**Introduction:** Transmembrane channel-like (TMC) genes 1 to 8 encode integral membrane proteins that are structurally related to voltage-gated potassium channels and TRP channels. Recent data indicate that TMC proteins 1 and 2 are responsible for mechanosensitive potassium influx into hair cells of the inner ear, forming the main transduction channels that enable hearing and vestibular function. At present, no data on TMC expression in skeletal and cardiac muscle is available. Thus, we analysed TMC gene expression of *M. soleus* (SOL), containing almost exclusively oxidative fibres, *M. tibialis anterior* (TBA), which almost consists of fast glycolytic fibres, and compared it to cardiac gene expression. In addition, we quantified transient receptor potential (TRP) V2 and V4 expression.

**Methods:** Total RNA was isolated from SOL, TBA and heart from 100 days-old female mice (*n=4*) and cDNA was synthesised by reverse transcription. Gene expression was quantified by PCR (40 cycles) using commercial TaqMan® assays. Data were normalized to the expression of the housekeeping gene porphobilinogen deaminase. Data are 2-ΔCt and are presented as means ± standard error. Data were analysed by ANOVA on ranks.

**Results:** Expression of TMC1 to TMC4 was below detection level in SOL and TBA (*n. d.*, see Table). In the SOL, expression levels ranked in ascending sequence were TMC5<TMC8<TRPV2<TRPV4<TMC6<TMC7. In the TBA, expression levels were TMC8<TRPV2<TMC6<TMC7<TRPV4. Cardiac expression levels were TMC3<TMC5<TRPV2<TMC8<TMC6<TMC7<TRPV4. In TBA and heart TRPV4 turned out to have the highest expression level of all tested ion channel genes, while TMC7 was the most abundant one in SOL (see Table).

**Conclusions:** We showed that members of the TMC gene family are differentially expressed in skeletal and cardiac muscles of mice. TMC1 to TMC4 expressions were near or below detection level. However, gene expression of TMC5 to TMC8 was in the same range than that of the well-characterized TRP channels V2 and V4. Protein expression and subcellular localization of TMC5 to TMC8 need to be confirmed and a potential role of this novel class of transmembrane proteins for ion transport in muscle cells needs to be elucidated.

<table>
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<th>SOL</th>
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<th>heart</th>
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<td>TMC1</td>
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<td>n. d.</td>
<td>n. d.</td>
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<tr>
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<td>0.4850±0.3430</td>
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</table>

**P172**

**H558R polymorphism in SCN5A associated with LQT syndrome patient**

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The congenital long QT syndrome 3 (LQT3) is characterized by delayed cardiac repolarization, leading to a prolonged QT interval in the surface ECG. Common symptoms are syncope, seizures, torsade-de-pointes arrhythmias and sudden cardiac death by ventricular arrhythmias. LQT3 syndrome is caused by gain-of-function mutations in the α-subunit of the voltage-gated cardiac sodium channel Nav1.5 (SCN5A). In contrast, SCN5A loss-of-function mutations cause Brugada syndrome an inherited arrhythmia which is thought to be responsible for a large fraction of sudden and unexplained cases of cardiac death. H558R is a common SCN5A polymorphism which is present in about 30% of the population...
in heterozygous manner, while the homozygous gene frequency is about 6%. H558R was previously considered as non-harmful, and in some studies, it has even been postulated that H558R can rescue inherited forms of Brugada syndrome. We found several index patients suffering from LQT syndrome which carried the H558R polymorphism in a heterozygous manner, while we did not find any mutations in other classical LQT genes in these cases. The aim of the current study was to analyze the functional consequences of the H558R exchange in Nav1.5, in order to provide answers to the questions why H558R is associated with LQT syndrome and why it can rescue Brugada syndrome. We functionally characterized the H558R polymorphism using the two-electrode voltage-clamp technique after expression in Xenopus oocytes. Here, we found that H558R causes a gain-of-function by increasing current amplitudes, shifting the voltage-dependence of activation to more negative potentials and by enhancing the recovery from inactivation. Therefore, we conclude that the gain-of-function by heterozygous H558R exchanges can provoke LQT syndrome in patients with a reduced cardiac repolarization reserve and that H558R can antagonize the loss-of-function by Brugada syndrome causing mutations.

This suggests that intermediates of CORM2 breakdown affect the channels investigated. The impact of CORM2 was studied in more detail for hERG1; even 1 µM CORM2 blocked 52±11% of the channels with \( \tau_o = 435±8 \, \text{s} \) (n=4). Voltage dependence and kinetics of channel activation, deactivation and inactivation were not affected; only the voltage dependence of recovery from inactivation was slightly shifted by −10 mV. The hERG1 channel mutations that strongly reduce sensitivity towards oxidative modification (Kolbe et al. 2010, J Physiol 588:2999) did not eliminate the CORM2 effect indicating that redox processes may not underlie hERG1 inhibition by CORM2.Predominantly neuronal hERG3, however, was significantly less strongly affected by CORM2 than hERG1 providing means to unravel the underlying molecular determinants. Considering an insensitivity of the above tested channels as a specificity-of-action criterion for CORMs, we sought for alternatives to CORM2. Hexacarbonyl-tris(2-ammoniumethanethiolato)dimanganese(I) (CORM-EDE1) may be a suitable alternative. It is highly water soluble, it is stable, and it releases CO upon illumination with visible light as evidenced with a myoglobin assay. CORM-EDE1 did not inhibit Na,1,5, K,1,5 or hERG1 and it promises to be a suitable photoCORM for physiological applications devoid of the side effects of CORM2.
used as a heterologous overexpression system. In addition, we also checked whether activation of MOP inhibits TRPV1 activity and found that TRPV1 channels are also inhibited by DAMGO, but to a much lesser extent compared to TRPM3 channels. In our analysis of PregS-sensitive DRG neurons, we found that in 69% of these cells the TRPM3 activity was inhibited by DAMGO, while in 31% of the cells it was not. Interestingly, only 15% of the DAMGO-insensitive TRPM3-expressing neurons (30 out of 196) responded to the TRPV1 agonist capsaicin.

Together, our data indicate that in a subset of nociceptive neurons TRPM3 channels are regulated by MOPs through β/γ subunits of PTX-sensitive G-proteins. Furthermore, we show that TRPM3 channels are expressed in functionally diverse DRG neurons. These findings might be important to understand the physiology of this channel and indicate that TRPM3 might be involved in the MOP-mediated peripheral analgesia.

References

P175
TRPC6 channels control CXCR2-mediated recruitment of neutrophils
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The recruitment process of neutrophils during an inflammation includes activation, adhesion to the endothelium and transendothelial migration as well as chemotaxis to the site of inflammation. Most of the underlying signal transduction pathways utilize Ca2+ transients often triggered by GPCR activation. To identify the involved Ca2+ entry channels we investigated the role of TRPC6, a member of the transient receptor potential (TRP) channel family that is involved in receptor-operated Ca2+ entry.

Neutrophil adhesion, arrest and transmigration were analyzed with intravital microscopy. The impact of TRPC6-deficiency on chemotaxis of murine neutrophils in 3-dimensional matrices was studied with time-lapse videomicroscopy. In chemotaxis assays fMLP (end-target chemoattractant) and KC/CXCL1 (intermediary chemoattractant) were applied. Intracellular Ca2+ concentration was analyzed with the fluorescent Ca2+-indicator Fura-2. Neutrophil adhesion on endothelial cells was studied with Atomic Force Microscopy (AFM).

Adhesion, arrest and transmigration of TRPC6-/- neutrophils were diminished in vivo when applying KC. In contrast, recruitment of TRPC6-/- neutrophils was not affected after application of fMLP. Using AFM we found that the loss of TRPC6 strongly reduced the maximum adhesion force between neutrophils and endothelial cells. Chemotaxis in gradients of KC was impaired, while fMLP-mediated chemotaxis was unaffected in TRPC6-/- neutrophils. The chemotaxis and adhesion defect of TRPC6-/- neutrophils in response to KC were accompanied by diminished Ca2+ influx and decreased CXCR2 signaling. Diminished Ca2+ transients after KC stimulation in TRPC6-/- neutrophils were likely responsible for reduced F-actin formation and integrin activation/adhesion.

Our findings indicate that TRPC6 channels are important regulators in neutrophil recruitment. TRPC6 is a component of intermediary chemoattractant-triggered adhesion and actin polymerization but has no influence on end-target chemoattractant-mediated recruitment of neutrophils.

P176
Heteromeric assembly of TRPV channel subunits
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The family of vanilloid transient receptor potential (TRPV) channels is further subdivided into two subgroups: TRPV1-4 and TRPV5/6. TRPV1-4 homomeric channels are unspecific cation channels with pCa2+/pNa+ values between 1 and 10 while TRPV5 and 6 are highly selective for Ca2+. As members of the TRP family TRPV channels are formed by 4 subunits with 6 transmembrane domains. It is well accepted that TRPV5 and 6 are forming heteromeric channels. On the other hand the formation of heteromeric channels within the other subgroup remains controversial. To address this question we constructed dominant-negative mutants of all 4 TRPV subunits. After coexpression with these mutant channel we investigated the suppression wild-type channels with the two-electrode voltage-clamp technique in Xenopus oocytes and with patch-clamp recordings in HEK293 cells. We found a strong assembly of TRPV1 and 2 subunits but no suppression of TRPV3 and TRPV4 currents by any dominant-negative mutant. For the characterisation of heteromeric TRPV channels we constructed concatemers. The expression of these heteromeric channels formed by two TRPV1 and two TRPV2 subunits revealed channels with characteristics dominanted by TRPV2 subunits in relation to their pH and capsaicin sensitivity.

In conclusion, we could confirm the formation of heteromeric channels by TRPV1 and 2 subunits. On the other hand, we could disprove the formation of other TRPV heteromers.
**P177**

**Prostate-specific antigen (PSA)/Kallikrein-related peptidase 3 (KLK3) regulates TRPV4 cation channel function**

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**Question:** Transient receptor potential vanilloid 4 (TRPV4), a member of the TRP channel superfamily, is a non-selective cation channel. It has a broad spectrum of physiological functions and is expressed in a wide range of tissues including the prostate. However, the functional significance of TRPV4 in the prostate is still unclear. KLK3 (kallikrein-related peptidase 3), also known as PSA (prostate-specific antigen), is a member of the family of kallikrein-related peptidases and has been linked to cancer-associated pathophysiology in particular in the prostate. Interestingly, proteases are thought to be involved in the regulation of TRPV4. Therefore, the aim of this study was to investigate whether KLK3 affects TRPV4 activity.

**Methods:** We determined TRPV4 activity by measuring whole-cell currents elicited by the selective TRPV4 agonist GSK1016790A in Xenopus laevis oocytes heterologously expressing human TRPV4 using the two-electrode voltage-clamp technique. Active KLK3 was purified from pooled human seminal fluid by immunoadfinity chromatography followed by anion exchange chromatography. To test the effect of proteases on TRPV4 channel activity, oocytes were preincubated for 30 min in the presence or absence of KLK3 or trypsin. To characterize the effect of KLK3 we used the KLK3-inhibiting antibody S57 (100 µg/ml), two KLK3-stimulating peptides (B2-NH2 and C4) (150 µg/ml) or the tyrosine kinase inhibitor bafetinib (10 µM).

**Results:** Exposing TRPV4 expressing oocytes to KLK3 (10 µg/ml) increased the GSK1016790A-induced TRPV4 currents about fourfold compared to those measured in TRPV4 expressing control oocytes. The serine protease trypsin, known to activate endogenous PAR (Protease-activated receptors)-like receptors in oocytes, partially mimicked the effect of KLK3. The stimulatory effect of KLK3 on TRPV4 currents was concentration-dependent, was enhanced by KLK3-stimulating peptides and was prevented by the KLK3-inhibiting antibody. Interestingly, the increase of TRPV4 currents by KLK3 was essentially abolished by the tyrosine kinase inhibitor bafetinib.

**Conclusions:** Our results demonstrate that KLK3 has a stimulatory effect on TRPV4 currents in the oocyte expression system. This stimulatory effect involves a tyrosine kinase signaling pathway and may be mediated by endogenous PAR-like receptors. KLK3 mediated stimulation of TRPV4 activity may be relevant for prostate physiology and pathophysiology.

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**P178**

**Ruminal epithelial cells express multiple TRP channels**

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**Introduction:** Ruminants absorb large quantities of cations such as Na+, NH4+, Ca2+ and Mg2+ across the epithelia of the forestomachs. Previous functional studies suggest that this may involve TRP channels.

**Methods:** Expression of mRNA for the bovine sequences of TRPA1, TRPM6 - M8 and TRPV1 - V6 was investigated via PCR using specific primers. After sequencing, the bovine representative of TRPV3 (2397bp) was selected for characterization by transient, tetracycline-inducible overexpression in HEK 293 cells. Single channel conductances were studied in inside-out patches using a 110 mM Na-glucosate pipette solution and bath solutions with varying concentrations of Na+ and Ca2+ glucosate both in cultured cells from native epithelia and overexpressing HEK 293 cells.

**Results:** PCR experiments show clear expression of TRPA1, TRPM6, TRPM7, TRPVM2, TRPV3 and TRPV4 in the native bovine rumen on the mRNA level, while expression of TRPM8, TRPV5 and TRPV6 could not be demonstrated. In cells from primary ruminal cultures, a plethora of channels with conductances ranging from <1 pS to ~100 pS were observed. When Ca2+ was elevated on the cytosolic side, the channels typically showed a slight elevation in single channel conductance with very variable changes in open probability. First results from HEK 293 cells overexpressing bovine TRPV3 show that heterologously expressed TRPV3 is correctly targeted to the plasma membrane. Diverse conductances of up to 100 pS were observed.

**Conclusions:** The ruminal epithelium expresses multiple channels from the TRP family. Further work is needed to identify which of these channels are functionally involved in nutrient transport.

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**P179**

**Characterization of a new TRPM3 agonist**

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The non-selective ion channels formed by TRPM3 proteins are expressed in several tissues and have been implicated in insulin release from pancreatic β-cells and in sensing noxious thermal stimuli in cutaneous nociceptor neurons. These channels can be activated by various chemical substances, including the steroid pregnenolone sulfate (PregS) and the dihydropyridine nifedipine (Nif). Studies using structural homologs of these compounds and modified TRPM3 proteins with various point mutations demonstrated that PregS and Nif do not bind to the same binding site while activating TRPM3. Rather, these substances synergistically activate the channels. In addition to a single central cation-conduction pathway, possibly involving channel opening or入门 suggested by structural data, the channel can be allosterically regulated by multiple other factors, including modulation by the phosphorylation state of the protein.
pore, TRPM3 contains a second, Na+-selective, ion permeation pathway ('ω-like' pore). The present study was conducted to better understand the structural requirements for agonism at TRPM3 channels. Using electrophysiological techniques and Ca2+-imaging we analysed the effects of different nifedipine analogues on the activity of endogenously expressed TRPM3 channels (murine β-cells and DRG neurons) and in the HEK cell recombinant overexpression system. We found one compound (DNN, De-nitro-nifedipine) that showed a strong agonistic potency at high concentrations (>25 µM), when solely applied. However, co-application of PregS and DNN at lower concentrations (3.5 µM, which applied alone only poorly activates TRPM3 channels) still resulted in a strong, supra-linear synergistic potentiation, indicating that PregS and DNN do not compete for the same binding site. We then tested the specificity of DNN i) by comparing its effects on primary β-cells and DRG neurons from wild-type and TRPM3-KO mice, and ii) by testing the sensitivity of several recombinant TRP channels (TRP1/2/7/8, TRPA1, TRPV1/4) towards DNN. Both approaches led to the result that DNN-induced currents as well as [Ca2+]i increase are TRPM3-specific. Moreover, we show that DNN-activated TRPM3 channels can be inhibited by Gi/o protein-coupled receptor activation and TRPM3 inhibitors in a similar way as TRPM3 channels activated by PregS. Further biophysical characterization revealed obvious differences in the I-V curve shapes of DNN- and PregS-activated TRPM3 channels, indicating differences in the agonist-specific properties of the ion-conducting pore. These data will help to identify more potent and specific pharmacological tools, with the ultimate goal to manipulate these channels in vitro and in vivo for experimental and therapeutic purposes in the context of diabetes and chronic pain.

P180
Regulation of the human short transient receptor potential channel 3 (hTRP3) by the serum and glucocorticoid inducible kinase 1 (SGK1)

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The transient receptor potential channels (TRP channels) are widely expressed in a large number of various human and animal cell types. Most of the TRP channels are permeable for Ca2+ and some also for Mg2+. TRP channels are divided into three main groups based on their structure: short, long and osm-like TRPs. The short TRP subfamily of cation channels contains mammalian TRPs, TRPL, and the Drosophila TRP. The mammalian TRP superfamily of cation channels contains four subfamilies (TRP1; TRP4, 5; TRP2 and TRP3, 6, 7, 8) based on sufficiently similar sequence and function. The human short transient receptor potential channel 3 (hTRP3) is expressed at the highest levels in brain, and at much lower levels in small intestine, colon, testis, prostate, ovary, placenta and lung. Cytosolic Ca2+ ([Ca2+]i) plays a crucial role in various cellular functions of virtually all cell types and is thus under tight hormonal control. However, cellular mechanisms governing the regulation of human TRP3 abundance in the cell membrane are poorly understood. Surface abundance of the epithelial Na+ channel is regulated by the serum and glucocorticoid inducible kinase SGK1. The present study has been performed to explore whether human TRP3 is regulated by SGK1 and the related kinases SGK2, and SGK3. To this end, cRNA encoding human TRP3 (hTRP3) has been injected with or without cRNA encoding wild type SGK1, SGK2, and SGK3 into Xenopus Oocytes. In the presence of Cl-, hTRP3 mediated Ca2+ entry leads to secondary activation of Ca2+-sensitive Cl- channels (ICl(Ca)). Coexpression of hTRP3 with SGK1 stimulates (ICl(Ca)) but not by SGK2, and SGK3. The observations suggest that SGK1 regulate hTRP3 and are thus likely to participate in the regulation of calcium homeostasis.

Figure 1

P181
Hyperpolarization-activated and cyclic nucleotide-gated channels in hippocampal neurons

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The hippocampal formation is considered a relay center for novel information in the vertebrate brain, participating in learning and memory functions of organisms. In general terms, initiation and coordination of signals in neuronal networks relies on a complex interplay of several ion channels. Amongst the different ion channel families, hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels play an essential role (Kaupp & Seifert, 2001). In the murine CNS, HCN channels are expressed in a distinct and differential manner. In contrast to typical voltage-dependent channels, they are activated at rather negative membrane potentials. Furthermore, HCN channel activation can be modulated via direct binding of cAMP. Here, we investigated the expression profiles of HCN channels in hippocampal neurons. We used independent methods, e.g. immunohistochemistry, immunocytochemistry, and quantitative PCR, to investigate the expression pattern of individual HCN isoforms. Of the four genes encoding HCN subunits, we found that HCN 1, 2, and 4 are expressed differentially in the murine hippocampus. Thus, we sought an approach allowing further study of these proteins in hippocampal cells. We used cultures of murine primary hippocampal
neurons (PHNs). These neurons maintain many biochemical and electrophysiological properties that have been identified in the intact hippocampus. We found that HCN channels exhibit distinct expression patterns in cultivated PHNs. Most notably, subcellular distribution patterns of HCN isoforms in PHNs resembled that found in intact hippocampal tissue. Our findings suggest that properties of individual signaling molecules, here HCN channels, can be studied in cultivated PHNs as a model. Finally, we established recombinant adeno-associated viral vectors (rAAVs) as an efficient tool to genetically modify hippocampal cell functions in forthcoming studies.


P182
Macroscopic and single-channel currents of heteroconcatameric olfactory CNG channels with functional and disabled ligand binding sites

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**Question:** Cyclic nucleotide-gated (CNG) ion channels – non-selective cation channels, activated by the binding of cAMP or cGMP – are key elements of sensory transduction cascades involved in visual and olfactory perception. Native CNG channels of olfactory sensory neurons are heterotetramers formed by the homologous CNGA2, CNGA4 and CNGB1b subunits in a 2:1:1 stoichiometry. Until now, it is an open question if functional channels require a defined arrangement of the subunits and how ligand binding to these different subunits leads to channel opening.

**Methods:** To address this, we forced the heterotetrameric channels to a defined order of the subunits utilizing a concatenation approach. We designed all twelve possible heteroconcatamers representing three possibilities for the arrangement of the monomers around the central pore. All concatamers were analyzed in inside-out patches of Xenopus laevis oocytes to study ligand-dependent activation induced by the binding of cAMP or cGMP.

**Results:** The amplitude of the macroscopic currents was similar to that observed for channels built of monomeric subunits. The Hill coefficients for cGMP exceeded those for cAMP (~1.8 versus ~1.5), but were equal for the same ligand with all concatamers. The EC_{50} values were moderately different among the concatamers without revealing obvious systematics. B1b-A2-A2-A4 proved to be the most and A2-A2-B1b-A4 the least ligand-sensitive channel. Like for channels built of monomers, all twelve concatamers exhibited a typical and complex single-channel behavior with a main single-channel conductance of about 12 pS and 15 pS at positive and negative voltages, respectively. We further studied the effect of disabled cyclic nucleotide binding sites (CNBD) of the individual subunits. We systematically introduced a single point mutation in the individual CNBDs (R538E in A2, R430E in A4 and R657E in B1b) that was previously shown to effectively decrease the apparent affinity for cGMP. In the background of A4-B1b-A2-A2, these mut-concatamers behaved similar to channels build of the respective mut-monomers, exhibiting EC_{50} values shifted to higher concentrations and the appearance of additional components in the concentration-activation curves. Compared to wt-channels, mut-channels exhibited a more flickery single channel behavior at sub-saturating ligand concentrations with open probabilities approximating unity at cGMP concentrations ≥ 5 mM.

**Conclusions:** Our observations suggest that all subunit types essentially contribute to the channel opening and that the relative subunit arrangement is not critical for very channel function.

P183
Functional interaction between volume-regulated anion channels and P2X7 receptor in RAW macrophages

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In the immune system, extracellular ATP, released from cells under pathological conditions like necrosis or hypoxia, serves as a danger-associated molecular pattern, informing cells about an upcoming threat by activating purinergic P2Y or P2X receptors. In the murine macrophage cell line RAW 264.7, ATP release is mediated by volume-regulated anion channels (VRAC). This can be induced by sphingosine-1-phosphate (S1P) which is produced by sphingosine kinases which are activated by several different inflammatory signaling molecules. Since LRRC8 proteins are supposed to be essential parts of VRAC, we tested if LRRC8 proteins are also essential for S1P-induced activation of VRAC in RAW macrophages. As measured by PCR, all five LRRC8 subtypes (LRRC8A-E) are expressed together in RAW cells. SB by application of siRNA against subtype LRRC8A for 3 or 4 days, the mRNA level and the amount of LRRC8A protein was decreased. Furthermore, hypoosmolarity- and S1P-induced whole cell currents measured by voltage clamp were decreased after treatment of RAW macrophages for 3 or 4 days with anti-LRRC8A siRNA. We conclude that LRCC8A is an essential part of the volume-regulated anion channel in macrophages which mediates S1P-induced ATP secretion for signaling via purinergic receptors.
As aqueous system the ocular lens needs a precise volume regulation for maintenance of transparency. The volume regulation requires ion transport mechanisms provided by different ion channels.

Therefore we studied ion channels in primary cultured human lens cells and in cells of the cell line HLE-B3 by means of whole-cell patch-clamp technique, immunostaining and PCR experiments. The differentiation of the cells was verified by gene expressing profiling for Crystallin-αB, Connexin-43, α-smooth muscle actin, Collagen-IA2, Integrin-αV and E-cadherin. For an overview of physiological ion channel activity, we first used a K+ containing solution. Adding the Ca2+-ionophore ionomycin (1 µM) resulted in outwardly rectifying currents with properties of Maxi-K+ channels in both primary cultured and HLE-B3 cells, which is in accordance with earlier publications (Shepard, Rae 1998 Curr Eye Res).

For further assessment of ion channel expression we replaced K+ by Cs+ at different Cl- conditions ([Cl\textsubscript{o}] = 146 mM, [Cl\textsubscript{i}] = 30 mM or [Cl\textsubscript{i}] = 111 mM, respectively). Further Ca2+-dependent currents were detected after ionomycin application in both cell types: one led to depolarization independent from Cl- concentrations and showed reversal potentials close to 0 mV and the other one changed from linear current/voltage relation to outward rectification depending on intracellular Cl-. Using a more physiological stimulus, ATP (45 µM) comparably increased the membrane conductance with a faster but transient activation compared to that achieved by ionomycin. Gene expression of anoctamin 2 (Ano2) and 4 (Ano4) could be detected in both HLE-B3 and primary lens cells. Confocal microscopy revealed the presence of Ano2 and Ano4 in the cell membrane of lens cells.

In summary we newly detected in lens cells Ca2+ dependent cation and Cl- conductance. Ano2 as a Ca2+ dependent Cl- channel and Ano4 as possible non-selective cation channel are promising candidates for the molecular identity of these channels.

Claudins are key regulators of transepithelial transport by sealing the paracellular cleft or forming size-, charge- and water-specific channels. This is achieved by a concerted interaction of different claudin molecules within the same (cis-interaction) and the neighboring (trans-interaction) plasma membrane resulting in the formation of a complex strand meshwork, the tight junction (TJ). In the kidney, the thick ascending part of Henle’s loop (TAL) was reported to co-express claudins 3, 10, 11, 16 and 19. However, some of these claudins fail to interact with each other in cis and/or in trans and do not insert into strands together.

Claudins consist of four transmembrane segments, a small intracellular loop and two extracellular loops. To examine which segments are involved in the ability of claudin interaction, a set of chimeras was created, consisting of mixed segments of TAL claudins. Chimeras were expressed in kidney cells either with an established TJ (MDCK C7) or without endogenous TJ (HEK 293). Protein localization, interaction and TJ strand formation were analyzed by western blotting, confocal laser-scanning microscopy, Förster/fluorescence energy transfer (FRET) and freeze fracture electron microscopy.

Whereas several chimeras failed to localize to the plasma membrane or were not capable of cis- or trans-interaction, others formed TJ strands. Our results suggest that both transmembrane segment 1 and extracellular loop 1 are involved in cis-interaction of claudins.

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**P186**
A novel principle of mineralocorticoid receptor-induced epidermal growth factor receptor expression involves specificity protein 1

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**Question:** The mineralocorticoid receptor (MR), a member of a superfamily of steroid receptors, acts as a ligand-bound transcription factor at hormone response elements (HRE). Until now, the glucocorticoid response element (GRE), which is shared by the MR and its closest relative, the glucocorticoid receptor (GR), was the only characterized MR-binding DNA element. Whereas the GR is involved in carbohydrate
and lipid metabolism, the MR plays a crucial role in electrolyte and water homeostasis. Also, the GR acts anti-fibrotic and anti-inflammatory, while the MR induces pathophysiological changes in the renocardiocirculatory system, e.g. inflammation, hypertrophy and fibrosis. Since MR and GR elicit different effects, the existence of additional MR-specific hormone response elements seems likely. In the past, we showed that some of the pathophysiological changes can be mediated by MR/aldosterone-induced upregulation of the epidermal growth factor receptor (EGFR) due to enhanced EGFR promoter activation. The aim was to identify a MR-specific hormone response element (MRE) on the EGFR promoter, and furthermore to characterize the principle mechanism of the MR/aldosterone-induced gene expression.

Methods and Results: In reporter gene assays we identified a 65 bp MRE on the EGFR promoter which was not activated by ligand-bound GR. Additionally, specificity protein 1 (SP1) was detected as a mandatory co-factor for MR/aldosterone-dependent MRE activation. We demonstrated a direct binding of SP1 to MRE in electromobility shift assays (EMSA) and transcription factor binding ELISA. Additionally, SP1 inhibition using specific siRNA or an inhibitor led to a reduction of MR/aldosterone-mediated MRE induction and thereby EGFR expression in different cell lines and also primary culture. Furthermore, mutations in the SP1-binding site lead to a strong reduction in MR/aldosterone-induced EGFR expression due to disturbed binding of SP1. Interestingly, a single nucleotide polymorphisms (SNP) was identified in the SP1-binding region of MRE. This SNP (-216 G/T) as well as another SNP (-191 C/A) influences the nature of MRE-SP1 binding and thereby alters the basal expression levels and MR-responsiveness of the EGFR promoter.

Conclusion: A mineralocorticoid response element could be localized on the EGFR promoter and our results indicate that SP1 is essential for the MR interaction with this MRE. Overall, a novel principle of MR-specific gene expression was explored that applies to the pathophysiological relevant expression of the EGFR. Further investigations aim to characterize the MR-SP1-MRE interaction in detail and to explore a putative correlation of the SNPs with renocardiovascular diseases.

An amino acid exchange (P209L) in the human BAG3 gene, caused by a single base mutation, gives rise to a severe dominant childhood muscular dystrophy, cardiomyopathy and respiratory insufficiency (Selcen et al., 2009). Aiming for deeper insights into the pathological mechanisms of the disease, we designed transgenic in vitro and in vivo models of the human mutation BAG3\(^{P209L}\). Generation of stable embryonic stem cell (ESC) lines with cardiomyocyte specific expression of either human BAG3\(^{wt}\)-eGFP or BAG3\(^{P209L}\)-eGFP revealed localization of BAG3\(^{wt}\)-eGFP to the Z-disc in ESC derived cardiomyocytes. In contrast, the expression of BAG3\(^{P209L}\)-eGFP led to Z-disc disruption in vitro, a phenotype similar to that found in patients. In cardiomyocytes, isolated from hearts of transgenic mice overexpressing BAG3\(^{wt}\)-eGFP or BAG3\(^{P209L}\)-eGFP, BAG3\(^{wt}\)-eGFP stringently localizes to Z-discs and intercalated discs and co-localizes with α-actinin, Myopodin and Filamin C, whereas cardiomyocytes from BAG3\(^{P209L}\)-eGFP mice show disruption of Z-discs in vivo. Measuring the protein exchange rate and mobility of BAG3\(^{P209L}\)-eGFP by using fluorescence recovery after photobleaching (FRAP) indicated an impaired recovery accompanied by a reduced mobile fraction of the protein. In addition, BAG3\(^{P209L}\)-eGFP binding to α-Hsp70, Filamin C and α-HspB8 was impaired as demonstrated by immunoprecipitation studies. Further studies will focus on unravelling the pathomechanisms of the disease.

P188

Biogenesis of large dense core vesicles in the adrenal chromaffin cells of newborn mice

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Chromaffin cells are neuroendocrine cells which secrete catecholamines and peptides and thus play an important role in stress induced responses. The hormones that they release in a Ca\(^{2+}\) dependent manner are stored in Large Dense Core Vesicles (LDCVs) with a diameter of about 120nm. Although adrenal chromaffin cells have served as a model system to study fast Ca\(^{2+}\) dependent exocytosis for many years, very little is known about the biogenesis of LDCVs or their recycling. It has been shown that LDCVs are generated at the level of the Trans Golgi Network (TGN) under the control of clathrometins. Much less is known about sorting of associated and integral membrane components to the membrane of LDCVs. A study suggested that in pheochromocytoma cells (PC12) LDCV membrane proteins converge briefly in a distinct trans-Golgi network sub-compartment before sorting into each respective organelle. Additionally, Synaptobrevin-2, an intrinsic component of the LDCV membrane, appeared to be processed through the plasma membrane before it can be incorporated in the vesicular membrane. These intriguing findings needs to be confirmed in chromaffin cells and substantiated by the study of the biogenesis and processing of other vesicular components such as Celubrevin, Synaptotagmin or VMAT.

P187

Myopathy causing BAG3\(^{P209L}\) protein leads to Z-disc disruption in cardiomyocytes and displays impaired mobility and altered binding characteristics

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Progressive muscle weakness, the key symptom of muscular dystrophies, is caused by mutations in genes encoding Z-disc proteins. The co-chaperone BAG3 (Bcl-2 associated anathogene 3) is strongly expressed in cross striated muscles, co-localizes with Z-discs and plays a key role in the turnover of muscle-proteins (Ulbricht et al., 2012).
References:


P189
Modulation of mineralocorticoid receptor activity by casein kinase II

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Introduction: Classically, the mineralocorticoid receptor (MR), a ligand-dependent transcription factor, contributes to electrolyte homoeostasis and blood pressure control. Additionally, in the reno-cardiovascular system activated MR mediates pathophysiological processes like inflammation, fibrosis and hypertrophy. However, the underlying mechanisms responsible for the switching from physiological to pathological MR actions are incompletely understood. It seems that alterations in the local tissue homeostasis (para-inflammation) as well as posttranslational MR modifications, like phosphorylation, are involved in the switching. Interestingly, casein kinase II (CK2), a ubiquitously expressed tetrameric multifunctional serine/threonine kinase, was identified to be activated under similar pathophysiological conditions as the MR. Until now, a direct link between CK2 and MR, including the precise CK2-induced phosphorylation sites of MR and the consequences for MR properties/functions, have not been analyzed. Therefore, we investigated the possible CK2-MR interaction and their consequences in cell culture.

Methods and Results: Coimmunoprecipitation experiments suggest that CK2 is associated with the MR in a protein complex. In the presence of the CK2 inhibitor TBCA basal as well as aldosterone-induced genomic MR activity measured by reporter-assay was concentration dependently reduced without affecting cell viability. Inhibition of the aldosterone-induced reporter activity by TBCA was more pronounced when using full length MR in comparison to MR<sub>ΔNTD</sub> which lacks the N-terminal domain (NTD), suggesting that potential CK2 responsive serine/threonine residues are located in the NTD. Mechanistically, nuclear MR shuttling was not affected by CK2 inhibition (time-lapse experiments), whereas MR-DNA binding was diminished compared to control conditions (transcription factor binding ELISA). To analyze the site(s) of CK2-induced MR phosphorylation, peptide microarrays were conducted, which showed that CK2 phosphorylates the MR at position serine 111 and 459 in the NTD. To verify the importance of these two CK2 phosphorylated serine residues, site-directed mutagenesis was performed by conversion of serine into alanine (A) or aspartate (D). MR mutants S111A and S459A showed an equal MR protein expression but a reduced basal and aldosterone-induced genomic MR activity compared to wild type MR. This effect was reversed in the MR S459D mutant.

Conclusion: Overall, our data indicate that MR and CK2 are associated in a protein complex whereby CK2-induced MR phosphorylation at e.g. serine 111 and 459. MR phosphorylation by CK2 causes an increased MR-DNA interaction leading to an increased basal as well as aldosterone-induced genomic MR activity. Consequently, CK2 acts as a positive regulator of transcriptional MR activity.

P190
DZNep directly targets putative cancer stem cells in biliary tract cancer cells by inhibition of polycomb repressive complex 2

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Question: Biliary tract cancer (BTC) is a deadly disease with very poor outcome and limited therapeutic options. The multiprotein polycomb repressive complex 2 (PRC2) is a major epigenetic regulator that silences genes by trimethylation of histone 3 at lysine 27. From previous studies it is known that aberrant PRC2 activity is associated with unfavorable clinical features in BTC. Therefore, targeting PRC2 might be a promising therapeutic approach. In this study we investigated the effect of PRC2 inhibitor DZNep using eight BTC cell lines.

Methods: Gene expression analysis was performed using quantitative real time reverse transcription PCR. We used the resazurin cell viability assay to quantify cell line dose- and time dependent cytotoxic effects of DZNep in vitro. The effects of DZNep on potential cancer stem cells were assessed using the functional stem cell characteristics
anchorage-independent growth and aldehyde-dehydrogenase (ALDH) activity.

**Results:** The PRC2 core components eed, ezh2 and suz12 were expressed in all eight BTC cell lines to variable extent. Treatment with different DZNep concentrations (range: 0.04 – 20 µM) caused a cell line- and dose dependent reduction of cell viability, ranging from 10% to 50% for the highest DZNep concentration. Additionally, DZNep affected gene expression in the EGI-1 cell line: mRNA levels of all three PRC2 core components were significantly reduced as well as the mRNA levels of the cyclines ccna2 and ccnb1. In addition, the expression of stem cell-related genes cd24, cd133, epcam and hotair were reduced. We also investigated the effect of DZNep on functional stem cell characteristics, i.e. enhanced ALDH activity that has been recently linked to cancer stem cell properties in BTC. In our study, DZNep reduced the fraction of ALDH-positive cells. Furthermore, DZNep significantly reduced sphere formation by 80%. Interestingly, the effect of DZNep on anchorage-independent growth seems to be stable, since pre-treatment of cells with DZNep and subsequent seeding in a semisolid agar without ongoing DZNep treatment also significantly reduced the ability of the cells to form spheres. Gene expression analysis of ALDH-positive cells as well as of spheres revealed that both of these subpopulations affected by DZNep showed enhanced expression of stem cell markers, suggesting that DZNep directly targets potential cancer stem cells.

**Conclusions:** In summary, we showed that DZNep might be a promising agent for inhibition of PRC2 and for targeting cancer stem cells in BTC.

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**P191**

Treatment of biliary tract cancer cells with polycomb repressive complex 1 inhibitor PTC-209 reduces putative cancer stem cell population and causes cell cycle arrest

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**Question:** The polycomb repressive complex 1 (PRC1) is a multiprotein complex that regulates diverse biological processes by mono-ubiquitinylation of histone 2 at lysine 119. This epigenetic modification causes gene silencing and plays a crucial in various types of cancer including biliary tract cancer (BTC). BTC is a fatal disease associated with high chemo-resistance and poor outcome. Hence, new therapeutic strategies are desperately needed. Very recently, the first small-molecule PRC1 inhibitor called PTC-209 has been developed. In this study, we examined the effect of PTC-209 on eight different BTC cell lines.

**Methods:** Expression of PRC1 core components bmi1 and ring1b was measured with quantitative real time reverse transcription PCR. Cytotoxic effects of PTC-209 were evaluated using resazurin cell viability and caspase activity assays. As for cell cycle analysis we performed propidium iodide staining and flow cytometry. Additionally, we measured aldehyde-dehydrogenase (ALDH) activity as a cancer stem cell (CSC) characteristic.

**Results:** All eight BTC cell lines expressed the PRC1 core components bmi1 and ring1b at differing levels. Using a dilution series of PTC-209 ranging from 0.04 to 20 µM we ascertained that – except for one cell line – the cell viability decreases in a cell line- and dose-dependent manner. To specify this cytotoxic effect we performed growth curve analysis and caspase activity measurements. Although we recognized a slight increase of caspase activity, growth curve analysis showed that the cytotoxic effect of PTC-209 was mainly caused by a growth arrest of the cells. Subsequent cell cycle analysis revealed a decrease of cells in the S and 4N phases, respectively, accompanied by an increase of cells in the 2N phase, suggesting that PTC-209 treatment caused a cell cycle stop at the G1 checkpoint. Additionally we investigated the effect of PTC-209 on ALDH-positive cells, which were recently identified as a subpopulation with CSC properties in BTC, and saw that treatment with PTC-209 significantly reduced the amount of ALDH-positive cells.

**Conclusions:** Up to now, there are no published data available that address the effect of PTC-209 on BTC cells. Our data suggest that PTC-209 not only causes cell cycle arrest of BTC cells, but also might target CSC in BTC, making it an attractive drug for future experiments.

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**P192**

The long non-coding RNA Mantis is highly expressed during vascular regeneration and essential for the VEGF-A-induced angiogenic function

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Although it is widely believed that long non-coding RNAs (lncRNAs) are highly important for gene expression features specific to humans, almost nothing is known about functions of vascular lncRNAs. We sought to identify lncRNAs with...
angiogenic potential regulated in the vascular system. Exon-arrays revealed thousands of IncRNAs to be expressed in human umbilical vein endothelial cells (HUVEC). A specific search for epigenetically suppressed endothelial IncRNAs yielded MANTIS as the most strongly regulated IncRNA. Analysis of its expression in vessels from monkeys subjected to high fat diet revealed a strong induction of the IncRNA Mantis in the regression phase after cessation of the diet. Down-regulation or inhibition of KDM5B histone demethylases strongly induced its expression. By chromatin immunoprecipitation an interaction between KDM5B and the Mantis promoter was confirmed. Molecular characterization revealed Mantis as a mRNA-like IncRNA with nuclear localization. RNAi of Mantis demonstrated an important function of this lncRNA for endothelial biology: Depletion of Mantis attenuated the VEGF-A-induced sprouting and serum-induced proliferation rate. Gene analyses uncovered that Mantis is required for proper responses to VEGF-A: Down-regulation of Mantis decreased the expression of shear stress-induced genes like KLF2 and KLF4 and of Angiotensinase C, an enzyme involved in Angiotensin metabolism. Our data establish Mantis as a novel lncRNA promoting endothelial function. As Mantis is partially suppressed in HUVEC, strategies to unlock its expression, like histone demethylase inhibitor, can be used to improve endothelial cell regenerative capacity.

P193
Function and cell-specific abundance of a new long non-coding RNA, WISP1-OT2
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Long non-coding RNAs (lncRNAs) are RNAs containing more than 200 nucleotides which do not code for proteins. The number of detected IncRNAs is continuously increasing and it is expected that IncRNAs play important roles in the expression of certain genes and thereby are of concern in cancer development. We identified a long non protein coding transcript of Wnt-inducible signaling pathway protein 1 (WISP1), named WISP1-OT2. WISP1-OT2 spans 2922 nucleotides. It consists of the posterior part of the fourth intron (924 nucleotides), the fifth exon containing 300 nucleotides of the coding region and the first 1698 nucleotides of the 3’untranslated region. Its role in physiological and pathophysiological processes and its occurrence in various cells, esp. malignant cells are unclear. WISP1-OT2 was overexpressed in Hek293T-cells and gene expression changes and cell survival were examined. Additionally, the interaction of WISP1-OT2 with proteins was determined and its occurrence in different renal cell carcinoma cell lines was studied. WISP1-OT2 overexpression reduces the expression of COL1A1, FOS, SERPINE and TNF mRNA. Furthermore, WISP1-OT2 overexpressing cells showed reduced extracellular collagen I and III contents. An effect of overexpression on necrosis and apoptosis was not observable. WISP1-OT2 binds besides others mainly to proteins belonging to the nuclear spliceosome C complex and/or to IGF2BP1-associated proteins. The occurrence of WISP1-OT2 was determined in different renal clear cell carcinoma cell lines. Increased expression of WISP1-OT2 was observed in 21 out of 23 renal clear cell carcinoma cell-lines compared to the expression in primary renal proximal tubule cells (RPTEC) whereas the expression of the full-length WISP1 transcript was decreased. In contrast, two different chromophilic renal cell carcinoma cell-lines showed much weaker expression of WISP1-OT2 compared to RPTEC cells.

In conclusion, it could be shown that WISP1-OT2-overexpression influences the expression of some genes but not cell survival. It interacts mainly with nuclear proteins and is found in increased abundance in renal carcinoma cell lines making its occurrence a probable hint for the detection of malignant cell alterations. The question remains whether WISP1-OT2-expression is promoted by cancer or whether it participates in the origin of carcinogenesis.

P194
Glycogen synthase kinase 3 regulates formation of fibroblast growth factor 23
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Question: Glycogen synthase kinase 3 (GSK3), a serine/threonine kinase phosphorylated and inactivated by insulin-dependent Pi3-kinase signaling, has been shown to be involved in renal phosphate handling as well as catecholamine release. Mice expressing GSK3α/β which is not phosphorylatable by PKB/Akt (gsk3wt/wt) are, therefore, phosphaturic. Bone-derived fibroblast growth factor FGF23 is a potent phosphatonin. Here, we explored the role of GSK3 in FGF23 formation.

Methods: Gsk3α/β mice were compared to wild type mice (gsk3wt/wt) before and after treatment with a β-blocker. In addition, mice were studied in metabolic cages. Serum FGF23 and calcitriol were analyzed by ELISA, serum and urinary phosphate and creatinine by a photometric method, and serum and urinary calcium by flame photometry.

Results: Serum FGF23 was significantly higher and calcitriol significantly lower in gsk3α/β mice compared to gsk3wt/wt mice. Despite normocalcemia and hypophosphatemia, renal phosphate and calcium loss were significantly higher in gsk3α/β mice than in gsk3wt/wt mice. β-blocker treatment normalized serum FGF23 and reduced renal calcium loss in gsk3α/β mice as well as renal phosphate wasting in both genotypes.

Conclusions: GSK3-controlled catecholamine release strongly impacts on FGF23 formation in bone resulting in phosphaturia. Hence, β-blocker treatment normalized both, the serum FGF23 level and renal phosphate loss in mice expressing PKB-resistant GSK3.
**P195**

**Dynamics of cyclic adenosine monophosphate signaling in cell lines and primary hippocampal neurons**

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With more than 800 members, G protein-coupled receptors (GPCRs) constitute the largest gene family encoding cell-surface receptors. They are targets for a wide range of drugs such as β-blockers, antihistamines and opiates. Once activated by specific ligands, GPCRs can control and modulate – amongst other pathways – the concentration of the intracellular second messenger cyclic adenosine monophosphate (cAMP). The molecular components contributing to cAMP signaling have been studied extensively, yet little is known about the time constants of individual steps during the signal transduction process. Notably, cAMP signaling is highly compartmentalized within cells. Therefore, studying the spatial and temporal distribution of cAMP signals as well as investigating the kinetics of GPCR-mediated signal transduction is an important issue in current research. Genetically encoded sensors have been developed for monitoring intracellular dynamics of cAMP.

Epac1-camps, one of the first optogenetic sensors, is a versatile tool to detect intracellular changes in cAMP (Nikolaev et al., 2004). However, expression of Epac1-camps in cells leads to a homogeneous distribution in the cytosol. For monitoring of local cAMP signals, we genetically modified the sensor to target the protein to distinct cellular compartments such as the plasma membrane and the nucleus. Based on the purified Epac1-camps proteins we examined biophysically whether the targeting sequences had any effect on the sensor properties. Further characterization of the different Epac1-camps versions was performed in living HEK293 cells. Since cAMP signals play an important role in neuronal function recombinant adeno-associated viruses (rAAVs) were generated allowing expression of the different Epac1-camps variants in primary hippocampal neurons to register and examine GPCR-mediated cAMP signals in these cells.


**P196**

**The C-terminal SH2/SH3 domains of Connexin 43 are pivotal for its migration enhancing activity**

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We have shown before that Connexin 43 (Cx43) expression enhances cell migration and cytoskeletal dynamics (filopodia formation) in a channel-independent way, but requires expression of its the C-terminal cytoplasmic part (aa257-382). In this study we aimed to identify the regions of the C-terminal part of Cx43 which mediate its migration enhancing effect.

Methods: Potential protein binding sites (SH2-, SH3- and PKA-binding domains) in the C-terminal region of Cx43 were mutated by site-directed mutagenesis and mutants (Cx43-SH2 (Y265D; Y267D) or Cx43-SH3 (P274A; P277A; P280A; P283A), Cx43-PKA (S364A; S369G, S373G)) were stably expressed in HeLa cells. Cx43 expressing HeLa cells (Cx43) served as controls. All Cx43-mutant proteins were covalently coupled to GFP. Protein expression and cellular localization were investigated by Western blot analysis and confocal microscopy. Serum-induced (10% NBCS) cell migration after serum starvation was analyzed by time-lapse microscopy using a wound healing assay. The amount of filopodia per single cell was determined after staining of the F-actin with AF546-phalloidin.

Results: The cellular expression and membrane distribution of all of the Cx43-mutant proteins did not differ from Cx43 controls (n=3 independent cell cultures). HeLa cells lacking Cx43-SH2 or -SH3 binding sites showed a significantly reduced migration compared to HeLa Cx43 control, while the expression of Cx43-PKA increased it (accumulated distance: mean ± SEM: Cx43: 317±28 μm; Cx43-SH2: 142±11 μm; Cx43-SH3: 178±13 μm and Cx43-PKA: 403±11 μm; n=4 independent cell cultures; p<0.03). The amount of filopodia was also significantly reduced in Cx43-SH2 or Cx43-SH3 cells whereas it did not differ from HeLa-Cx43 control in HeLa-Cx43-PKA cells (amount of filopodia with a length of >4 μm per single cell; mean ± SEM: Cx43: 13±2; Cx43-SH2: 3±0.4; Cx43-SH3: 5±2 and Cx43-PKA: 10±0.3; n=3 independent cell cultures; p<0.02).

Conclusion: Our results indicate that the SH2 and the SH3 domains which are known binding sites for Src kinase are pivotal in mediating the Cx43-dependent migratory activity whereas binding of PKA might inhibit it.

**P197**

**Translocation and function of myosin 1b and myosin 1c during exocytosis in alveolar type II cells**

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We studied the role of myosin 1b and 1c for exocytosis of surfactant-containing secretory vesicles in primary isolated alveolar type II cells. Class 1 myosins have an actin-binding head and lipid-binding C-terminal domain and function as molecular motors on the interface between actin network and membranes. Different myosin 1 isoforms have different tension sensing properties and likely mediate different functions in the cell. Myosin 1b and 1c are expressed in type II cells and localize to fused secretory vesicles. Live cell microscopy experiments on cells expressing fluorescently coupled myosin 1b and 1c constructs showed that they have different recruitment kinetics to fused vesicles. Myosin 1c translocation closely resembled the translocation of fluorescently labelled pleckstrin homology domain (indicator for PIP2) to the vesicle membrane, whereas myosin 1b translocation was slower. Inhibition of myosin 1c using a
dominant-negative tail domain decreased exocytosis after ATP stimulation, whereas dominant-negative tail domain of myosin 1b had no such effect. We confirmed the inhibitory effect by using myosin 1c inhibitor pentachloropseudilin, which also significantly lowered exocytosis in type II cells. Fused secretory vesicles acquire actin coats, which compress and thereby enable efficient surfactant extrusion. The investigation of the influence of myosin 1c and 1b on these processes showed that inhibition of myosin 1c slowed down vesicle compression. Taken together, myosin 1c and 1b are both present on secretory vesicle membrane after fusion, however myosin 1c appears to be the one with a more prominent function for exocytosis in alveolar type II cells.

**P198**
LncRNA 641 promotes endothelial angiogenic capacity by protecting PAFAH1B1 from miRNA-mediated degradation
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Platelet-activating factor acetylhydrolase 1B (PAFAH1B1) is an enzyme involved in the inactivation of platelet activating factor and in cell motility. We hypothesized that PAFAH1B1 may promote endothelial cell angiogenic capacity and sought for strategies to enhance its expression in human umbilical vein endothelial cells (HUVEC). PAFAH1B1 expression was readily detectable on HUVEC and overexpression of the protein increased endothelial cell angiogenic capacity as determined by spheroid outgrowth assay. Bioinformatic analysis suggested that microRNA-139-5p binds PAFAH1B1 mRNA. Moreover, the highly conserved long non-coding RNA (lncRNA) 641, which has sequence similarities to PAFAH1B1 was also predicted to be an interactor of microRNA-139-5p. On this basis we speculate that lncRNA 641 may act as a microRNA sponge to stabilize PAFAH1B1.

By RNA pulldown experiments, LncRNA 641 was indeed observed to bind to the miRNA-139-5p. Moreover, LncRNA 641 stabilized PAFAH1B1 mRNA. Importantly, overexpression of LncRNA 641, although it is not protein coding, also promoted endothelial cell angiogenic capacity.

We conclude that the LncRNA 641 acts as a sponge for miRNA-139-5p and thus maintains PAFAH1B1 mRNA to promote angiogenesis.

**P199**
ATP induced intracellular calcium signaling in Fam20a deficient proximal tubular cells
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**Question:** Proximal tubular cells play an important role in regulation of calcium homeostasis. It has been shown that mutations in FAM20A are related to nephrocalcinosis and ectopic calcification in other organ systems (enamel renal syndrome). To investigate if Fam20a also influences the intracellular calcium regulation we examined the effect of ATP on intracellular calcium activity in two different Fam20a deficient proximal tubular (PT) cell culture models.

**Methods:** LLC-PK1, cells, an established porcine PT cell line was cultivated on glass cover slips and control cells were compared with siRNA knockdown cells. In addition proximal tubular fragments of 4 Fam20a knockout (KO) and 4 wildtype (WT) mice were isolated, manually sorted, enzymatically digested, seeded and cultivated on glass cover slips (primary culture for 5-6 days). FURA 2-AM microfluorimetry was performed to determine the intracellular calcium activity which is represented as fluorescence ratio at 340nm/380nm excitation wavelength. Cells were stimulated by ATP (30 μM) under normal extracellular calcium concentration (1.3 mmol/l) and under virtually calcium free conditions. 9-10 cells per cover slip were analyzed, averaged and represent one experiment (n).

**Results:** LLC-PK1 knockdown cells (n=17) showed a significantly higher intracellular calcium activity after ATP stimulation than control cells (n=18). Starting from a similar baseline ratio the area under the curve (AUC) of LLC-PK1 knockdown cells was increased by 47%. In contrast primary cultured proximal tubular cells did not display any difference in baseline ratio, AUC or curve shape under ATP stimulation in the presence of physiological calcium (KO: n=6; WT: n=7). Under calcium free conditions the ATP induced calcium transient was characterized by a short and steep increase representing intracellular store release. However, there was no difference in peak height and AUC between both, knockout and wildtype cells (KO n=7; WT n=9).

**Conclusion:** In LLC-PK, cells with Fam20a knockdown a significantly higher ATP response could be observed compared to control cells. This difference was not apparent in PT primary cells from Fam20a KO mice in comparison to WT. Further studies are required to identify the origin of difference between the two cell models with respect to Fam20a function.
Calcium (Ca\textsuperscript{2+}) takes part in numerous physiological functions and influences a plethora of signaling cascades. In differentiated skeletal muscle, intracellular Ca\textsuperscript{2+} concentrations rise dramatically upon membrane depolarization, constituting the link between excitation and contraction (EC). This transient rise in [Ca\textsuperscript{2+}]\textsubscript{i} emerges from Ca\textsuperscript{2+} released mainly by the type 1 ryanodine receptor (RyR1) of the sarcoplasmic reticulum (SR), the dominant Ca\textsuperscript{2+} store in skeletal muscle. However, during skeletal myogenesis, both, RyR1 as well as the inositol 1,4,5-triphosphate receptor (IP3R) are expressed. While the IP3R-mediated, slow Ca\textsuperscript{2+} transients, have been implicated in developmental cell signaling, the potential extra-contractional role(s) of RyR1 remain obscure. Here we use a homozygous mouse RyR1 knockout model (so called “dyspedic”) to investigate the consequences of absence of functional RyR1 expression and, consequently, the lack of RyR1-mediated Ca\textsuperscript{2+} signaling during myogenesis, for skeletal muscle gene expression. While heterozygous mice of the model are not discernible from wt littermates, homozygous dyspedic mice die at birth from asphyxia, since their skeletal muscle does not support EC coupling. Furthermore, they display abnormal spine curvature, subcutaneous hematomas, small limbs, and enlarged neck. Skeletal muscles from front and hind limbs of dyspedic foeti (day E18.5 - E19.5) were subjected to gene microarray analysis, revealing 320 significantly (FDR-adjusted P value < 0.05; expression level positively or negatively regulated by at least 50\%) regulated genes. Interestingly, the expression of two genes, myosin light chain 2 (Myl2) and smoothelin-like 1 (Smtnl1), essential for muscle organization and contraction, was approximately 10 fold down-regulated. Moreover, the expression of multiple members of the Wnt and mitogen-activated kinase (MAPK) signaling pathways were also significantly down-regulated, including the immediate early genes c-Fos, c-Jun and JunD, acting as dimerization partners in the composition of the pleiotropic transcriptional regulator AP-1. Several markers for muscle differentiation like Fgf6, Cd44 and Fhl1 are also negatively regulated. On the contrary, a cluster of genes encoding extracellular matrix proteins is highly up-regulated. Taken together these findings suggest an important, non-contractional role of RyR1 or RyR1-mediated Ca\textsuperscript{2+} signaling in the later steps of muscle development and myocyte differentiation.
P202
Hyperstimulation of vascular nerve varicosities can induce tetrodotoxin- but not nifedipine-resistant contractions in rat mesenteric arteries

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**Question:** Time variant contractions of rat mesenteric arteries have recently been shown by us to be due to cyclic release of noradrenaline from nerve varicosities. Here we ask, if all vasomotor activity observed during stimulation is due to perivascular nerve activity.

**Methods:** Using rat mesenteric arteries (0.3 to 0.5 mm inner diameter) in a wire myograph setup, regular tonic contractions were observed during application of a vasoconstrictor like noradrenaline or high potassium solution.

**Results:** Using tetraethylammoniumchloride (TEA: 10 mM) and electrical field stimulation (EFS: square pulses, 1 to 10 ms, 30 to 70 mA), single and rhythmical contractions could be observed. The direct EFS- induced activity could be regularly inhibited by tetrodotoxin (TTX: 0.1 µM). Longer phasic activity often changed into a tetanic tone in the mesenteric vessels. The application of TTX, however, did not or only partially inhibit these contractions, indicating a functional uncoupling of varicosities from the perivascular nerves. The L-type calcium channel inhibitor Nifedipine was capable to inhibit both the regular and hyperstimulation induced activities.

**Conclusions:** These findings might be important for treatment of vascular events during a pathophysiological or artificial adrenergic stimulation.

P203
Effects of unilateral and bilateral carotid baroreflex stimulation on cardiovagal and blood pressure response in young healthy men

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Left and right carotid baroreflex participate in the control of heart rate (HR) and blood pressure, but the contribution from each side is unclear. Carotid baroreceptors in 10 healthy men aged 20 ± 0.3 years were stimulated by applying subatmospheric pressure (NS) bilaterally and unilaterally on the carotid sinus areas in the neck in 10 mmHg steps from -10 to -60 mmHg. We measured the latency of the response from the onset of neck suction to the maximal RRI interval (RRI) and minimum of systolic blood pressure (SBP) during the suction time and additionally the gain of the response (maximum change in RRI divided by neck chambers pressure (NS) and minimum of change in blood pressure divided by NS pressure). We recorded continuously: arterial blood pressure (BP) by Finapres method, heart rate (HR) by ECG. The right carotid baroreflex stimulation resulted in greater cardiovagal response and a shorter by 63% latency of the maximum RRI response compared to the left side and to bilateral carotid baroreflex response. The effects from left carotid NS stimuli showed longer cardiovagal latency and not significantly greater blood pressure gain and greater SBP decreases compared to the right side and bilateral carotid baroreflex response. The concomitant baroreflex loading resulted in inhibitory interaction with respect to: RRI cardiogaval maximum response and gain, SBP maximum response and SBP gain and the maximum response latencies. We suggest that in several pathological conditions with baroreflex failure e.g.: hypertension, carotid atherosclerosis the presence of abnormalities in the right or left carotid artery might affect baroreflex control of arterial blood pressure and heart rate by the change of the functional balance between right and left side carotid baroreflexes. We suggest that the observed asymmetry might be considered in the interventional management of hypertension by the carotid baroreflex activation.

P204
Bayesian analysis of impedance spectroscopy data and model enhancements

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**Question:** The determination of transendothelial resistance by impedance spectroscopy is a versatile approach to analyze the molecular mechanisms underlying the maintenance of the endothelial barrier function under physiological conditions and to identify determinants of barrier failure under pathophysiological conditions. Impedance spectroscopy measures the absolute value and phase of the complex conductivity of living cells as function of frequency of the applied voltage from Hz to MHz. Interpretation of these measured raw data is accomplished by application of equivalent electronic circuits for all components of the measuring device, i.e. endothelial monolayer on a filter support, cell medium, and electrodes. Most frequently used devices allow direct data analysis with predefined models, but they miss several options: Models of underlying equivalent circuits are limited and cannot be extended easily. In addition, parameters are estimated without information about their uncertainties and possible correlations.

**Methods & Results:** Here absolute values and phase of raw impedance data between 3 Hz to 100 kHz were collected from a commercial device (cellZscope®, nanoAnalytics, Münster) and processed with a newly designed formalism based on so-called Bayesian data analysis. First, simulated test data verified the parameter estimation by this algorithm. Adding phase information further reduced the uncertainties of the calculated parameters. In addition, uncertainty of parameters reduced with an increasing number of data points. In a second step, the algorithm was applied to data from barrier function measurements of human endothelial monolayers (HUVEC). In these experiments monolayer permeability was challenged by thapsigargin (0.1 µM), an inhibitor of the Ca2+-ATPase of the endoplasmic reticulum, which induces a
robust increase in permeability, thrombin (0.2 U/ml), which elicits hyperpermeability via a G-protein coupled receptor (GPCR), and ATP (10 µM), which stabilizes endothelial barrier via GPCR. Under all three conditions tested, Bayesian data analysis was in good accordance with the measured raw impedance data. The inclusion of phase data improved the data fit and reduced the correlations between estimated parameters. In this procedure, precise inclusion of measurement errors of impedance and phase data is of fundamental importance for the valid model parameter estimation and finally for the permeability calculation.

Conclusions: Taken together, we have established and proven a new algorithm based on Bayesian data analysis to estimate model parameters and their uncertainties for impedance data including phase information. This formalism allows further model extensions for impedance data and the coupling with models of molecule flux measurements.

P205
Vasorelaxant effects of rat aorta by leaves of the Moringa tree are endothelium mediated but atropine independent
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Question: Recently, we published that the leaf extract of the Moringa tree exerts motoric effects on gastric smooth muscle. In this study we focused the investigation on possible effects on rat aorta.

Methods: Using conventional organ bath technique, helical strips of aortic smooth muscle were prepared and isometric tension development was measured. 150 mg dry weight of Moringa leaves was added to 2 ml of DMSO. The mixture was agitated for 10 minutes and quantities were stored in the fridge (-20°C).

Results: After equilibration of the tissues, aortic strips were either activated with high potassium solution (20 mM) or Noradrenaline (NA: 1 µM). Using the extract in concentrations between 0.5 and 5 µg per 40 ml (organbath volume), relaxations were observed on both, high potassium or NA preactivations. These relaxations ranged between 20% and 55% of the preactivations. Acetylcholine (ACh: 1 µM) induced comparable relaxations. In endothelium denuded preparations both, Moringa extract and ACh induced no relaxations. In those preparations with endothelium, in the presence of L-NAME 0.1 mM, Moringa had no effect. However, in the presence of atropine (10 µM) the relaxant effect of Moringa was unaltered. Preapplication of ODQ (10 µM) inhibited like L-NAME the vasorelaxant effects of Moringa.

Conclusions: It is concluded that Moringa, used as a food additive, might exert vasorelaxant and blood pressure lowering effects by induction of liberation of EDRF. This induction is unlikely to be initiated by muscarinic receptors.

P206
Adenosine contributes to active hyperemia in the murine skeletal muscle through activation of K_{ATP}-channels
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Active hyperemia is an important physiologic response but the mechanisms involved are still obscure. Adenosine has an important role in active hyperemia in the heart and may also contribute in skeletal muscle. Therefore we investigated the role of adenosine as a dilator in the microcirculation and its interaction with endothelial NO and prostaglandins during active hyperemia in skeletal muscle in mice by intravitral microscopy. Arterioles with a maximal diameter between 20 and 45 µm where investigated before and during stimulation of the motor nerve (100 Hz for 1, 15, 30 or to 60 s with a burst of 0.5 s duration followed by a break of 2.5 s). Adenosine receptors were blocked by DPSPX (1,3-Dipropyl-8-(p-sulfophenyl)xanthine, 10 µM) or K_{ATP}-channels by glibenclamide (Gb, 10 µM). These blockers were applied with or without inhibitors of NO synthase and cyclooxygenase (30 µM N-nitro-L-arginine, 3 µM indomethacin, LN+indo). Stimulation of the skeletal muscle induced an immediate arteriolar dilation whose amplitude and duration increased with prolonged stimulation. For example, 30 s stimulation induced a dilation of 35±7% that was abrogated by blockade of nicotinergic receptors (pancuronium, 2.7 µM). This indicates that the motor nerve induced muscular contractions are required for dilation in this setting. In the presence of DPSPX, a tendency for an attenuation of the dilation (60 s stimulation) was observed (control: 94±3%; DPSPX: 73±10%). Additional blockade of NO synthase and cyclooxygenase reduced the dilations significantly (47±11%). LN+indo alone did not affect dilations (control: 90±6%; LN+indo: 78±12%), however, additional DPSPX reduced dilations (42±18%, p<0.05). Glibenclamide alone was likewise without significant effect (control: 89±4%; Gb: 75±10%), but additional LN+indo blunted the dilations (27±13%). DPSPX or Gb strongly reduced dilations induced by adenosine, whereas acetylcholine dilations remained unaffected indicating specific effects of the blockers.

We conclude that stimulation of the motor nerve and contraction of skeletal muscle in the cremaster muscle provides a convenient model to study active hyperemia. Adenosine induces vasodilation through DPSPX-sensitive receptors which engage K_{ATP}-channels. This adenosine/K_{ATP}-channel mediated mechanism is involved in active hyperemia and is most obvious after blockade of endothelial NO and prostaglandins. Our results suggest that distinct dilator mechanisms can substitute each other during active hyperemia.
**P207**

Multimodal assessment of endothelial dysfunction in *ex vivo* vessels from ApoE/LDLR-/- mice

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The epithelial sodium channel (ENaC) is, besides epithelial tissues, expressed in endothelial cells (ECs). A positive correlation between membrane insertion of endothelial ENaC (ENaC) and the mechanical properties of ECs was found: The more ENaC, the stiffer the EC cortex, a region 50-150 nm beneath the plasma membrane. A stiff cortex in turn results in a decreased secretion of nitric oxide which is the hallmark for endothelial dysfunction. This in turn might lead to cardiovascular disease. Aim of the present work was to establish a combined method to investigate the mechanical properties of ECs from *ex vivo* mouse aortae with an Atomic Force Microscope (AFM) and additionally the biochemical composition with Raman spectroscopy (RS) within the same aortic area. Aortae of either wildtype (WT) or ApoE/LDLR-/- mice were used, whereas the latter represent a reliable model of atherosclerosis and thus severe endothelial dysfunction. *Ex vivo* patches of dissected aortic rings were fixed onto glass dishes and studied with a combined setup of AFM and RS (confocal Raman imaging system; WITec alpha 300). To perform stiffness measurements, a cantilever is lowered onto the EC and indents the membrane upon contact. A laser hits the cantilever and is reflected from its back. The resulting change in reflection of the laser is detected by a photodiode. It reflects the stiffness of the EC and can be transformed into so called force vs. distance curves (FDCs). The cortical stiffness was determined in an area of 12µm x 12µm. Within this area 16 spots were chosen (6 FDCs at each spot). Additionally, “digital pulsed force mode” (dPFM) was used to perform a map of the same area to obtain topography, stiffness values and adhesion. For RS, the scattered light of a laser is detected which is shifted in energy because of interactions between light and matter (Raman scattering). The shift density can be plotted against the frequency, resulting in a specific Raman spectrum of the biochemical compound. We obtained Raman maps of 15µm x 15µm (60 x 60 pixels), with the same center as during AFM measurements. To study whether ENaC is involved in the development of atherosclerosis, the specific ENaC-blocker benzamil (1 µM) was applied. Since ENaC mediates cortical stiffness and thus endothelial dysfunction, differences in the biochemical composition and the mechanical stiffness of ECs between both mouse-types were expected. Taken together, a combined approach of AFM and RS was established and seems useful to identify biochemical and mechanical properties in *ex vivo* artery to study pathways leading to endothelial dysfunction and atherosclerosis including ENaC.

**P208**

Red blood cell aggregation in stented human culprit native coronary arteries and saphenous vein grafts

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**Background:** Stent implantation into atherosclerotic coronary vessels induces the release of particulate debris and soluble vasoactive substances, which affect downstream microvascular function. We have now analyzed, if there are rheological changes of coronary blood during stent implantation, which additionally might impair the microvascular perfusion.

**Methods:** Symptomatic, male patients with stable angina pectoris and a stenosis in their RCA (*n = 26*) or SVG-RCA (*n = 25*) were enrolled. Plaque volume and composition were analyzed using intravascular ultrasound before stent implantation. Coronary aspirate was retrieved during stent implantation with a distal occlusion/aspiration device. RBC aggregation was determined using the erythrocyte adhesiveness/aggregation test by proportion of the RBC free area of the total area spreaded drop of blood quantified under 100x magnification.

**Results:** In patients with comparable plaque volume and composition in RCAs and SVG-RCAs, RBC aggregation was increased in coronary aspirate of RCAs and SVG-RCAs after stent implantation (RCA: 25.7±2.1 vs. 32.2±2.1%; SVG-RCA: 28.9±1.9 vs. 33.3±2.0%, *P < 0.01*), but not different between RCAs and SVG-RCAs.

**Conclusion:** Stent implantation into atherosclerotic coronary arteries leads to increased RBC aggregation in coronary aspirate. Potentially, this is an additional risk component for impaired microvascular perfusion.

**P209**

Maternal vitamin D deficiency and fetal programming


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**Question:** Cardiovascular disease originates partially from environmental and nutritional conditions in early life. Lack of micronutrients such as 25 hydroxy vitamin D3 (25OHD) during pregnancy may be an important treatable causal factor. Here, we studied the effect of maternal 25OHD deficiency on the offspring.

**Methods:** A prospective observational study was performed analysing the association of maternal 25OHD deficiency during pregnancy with birth outcomes considering confounding. To show that vitamin D deficiency may be the cause of
the observed associations, mice were set on either 25OHD-
sufficient or insufficient diets before and during pregnancy. 
Growth, glucose tolerance, and mortality were analysed in 
the F1 generation.

**Results:** The clinical study showed that severe 25OHD de-
ciciency was associated with low birth weight and low ges-
tational age. ANCOVA models indicated that established 
confounding factors such as offspring sex, smoking during 
pregnancy and maternal BMI did not influence the impact 
of 25OHD on birth weight. However, there was a significant 
interaction between 25OHD and gestational age. Maternal 
25OHD deficiency was also independently associated with 
low APGAR scores 5 minutes postpartum. The offspring of 
25OHD deficient mice grew slower after birth, had an im-
paired glucose tolerance shortly after birth and an increased 
mortality during follow-up.

**Conclusions:** Our study demonstrates an association be-
tween maternal 25OHD and offspring birth weight. The effect 
of 25OHD on birth weight seems to be mediated by 
vitamin D controlling gestational age. Our study emphasizes 
the need for novel monitoring and treatment guidelines of 
25OHD during pregnancy to improve cardiovascular out-
come.

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**P210**

Camphenol is a potent inhibitor of vascular smooth muscle 
tone

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**Question:** Camphenol, a Campher containing drug is used 
in dentistry in various countries of the world. It is mainly used 
in favour of its antibacterial properties. The question is, if this 
drug has any vasomotor activity.

**Methods:** Using conventional organ bath technique, helical 
strips of rat aortic smooth muscle, A. maxillaris and longitudinal 
V. porta were prepared and isometric tension development 
was measured.

**Results:** Camphenol was either applied directly 10 to 30 µl 
into the organ bath (30 ml), or dilutions of 1/10 or 1/100 were 
prepared with ethanol. The vessels were preactivated either 
with noradrenaline (NA: 10 µM) or high potassium solution 
(80 mM) or investigated with respect to their spontaneous 
activity. The drug used in the concentration range between 
1/100000 and 1/1000 of its original concentration induced 
vasorelaxations and inhibited spontaneous, NA-induced or 
high potassium solution-induced tone with a half-maximal 
activity. The drug used in the concentration range between 
1/10000 and 1/1000 of its original concentration induced 
vasoconstriction at a dilution of 1/1000. Campher, the main in-
gredient of the drug, again induced vasorelaxation but to a 
lesser extend (30%). Increasing the extracellular Ca²⁺ con-
centration 10-fold, partially reversed the inhibitory effects of 
Camphenol.

**Conclusions:** The results indicate that Camphenol exerts 
very powerful vasorelaxant effects that might be of relevance 
for the systemic blood pressure when used non-diluted 
during surgical interventions. The vasorelaxant activity on all 
types of activations and partial reverse by increase of extracellu-
lar calcium indicate a direct effect on transmembrane-
ous calcium influx by Camphenol.

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**P211**

CFTR is involved in polyphenol-induced swelling of the endo-
theial glycocalyx

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The luminal side of the endothelial cells is lined by a viscous 
liquid surface layer rich in carbohydrates, the endo-
theial glycocalyx (eGC). The prominent constituents of the 
eGC are the proteoglycans, formed by a core protein and 
one or more attached carbohydrate side chains. The eGC is 
a complex interface between blood and tissue with specific 
functions, mainly the protection of the endothelium and the 
regulation of vascular permeability. It is not a static structure 
but, under physiological conditions, in a dynamic equilibri-
um with ongoing changes in the composition, thickness and 
its functional properties. It was shown that polyphenol-rich 
compounds induce swelling of the eGC and polyphenols are 
also known as modulators of specific ion channels. Our goal 
was to investigate whether the chloride channel CFTR(cystic 
fibrosis transmembrane conductance regulator) is involved 
in the polyphenol-induced GC-swelling and thus in the reg-
ulation of vascular function.

**Methods:** The impact of the polyphenols resveratrol, (-)-epi-
catechin, and quercetin on nanomechanics of living endo-
theial GM7373 cells was monitored by nanoindentation. To 
study the role of CFTR, specific Cl--channel inhibitors (NPPB 
and CFTRinh172) were applied.

**Results:** The tested polyphenols lead to eGC-swelling with 
a parallel decrease in cortical stiffness. EGC-swelling, but 
not the change in cortical stiffness, was prevented by inhi-
bition of CFTR. Polyphenol-induced eGC-swelling could be 
mimicked by cytochalasin D, an actin depolymerizing agent.

**Conclusion:** In the vascular endothelium polyphenols soften 
cortical actin and activate CFTR which in turn induces eGC-
swelling. Our findings imply that CFTR plays an important 
role in the maintenance of vascular homeostasis and may 
explain the vasoprotective properties of polyphenols.

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**P212**

ACE-inhibiting potential of isoleucine-tryptophan obtained 
by food hydrolysis and its production by a biotechnical ap-
proach

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As a worldwide problem hypertension is a silent killer be-
cause it occurs over many years without any obvious 
symptoms. The Renin-Angiotensin System is an important 
factor in blood pressure regulation. Thus, inhibition of the
Angiotensin Converting Enzyme (ACE) is one of the most important options used in antihypertensive therapy. There are several pharmaceutical ACE inhibitors, but all of them with side effects, thus their use is limited to therapy, but cannot be extend to prevention. Here natural ACE inhibitors, for example biologically active peptides might be an option. Our group has shown that tryptophan-containing peptides are strong ACE inhibitors. The most potent, isoleucine-trypophan (IW), is sequenced in the whey protein alpha-lactalbumin. Our studies have shown, that the IW significantly decreases blood pressure in spontaneously hypertensive rats. Therefore, IW and other tryptophan-containing peptides are promising natural ACE inhibitors.

The main aims of the project were:

- Search for further natural resources of potent ACE inhibiting peptides by testing tryptophan-containing plant hydrolysates.
- Develop innovative strategies of IW production.

Different plant hydrolysates were tested for their ACE inhibiting potency in vitro using rabbit lung ACE, human plasma ACE and recombinant human ACE. Suitable candidates were consecutively tested also in Human Umbilical Vein Endothelial Cells (HUVEC) and aortic rings of rats. De novo production of IW was done by using recombinant proteins. Therefore a repetitive IW DNA-sequence was designed and ligated into a suitable expression vector. After purification of the IW-containing protein, the repetitive IW-section was separated from the affinity tag. Consecutively, the peptide was extracted from the IW-section by hydrolysis. For the plant hydrolysates soy was the most promising source for ACE inhibiting peptides. However, strongest inhibitory effects were determined with the control hydrolysate of whey protein which has the highest IW concentration. Because IW appears to be the major ACE inhibitor in these hydrolysates, but food resources for IW are limited. It is necessary to develop a new and feasible strategy to produce IW de novo in sufficient amounts. Preliminary experiments show that production of IW using recombinant proteins may be such an approach. The future perspective is to produce IW in sufficient amounts as a supplement for daily nutrition with the aim of baseline ACE inhibition.

P213
The impact of connexin40 in hyperemic response in isolated murine hearts is uncovered after NOS inhibition

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**Question:** Gap junctions are composed of connexins and of these proteins connexin40 (Cx40) is essential for intact transmission of dilatary signals along the vessel wall. A Cx40-dependent mechanism is required for an intact functional hyperemia in murine skeletal muscle, but its role in the coronaries is unknown.

**Methods:** To analyze the role of Cx40 in active and reactive hyperemia in the heart, we perfused wildtyp (WT) and Cx40-deficient hearts (Cx40-/-) paced at 600 bpm using a modified Langendorff setup at constant pressure (100 mmHg) measuring coronary flow via a transonic flow probe. Vascular reactivity of the coronary circulation system was assessed by applying increasing amounts of the vasodilator acetylcholine (ACh). Active hyperemia was provoked by enhancing the heart rate through external pacing (300-800 bpm) and reactive hyperemia was initiated by flow stoppage for up to 30 s. To study the contribution of nitric oxide the protocol was repeated in the presence of 100 µM N(omega)-nitro-L-arginine (LNA) a nonselective nitric oxide synthase inhibitor.

**Results:** Cx40-/- mice exhibit cardiac hypertrophy (175±15 vs. 127±6 mg) due to their hypertension as reported previously. Absolute basal coronary flow was similar in WT and Cx40-/- (2,1±0,2 vs. 2,1±0,3 ml/min) and reduced by LNA only in WT (to 1,55±0,22 ml/min). ACh enhanced flow in both genotypes similarly. This was not affected by LNA in WT, but ACh-induced flow increase was attenuated in Cx40-/- (from 1,1±0,19 to 0,8±0,2 ml/min at the highest dosage). Maximal flow change during reactive hyperemia was similar and decreased by LNA in both genotypes. Flow repayment was also attenuated by LNA in both genotypes, but this attenuation was enhanced in Cx40-/. Thus repayment flow was significantly reduced in Cx40-/- compared to WT after NOS blockade. In both genotypes, flow increased with heart rate indicating preserved active hyperemia. In WT, NOS inhibition increased coronary flow at the highest heart rate (800 bpm) while this effect was not detectable in Cx40-/- . Due to the divergent effect of LNA, coronary flow was reduced at higher heart rates in Cx40-/- compared to WT in the absence of NO.

**Conclusion:** In summary, deficiency of Cx40 did not alter coronary hyperemic responses. The preservation of the coronary dilation in Cx40-/- is most likely due to a compensatory enhanced NO release because impaired dilations are uncovered after NOS inhibition. We conclude that endothelial cell coupling through Cx40 supports hyperemia in the coronary microcirculation at conditions with compromised NO release.

P214
Nitric oxide stabilizes the neurogenic tone of venous smooth muscle

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**Question:** Physiologically, vascular smooth muscle tone is controlled by a repetitive activation of sympathetic varicosities. This study was undertaken to characterize the dynamic function of autonomic varicosities under perpetual neural input and to relate it to vascular signaling pathways.

**Methods:** Electrical field stimulation (EFS) of ring preparations from vena cava was applied and adapted to intensities which led to neuronally mediated contractile responses as was indicated by their sensitivity to tetrodotoxin (10^-6M) or the alpha-receptor antagonist urapidil (10^-5M). Repetitive stimulation protocols were standardized and the presence of a functional endothelium was tested by quantification of
the inhibitory action of acetylcholine on agonist-induced contractions. The temporal pattern of contractile responses was studied under control conditions and in the presence of inhibitors of rho-kinase, inhibitors of calcium storage pathways and after blocking nitric oxide synthase.

**Results:** Repetitive EFS every ten seconds for five minutes led to a tetanization of contractions, giving contractile forces of 48% (SD: 9%; n=10; p<0.05) of a control contraction evoked by 60mM potassium chloride. Under control conditions, the tetanic contractions showed an initial increase in force followed by a slow and minor decline during the first five-minute stimulation (relative decline: 27%; SD 11%; time to 25% force decline: 227s n=9). With repetitive episodes of EFS, this pattern changed: The time to peak was reduced to 52% after five subsequent stimulations (SD: 12%; n=10; p<0.05), the maximum force increased and the peak was then followed by a remarkable force decline (relative decline: 65%; SD: 21%; time to 25% force decline: 80s). Inhibitors of intracellular calcium storage (thapsigargin, caffeine) and to inhibitors of rho-kinase (H-1152) diminished the amplitudes of contractions but failed to change the dynamic pattern changes. Inhibition of NO synthesis with L-NAME 10^-3 mol/l increased the mean contractile responses to EFS to 297% of control levels (SD: 107%; n=9; p<0.05). Furthermore, L-NAME led to a rapid transformation of the tetanization pattern and abolished plateau contractions independently of the number of foregoing EFS trains in favor of sharp peaked tetanic responses.

**Conclusions:** Nitric oxide is released during neuronally mediated contractions of venous smooth muscle. Besides its inhibitory effect on the amplitude of contractile force, endogenously released NO is involved in shaping the temporal characteristics of changes in venous tone as a response to changes in sympathetic activity. Altered endothelial function during critical illness may therefore contribute to maladaptive reactions of the venous capacitance system and thereby promote the appearance of circulatory failure.

**P215**
**Targeting the brain with protein mimicking nanoparticles**

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The brain is the most challenging target for drug delivery due to the fact that the blood brain barrier (BBB) restricts the entry of molecules from the blood stream into the brain parenchyma. Consequently, 100% of large-molecules and 98% of small molecules cannot be delivered from blood to brain in pharmacologically effective concentrations which largely restricts the successful treatment of central nervous system diseases.

In drug delivery, proteins like Serum Albumin are of high clinical interest as endogenous drug transporters. For this reason, we intended to design dendrimer-based protein-like analogs. The herein designed dendrimeric nanoparticles form a shell structure with peripherally localized end-groups, presenting a patched surface. “Patchwork” surfaces of hydrophobic and hydrophilic areas are characteristic for proteins and play an important role in protein-membrane interactions.

In two studies presented here, the potential of a Polyphenylene Dendrimer (PPD3) (a) and Streptavidin-Dendrimers (b) to target brain endothelial cells and to overcome the BBB was investigated.

The PPD3 uptake into endothelial cells was analyzed by confocal live cell imaging and revealed a vesicular localization. The observed PPD3 uptake was quantified by cell cytometry and was shown to be taken up in a concentration-dependent manner. Quantification of live and dead endothelial cells indicated a very low cytotoxicity of PPD3. Additionally, the impact of the this dendrimeric transporter transporter on barrier properties was analyzed by electrical cell impedance sensing (ECIS).

Furthermore, the uptake of Streptavidin-Dendrimers was investigated by immunohistochemistry followed by confocal imaging and demonstrated that nanoparticles target cells of the neurovascular unit by the endosomal pathway. An in vitro co-culture model was used to study a potential transcytotic passage of these nanoparticles. Uptake and transport studies revealed a charge-dependent uptake and delivery of nanoparticles across a BBB in vitro model. In addition the investigated nanoparticles present a high cellular compatibility. Even with nanoparticle concentrations ten times above the effective concentration no cytotoxic side effects or a disruption of the transendothelial resistance could be observed.

In our studies we could show the high potential of dendrimer-based nanoparticles to target brain endothelial cells with high efficiency, low cytotoxicity and minor influence on barrier integrity at relevant concentrations for the first time.

**P216**
**Effects of nanoparticle surface charge on endothelial gene transfer using lentiviral magnetic microbubbles**


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**Question:** Targeted modulation of gene expression is a desired promising concept for therapeutic applications in blood vessels. Site-specific delivery and efficiency of gene transfer remain the two most important challenges. Here, we investigated whether the gene transfer to vascular cells in vitro and in vivo is dependent on the surface charge of
magnetic microbubbles (MMBs) enriched with lentiviruses (LVs). **Methods:** Magnetic moments and velocities of MMBs were assessed by magnetic responsiveness measurement. Coupling of LVs to MMBs was verified by flow cytometry using LVs containing a membrane GFP-fusion protein. Transduction efficiencies of endothelial cells (EC) under static as well as flow conditions were quantified by their resulting expression of GFP or luciferase reporter genes using flow cytometry or bioluminescence measurement, respectively. In vivo, LV-coupled MMBs were targeted to the dorsal skin in mice by combined magnetic field (MF) and ultrasound (US; 1 MHz, 2W/cm², DC50%, 30s) exposure. Reporter gene expression (GFP) in different organs was assessed by qRT-PCR. Residual viral particle amount in body fluids was analyzed with p24 ELISA and reporter gene expression in cell culture. MNP biodistribution was measured by magnetic particle spectrometry.

**Results:** Microbubbles containing negatively charged SO-Mag5 magnetic nanoparticles (MNP) exhibited a higher magnetic moment and velocity towards MF (94.7±0.5 fA/m² and 19.1±0.7 μm/s) compared to positively charged PEI-Mag4 MMB (32.1±0.1 fA/m² and 11.4±0.2 μm/s; n=3), whereas both MMBs were equally effective in binding GFP-coupled LV (n=4). SO-Mag5 MMBs yielded a significantly higher transduction efficiency than PEI-Mag4 MMBs under MF and US application to cultured EC, while both, SO-Mag5 or PEI-Mag4 MMBs, were more efficient than LV alone (31.8±1.7% and 23.2±5.9% vs. 1.8±0.5% GFP-expressing cells, respectively; p<0.05; n=4). In vivo, MF and US exposure resulted in an effective targeting of MMB to vessels of the dorsal skin (n=4) as assessed by vascular GFP expression. No differences between PEI-Mag4 and SO-Mag5 MMB could be observed so far. Intraperitoneal injection of MMB without magnetic targeting resulted in accumulation of MNP mainly in lung and liver (19±4% and 41±9% of administered dose respectively, n=5) 1h after treatment and was reduced to 0.2±0.07% and 0.3±0.09% of administered dose respectively 96h after treatment (n=4). No residual LVs were detected in body fluids 48–72h after LV-MMB application (n=3).

**Conclusion:** Gene transfer via MMBs to EC in vitro could be significantly improved by using negatively charged MNP. A combination of magnetic targeting and application of US yielded site-specific targeting of skin vessels in vivo. Thus, MMB mediated lentiviral gene transfer may represent a valuable tool for therapeutic gene delivery in the future.

**P217**

**Porcupine controls stability and composition of hippocampal AMPA receptors**

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AMPA-type glutamate receptors (AMPARs) are the major excitatory neurotransmitter receptors in the mammalian central nervous system. AMPAR complexes assemble as heterotetramers of the four ion channel pore-forming subunits GluA1-4 and a variety of auxiliary subunits that modulate receptor trafficking and gating. In addition to the well-studied transmembrane AMPA receptor regulatory proteins (TARPs) and cornichons (CNIH-2/3), recent mass spectrometric analyses revealed a surprisingly high number of additional AMPAR-associated proteins. Understanding how this large and diverse protein collection modulates AMPARs is an important challenge.

Here, we use a cell stabilization assay in combination with a FLIPR assay to systematically survey the collection of AMPAR interacting proteins.

We find that besides the well-characterized TARPs, CNIH-2 and GSG1like only porcupine (PORCN) and abhydrolase domain-containing protein 6 (ABHD6), but not representatives from the other seven protein families evaluated, increased levels of co-expressed GluA1. In heterologous cells, both PORCN and ABHD6 retain GluA1 intracellularly. Both PORCN and ABHD6 are selectively enriched in hippocampal neurons. Correspondingly, we find that PORCN controls hippocampal AMPARs, as PORCN knockdown destabilizes AMPAR complexes and thereby diminishes synaptic transmission. PORCN knockdown decreases AMPAR currents and accelerates their decay kinetics, and this is associated with selective depletion of TARP g-8 from AMPAR complexes.

This work defines a novel functional role for PORCN in controlling the level and composition of hippocampal AMPAR complexes.

**P218**

**Cell type-specific differences in activity-dependent postnatal apoptosis in neocortical cultures**

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A wave of neuronal apoptosis occurs in all mammals, ensuring the proper development of the nervous system. This physiological process, which triggers the loss of up to 50%
of the neurons in certain brain areas, happens mostly during the last trimester in humans and the first two postnatal weeks in rodents. While the regulation of early apoptosis is well understood in the peripheral nervous system, the mechanisms underlying developmental cell death in central neurons remain rather elusive.

Here, we studied the developmental profile of neuronal apoptosis using immunocytochemistry in two culture models: dissociated cortical cultures and organotypic cortical slices from neonatal mice. We were particularly interested in a transient neuronal subpopulation, the Cajal-Retzius neurons (CRNs), which disappear by the end of the second postnatal week in vivo. Our current data from two independent cortical culture models suggest that the time course and rate of postnatal apoptosis vary between different neuronal subpopulations in vitro, similarly to what has been described in vivo. Previous work from our group and others suggest that electrical activity is a major regulator of neuronal apoptosis in the brain. Based on this, we first studied how chronic blockade of electrical network by application of tetrodotoxin affects the rate of cell death in two different neuronal populations. Our results show that electrical blockade promotes apoptosis in NeuN-positive cells whereas it rescues CRNs from apoptosis. We also studied the effect of chronic application of gabazine on the electrical activity pattern using multi-electrode recordings, and then investigated its effect on the rate of apoptosis.

In summary, our data demonstrate that our neuronal populations in vitro are dissimilarly affected by modulations of the electrical activity patterns. Thus, distinct mechanisms seem to control cell survival and cell death in the different populations of neurons. To conclude, this in vitro study can provide further insights into the mechanisms underlying the neurodevelopmental activity-dependent apoptosis occurring in rodents as well as in humans.

### P219

**Physiological importance of the extracellular matrix stiffness for Schwann cells studied in primary dorsal root ganglion co-culture**

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Schwann cells are of particular physiological importance for the peripheral nervous system. They form the myelin around the axons of peripheral nerve fibres which enables their physiologically essential high conduction velocity. In addition, Schwann cells are involved in the secretion of the extracellular matrix which mediates numerous Schwann cell functions ultimately regulating the nerve fibre’s activity. There is increasing evidence that diverse cell types are able to feel and respond to the stiffness of their extracellular matrix, and that this ability is of particular physiological importance. Indeed, by using atomic force and confocal scanning microscopy simultaneously we have recently demonstrated that a stiff matrix provides Schwann cells with physiologically crucial mechanical, structural and functional integrity. Matrix stiffness increases along the morphogenesis of peripheral nerve fibres. We went on to hypothesise that Schwann cells are able to feel and respond to changes in the stiffness of their matrix and that this ability is essential for their morphogenesis and physiological functions. To test this hypothesis we established a primary Schwann cells-neurons co-culture from dorsal root ganglions and designed an extracellular matrix with tunable stiffness values. Our preliminary data indicate that Schwann cells feel the stiffness of their matrix and that the stiffness determines their morphology (Fig. 1). In the next step we will study the physiological response of Schwann cell to the matrix stiffness comprehensively.

![Figure 1](image-url)

### P220

**Cooperative action of TNFα, Interleukin-6, and microglia in the generation of spinal hyperexcitability**

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Spinal application of Tumor-Necrosis-Factor α (TNFα) enhances the responses of spinal cord neurons to innocuous and noxious mechanical stimulation of the normal knee joint suggesting a role of spinal TNFα in the generation of spinal sensitization. A similar effect is achieved by the spinal application of IL-6 together with its soluble receptor (IL-6sR), and, interestingly, the effects of TNFα and IL-6/IL-6sR do not add if TNFα and IL-6/IL-6sR are co-administered. Furthermore, the application of TNFα increases the spinal release of IL-6. Finally, TNFα-induced spinal hyperexcitability could be inhibited by neutralizing available IL-6/IL-6sR complexes with soluble gp130 (sgp130). Thus we hypothesize that TNFα and IL-6 cooperate.

We used in-vivo recordings from spinal cord neurons, immunohistochemistry on spinal cord sections, and ELISA to explore the possible interactions of TNFα and IL-6 and the possible recruitment of microglial cells as a possible source for IL-6 and IL-6sR which is required for IL-6 effects on neurons. Application of IL-6 alone to the spinal cord surface did not increase spinal neuronal activity to the same extent as TNFα did. Interestingly, TNFα-induced spinal sensitization was prevented by Minocycline which generally blocks
AMYLOID-BETA-INDUCED CHANGES IN VESICULAR TRANSPORT OF BDNF IN HIPPOCAMPAL NEURONS

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The neurotrophin brain derived neurotrophic factor (BDNF) is an important growth factor in the central nervous system. Deficits in transport of this secretory protein could underlie neurodegenerative diseases. Investigation of disease-related changes in BDNF transport might provide insights into the cellular mechanism underlying e.g., Alzheimer’s disease. In order to analyse the role of BDNF transport in Alzheimer’s disease, live cell imaging of fluorescently labeled BDNF was performed in hippocampal neurons of different Alzheimer’s disease models. BDNF and APP colocalize with low probability in vesicular structures, however the deficits in anterograde as well as retrograde transport of BDNF were mediated by factors released from hippocampal neurons into the extracellular medium. Transport of BDNF was altered at a very early time point after onset of human APP expression or after acute (5min) amyloid-beta(1-42) treatment, while the activity-dependent release of BDNF was unaffected. Taken together, extracellular cleavage products of APP induced rapid changes in anterograde as well as retrograde transport of BDNF-containing vesicles while the release of BDNF was unaffected by transgenic expression of mutated APP.

MUNC13-3 IS INVOLVED IN MEDIATING TIGHT Ca²⁺-INFLUX RELEASE COUPLING DURING POSTNATAL SYNAPSE DEVELOPMENT

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The coupling distance between presynaptic Ca²⁺ channels and the sensor for vesicular transmitter release is a major determinant of synaptic fidelity (1,2). It has been shown that coupling is significantly strengthened during postnatal synapse maturation (3,4). The underlying mechanisms, however, remain largely unclear. We present evidence that Munc13-3, a cytomatrix-protein at the active zone, is involved in narrowing the channel to sensor distance. The coupling distance was probed at immature and mature parallel-fiber (PF) to Purkinje neuron synapses in Munc13-3 mutant (Munc13-3-/-) mice and their wild-type siblings (Munc13-3+/+) making use of the differential effects of the slow exogenous Ca²⁺ buffer EGTA on release in tight vs. loose coupling regimes. If coupling is tight, the slow Ca²⁺ binding kinetics of EGTA do not permit substantial interference with release, while EGTA reduces release, if coupling is loose. We found that EGTA similarly reduced release from immature PF terminals in both genotypes, which is consistent with loose coupling in young mice. Release from mature terminals, however, was affected by EGTA only in Munc13-3-/- but not in Munc13-3+/+, suggesting that the developmental shift from loose to tight coupling requires Munc13-3. Munc13-3 has been implicated in a superpriming process (5), which increases the Ca²⁺-responsiveness of already release-competent vesicles. It remained unclear, however, whether superpriming increased the Ca²⁺-sensitivity of the release machinery (molecular superpriming) or tightened coupling (positional superpriming). Our data indicate that Munc13-3 mediates positional superpriming.

THE PHYSIOLOGICAL ROLE OF AUTOPROTEOLYSIS OF THE ADHESION-GPCR CIRL/LATROPHILIN

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The calcium-independent receptor of a-latrotoxin (CIRL) or latrophilin is an adhesion G protein-coupled receptor (aGPCR), which constitute the second largest class of GPCR with over 30 homologs in mammalian species. CIRL is, like nearly all aGPCR, characterized by the GAIN (GPCR autoproteolysis inducing) domain (Araç et al., 2012), which promotes self-cleavage of the receptor at the GPS (GPCR autoproteolysis site) during biosynthesis into an intracellular C-terminal (CTF) and extracellular N-terminal fragment (NTF; Krasnoperov et al., 1997). The physiological function of this autocatalytic event has remained unclear and puzzling thus far, particularly as the two cleavage fragments stay non-covalently associated at the plasma membrane (Krasnoperov et al., 1997). The genome
The mammalian neurotrophin brain-derived neurotrophic factor (BDNF) is an important modulator of a variety of brain functions. The protein is secreted in an activity-dependent manner and is involved in synaptic plasticity processes. The second messenger cAMP is another important modulator of synaptic plasticity. While involvement of cAMP in the secretion of peptides in neuroendocrine cells as well as neurons has been studied, little is known about the influence of cAMP and its downstream pathways on fusion pore opening of BDNF-containing secretory granules.

In our study, we now have investigated the relevance of the second messenger cAMP and its downstream pathways for the release of BDNF-containing secretory granules in dissociated hippocampal neurons from mice. Neurons were transfected with GFP-tagged BDNF and fusion pore opening of BDNF-containing secretory granules was analysed by combining whole cell patch-clamp recording and live cell imaging. Measurements were performed in the presence of the fluorescent quencher bromphenol blue to investigate fusion pore dynamics of BDNF-containing secretory granules. Fusion pore opening was induced by repetitive backpropagating action potential (bAP) firing upon electrical stimulation. While bAP-induced fusion pore opening of BDNF-containing secretory granules was facilitated by simultaneous electrical stimulation and application of cAMP analog 8-Br-cAMP, fusion pore opening of BDNF-containing granules was inhibited by 8-Br-cAMP application, when delivered before electrical stimulation. This reduction of fusion events was also observed upon application of the selective Epac agonist 8-Br-2-O-Me-cAMP-AM which was applied before electrical stimulation. Simultaneous electrical stimulation and application of the Epac agonist as well as application of the selective PKA agonist 6-Phe-cAMP irrespective of timing of application increased release probability of BDNF-GFP.

Our results show that the cAMP-effectors PKA and Epac can exert opposing effects on bAP-induced BDNF-release from hippocampal neurons and that the negative effect mediated by Epac signaling is critically dependent on timing of the Epac activation.

**References:**


**P224**

**Opposing effects of cAMP-effectors PKA and Epac on activity-dependent BDNF secretion in dissociated hippocampal neurons**

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The mammalian neurotrophin brain-derived neurotrophic factor (BDNF) is an important modulator of a variety of brain functions. The protein is secreted in an activity-dependent manner and is involved in synaptic plasticity processes. The second messenger cAMP is another important modulator of synaptic plasticity. While involvement of cAMP in the secretion of peptides in neuroendocrine cells as well as neurons has been studied, little is known about the influence of cAMP and its downstream pathways on fusion pore opening of BDNF-containing secretory granules. In our study, we now have investigated the relevance of the second messenger cAMP and its downstream pathways for the release of BDNF-containing secretory granules in dissociated hippocampal neurons from mice. Neurons were transfected with GFP-tagged BDNF and fusion pore opening of BDNF-containing secretory granules was analysed by combining whole cell patch-clamp recording and live cell imaging. Measurements were performed in the presence of the fluorescent quencher bromphenol blue to investigate fusion pore dynamics of BDNF-containing secretory granules. Fusion pore opening was induced by repetitive backpropagating action potential (bAP) firing upon electrical stimulation. While bAP-induced fusion pore opening of BDNF-containing secretory granules was facilitated by simultaneous electrical stimulation and application of cAMP analog 8-Br-cAMP, fusion pore opening of BDNF-containing granules was inhibited by 8-Br-cAMP application, when delivered before electrical stimulation. This reduction of fusion events was also observed upon application of the selective Epac agonist 8-Br-2-O-Me-cAMP-AM which was applied before electrical stimulation. Simultaneous electrical stimulation and application of the Epac agonist as well as application of the selective PKA agonist 6-Phe-cAMP irrespective of timing of application increased release probability of BDNF-GFP.

Our results show that the cAMP-effectors PKA and Epac can exert opposing effects on bAP-induced BDNF-release from hippocampal neurons and that the negative effect mediated by Epac signaling is critically dependent on timing of the Epac activation.

**References:**


**P225**

**The thrombomodulin protein C system modulates mitochondrial function and cardiolipin synthesis during experimental autoimmune encephalomyelitis**

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**Question:** Markers of ROS (reactive oxygen species) are increased in MS lesions and in the brain of mice with EAE (experimental autoimmune encephalomyelitis). Inhibition of ROS formation and mitochondrial dysfunction reduces the severity of EAE. Recently, antioxidant properties of the cytoprotective thrombomodulin (TM) - protein C (PC) system have been identified. Of note, the TM-PC system ameliorates EAE through a poorly defined mechanism. We hypothesized that the anticoagulant and cytoprotective TM-PC modules EAE through regulation of mitochondrial function and ROS-formation.

**Methods:** EAE was induced in wild-type (Wt) mice, Thbd<sup>P</sup>P mice (low levels of protein C activation), and double mutant Thbd<sup>P</sup>P x hPC<sup>trans</sup> mice in which levels of aPC were restored (Thbd<sup>P</sup>P x hPC<sup>trans</sup>). EAE scores were determined and tissue samples were collected for morphological and ex vivo expression studies. Further oxygen consumption of mitochondria was analysed by high resolution respirometry. The cardiolipin species/content was determined by HPLC-MS/MS technique.

**Results:** In Thbd<sup>P</sup>P mice the disease course of EAE was markedly aggravated as compared to wt littermates. In Thbd<sup>P</sup>P x hPC<sup>trans</sup> mice the aggravated disease course was partially corrected (~50% reduction). Histological analyses
of spinal cord by Luxol Fast Blue staining demonstrated that increased levels of aPC (Thbd Pro/Pro x hPChigh) reduced myelin loss. These changes correlated with histological evidence of ROS-dependent damage (nitrotyrosine and 8-hydroxy-2-guanosine). Our data of isolated brain mitochondria revealed a decreased respiration rate and a deficiency of complex II in wt EAE mice. In Thbd Pro/P EAE mice the function of the electron transport chain was further decreased by an additional defect in complex I. The cardioliopin (CL) composition and the CL content, the latter reflecting mitochondria mass, were drastically decreased in Thbd Pro/P EAE mice, indicating a quantitative and qualitative mitochondrial defect in Thbd Pro/P mice with EAE. Compensating for the mitochondrial defect by genetically depleting the mitochondrial redox-enzyme p66Shc partially reversed the aggravated disease course of EAE in Thbd Pro/P mice, establishing a causal link between mitochondrial dysfunction, loss of TM-PC function, and severity of EAE.

**Conclusion:** These studies strongly suggest that Thbd-dependent PC activation modulates EAE. Furthermore, aPC ameliorates the disease course of EAE by inhibiting the activity of the redox enzyme p66Shc. These results provide novel insight into the neuroprotective effects of aPC.

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**P226**

**Studying mechanosensitive functions of Adhesion-GPCR in chordotonal organs of Drosophila melanogaster**

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Larval chordotonal neurons are neuronal elements essential for the perception of proprioceptive, touch and sound stimuli rendering them universal mechanosensors. Chordotonal neurons are moniciliated, bipolar nerve cells that display anatomical and physiological properties similar but not identical to inner hair cells of the mammalian inner ear. This presents chordotonal organs as interesting sites to study the molecular machinery involved in the perception of mechanical challenges. Here we have developed a preparation to directly record from chordotonal neurons and correlate the molecular machinery involved in the perception of mechanical signals perceived by chordotonal neurons. Previously, different biological roles of Latrophilins have been reported e.g. planar cell polarity, synaptogenesis and fertility. While the depolarization-induced secretion of peptides in neuroendocrine cells as well as neurons has been studied, little is known about the involvement of cAMP and its downstream pathways on the activity-dependent secretion of BDNF.

The neurotrophin BDNF (brain-derived neurotrophic factor) is an important neuromodulator in the mammalian central nervous system. The protein is secreted in an activity-dependent manner and involved in synaptic plasticity processes. The second messenger cAMP is another important modulator of synaptic plasticity. While the influence of cAMP on the secretion of peptides in neuroendocrine cells as well as neurons has been studied, little is known about the involvement of cAMP and its downstream pathways on the activity-dependent secretion of BDNF.

In this study, the influence of cAMP on BDNF-release was investigated by live cell imaging of hippocampal neurons transfected with GFP-tagged BDNF. Patterns of electrical activity triggering BDNF-release were identified by combining whole cell patch-clamp recordings and time-lapse video microscopy to monitor the release of BDNF-GFP. Repetitive depolarizing stimulation as well as the generation of repetitive action potentials (APs) led to a robust BDNF-release from neuronal processes. While the depolarization-induced secretion was independent of TTX, backpropagating AP-induced release could be blocked by either TTX or the PKA-inhibitor Rp-cAMPS. Unexpectedly, depending on the time...
point of administration of membrane-permeable 8Br-cAMP during or before electrical stimulation, AP-induced BDNF release was either enhanced or abolished. In comparison, BDNF-release induced by elevated extracellular potassium, leading to a long-lasting depolarization of the neurons, was not affected by the administration of cAMP. Altogether, these results indicate that BDNF release can be achieved via physiological levels of electrical stimulation. A positive vs. negative regulation by a cAMP-dependent mechanism is critically dependent on the dosage or the timing of the cAMP-elevation in relation to the electrical stimulation.

P229
Mitogen-activated protein kinase phosphatase-1 and MAP kinases are involved locally in axonal peripheral nerve degeneration

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**Question:** Mitogen-activated protein kinases (MAPKs) control a diverse array of cellular processes and abnormality in MAPKs is implicated in many disorders. MAPKs are inactivated by dual specificity protein phosphatases (DUSPs), such as MKP-1. Play Mitogen-activated protein kinase phosphatase-1 (MKP-1), and MAPKs regulation a role in the injured peripheral nerve (PN) during axonal degeneration?

**Methods:** To induce axonal degeneration, fresh on ice prepared peripheral nerve (PN) in turkey legs was crushed by tweezers followed by 1.5-3 h incubation on ice. Protein expression of MKP-1, MAPKs (ERK/P38/JNK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were investigated by western blotting. The formation and structure of axons in distal, lesion and proximal side was histostained by means of toluidine blue.

**Results:** In the injured PN, MKP-1 protein expression increased significantly in the distal side in comparison to the proximal side. Protein expression of phosphorylated P38 was significantly down regulated in the lesion and the distal side in comparison to the proximal side of injured PN. Although protein expression of total P38 and GAPDH stayed relatively unchanged in all tested parts in the injured nerve (proximal, lesion, and distal side). Toluidinestaining of the injured PN to verify axonal degeneration revealed strongly demyelinated axons in distal (40-50%) and lesion (10-20%) of the injured PN, but was largely unchanged in the proximal side (1-3%) of the injured PN.

**Conclusion:** MKP-1, which regulates MAPKs is involved locally in axonal degeneration and can be used as biomarker for axonal degeneration in PN.

Keywords: axonal degeneration, Nerve injury, Mitogen-activated protein kinase phosphatase-1 (MKP-1), MAP kinases
of BDNF-containing secretory granules and BDNF-GFP content release were strongly affected following CAPS1 knockdown. Furthermore, although the average size and protein content of BDNF-GFP secretory granules remained unchanged after CAPS1 knockdown, the intragranular pH value was significantly higher in hippocampal neurons transfected with CAPS1-shRNA. We also determined the role of CAPS1 in neurotransmitter release by destaining of synaptic boutons loaded with the styryl dye FM®1-43. While density and size of synaptic boutons was unaffected after CAPS1 knockdown, the efficiency of synaptic vesicle release was significantly reduced.

Our results demonstrate that endogenous CAPS1 plays an important role in regulating both, neurotransmitter release from synaptic vesicles as well as protein release from single secretory granules and that CAPS1 has a previously unrecognized additional function in regulating intragranular pH.

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Glia and stem cells

P232
Galanin and its receptors in murine microglia cells
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Microglial cells are monocyte-derived immune cells in the central nervous system. In case of neuronal damage, occurrence of pathogens or neurodegenerative diseases, affected areas exhibit inflammation parallel to the activation of microglia. The neuropeptide galanin is up-regulated during inflammatory processes in the brain. Observations show that Microglia is able to reveal galanin-dependent release of cytokines. Migration of microglia cells is driven by ATP.

In the presented experiments, we characterized galanin receptors using RT-PCR as well as the consequences of galanin on phagocytosis in murine microglia and BV-2 cells. In a murine microglial cell line BV-2, RT-PCR revealed mRNA transcripts for galanin receptor GalR2 and GalR3, but not for GalR1. BV-2 cells exposed to galanin and polysterene beads (microspheres, MB) show increased phagocytosing behaviour. In primary microglial cells, galanin decreases the uptake of MBs compared to basal phagocytic activity. The GalR3 specific antagonist SNAP 37889 induced cell blebbing, indicating cell death. For further investigation of the receptors in phagocytosis we used GalR3 knock-out mice. The comparison between BV-2 cells and primary microglial cells indicates that depending on the galanin receptor expression pattern, galanin either facilitates or suppresses phagocytosis. Furthermore, our observations support the assumption that galanin modulates neuroinflammatory responses by affecting phagocytosis.

P233
NMDA and AMPA receptors mediate somatic calcium influx in human induced pluripotent stem cell-derived neurons
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Introduction: Human induced pluripotent stem cells (hiPSCs) have become a popular in-vitro model in the study of neuropsychiatric disease. A neural induction protocol using triple-SMAD (TGFβ receptor signalling) inhibition was used in order to generate a stable, self-renewable neuronal precursor cell (NPC) line. Terminal differentiation by means of basic fibroblast growth factor (bFGF) withdrawal led to the generation of glutamatergic and GABAergic neurons.

Methods: FURA-2-AM calcium microfluorimetry was performed around day 50 of terminal differentiation. High potassium (140 mM) solution, glutamate and the competitive NMDA and AMPA receptor antagonists, APV and CNQX respectively, were used at different concentrations for pharmacological characterization. Superfusion media containing Mg2+ but excluding glycine were used to rule out any NMDA receptor mediated contribution to the calcium signal. Tyrode’s solution substituted with 10 mM glycine and devoid of Mg2+ was used when applying NMDA and APV.

Results: Within the cellular networks obtained from six independent neuronal differentiations, about 50% of the cells within the networks responded with a significant rise in intracellular calcium upon stimulation with 100 µM glutamate independent of the superfusion media used. When super-fused with Tyrode’s solution containing Mg2+ but no glycine, glutamate-evoked Ca2+ transients were completely blocked by 30 µM CNQX but not affected by APV. Under these conditions, application of 100 µM NMDA had no effect. In a Mg2+-free and glycine substituted solution, however, 100 µM of NMDA led to a significant increase in intracellular calcium
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Comparable to that evoked by glutamate. This calcium influx was completely diminished in the presence of 100 µM APV. Glutamate evoked calcium transients under regular Tyrode superfusion displayed concentration dependence with an EC50 value of about 17 µM (log10 EC50 = -4.78 ± 0.26).

**Conclusions:** Through calcium imaging we could confirm the presence of AMPA and NMDA receptors after 50 days of terminal differentiation. The hiPSC-derived neurons displayed similar functional properties as bona fide cortical neurons.

**P234**
The Wnt adaptor protein ATP6AP2 regulates multiple stages of adult hippocampal neurogenesis

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In the mammalian hippocampus, canonical Wnt signals provided by the microenvironment regulate the differentiation of adult neural stem cells (aNSCs) towards the neuronal lineage. Wnts are part of a complex and diverse set of signaling pathways and the role of Wnt/Planar Cell Polarity (PCP) signaling in adult neurogenesis remains unknown. Using in vitro assays on differentiating aNSCs, we identified a transition of Wnt signaling responsiveness from Wnt/β-Catenin to Wnt/PCP signaling. Retroviral knockdown strategies against ATP6AP2, a recently discovered core protein involved in both signaling pathways, revealed that its dual role is critical for granule cell fate and morphogenesis. We were able to confirm its dual role in neurogenic Wnt signaling in vitro for both canonical Wnt signaling in proliferating aNSCs and non-canonical Wnt signaling in differentiating neuroblasts. While LR6 appeared to be critical for granule cell fate determination, in vivo knockdown of PCP core proteins FZD3 and CELSR1-3 revealed severe maturational defects without changing the identity of newborn granule cells. Furthermore, we found that CELSR1-3 control distinctive aspects of PCP-mediated granule cell morphogenesis with CELSR1 regulating the direction of dendrite initiation sites and CELSR2/3 controlling radial migration and dendritic patterning. The data presented here characterize distinctive roles for Wnt/β-Catenin signaling in granule cell fate determination and Wnt/PCP signaling in controlling the morphological maturation of differentiating neuroblasts.

**Figure 1**

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**P235**
Ouabain modulates release of 4-HHE-modified proteins in microglia cells

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Microglia are antigen-presenting immune cells in the central nervous system (CNS) and build the first line of defence in the brain against injury and infection. Activated microglia release pro-inflammatory cytokines and are able to do an oxidative burst fighting against pathogens. However, by this inflammatory response also healthy cells can be damaged and in addition microglia must handle with increasing oxygen radicals.

During oxidative stress, 4-hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE) are the major lipid peroxidation products of polyunsaturated fatty acids. 4-HNE and 4-HHE react with nucleophilic sites in DNA and proteins, and, accordingly, affects activity of enzymes, transporters, and ion channels.

In this study the generation of the lipid peroxidation product 4-HHE in BV2-microglia was investigated. Microglial cells were incubated with ouabain, an inhibitor of the sodium-potassium- pump. After 24h incubation the 4-HHE amount was documented using an antibody against HHE-protein adducts in combination with a second fluorescent antibody by a confocal laser scanning microscope. The amount of HHE-spots in the cells was quantified.
One neuropathological characteristic of Alzheimer’s disease is an atrophic change in the hippocampus, a brain region known to be important for formation of spatial memories. Despite the typical neurodegeneration during Alzheimer’s disease (AD), recent studies suggest an enhanced neurogenesis in the hippocampus at early stages of AD which is accompanied by an incomplete neuronal differentiation of hippocampal neurons. While the increased neurogenesis is supposed to be mediated by amyloid-β peptide, which is a central peptide in the pathogenesis of AD, the incomplete neuronal differentiation could be mediated by a lack of the neurotrophic factor BDNF.

In order to analyse the interaction between amyloid-β and BDNF signalling in hippocampal neurogenesis, organotypic hippocampal slice cultures (OHSC) were prepared from P5 mice according to Stoppini method. Proliferation of cells in the OHSC was detected using bromodeoxyuridine (BrdU). Identification of stem cells (nestin+), neuroblasts (BrdU+/DCX−), newborn neurons (BrdU+/DCX−/NeuN+) and mature neurons (BrdU+/DCX−/NeuN− and BrdU+/calbindin−) was achieved by immunohistochemical stainings of cryosectioned slices with an antibody directed against the respective protein.

Based on these stainings two neurogenic regions were identified in the OHSC: the dentate gyrus (DG) and the posterior periventricular zone (pPV), a part of the postnatal subventricular zone. Both regions were rich in proliferating cells and showed the presence of immature neurons. Newborn neurons in DG differentiate into excitatory granule neurons while newborn neurons in the pPV differentiate into inhibitory GABAergic neurons, respectively. Treatment of OHSC with amyloid-β oligomers, but not monomers, resulted in an increased number of newborn neurons in the DG, while the number of newborn neurons in the pPV remained unaltered. In the DG, the generation of new neurons induced by amyloid-β treatment was inhibited by simultaneous application of amyloid-β with TrkB-IgGs, a BDNF scavenger, which specifically inhibits BDNF/TrkB signalling. However, amyloid-β oligomers in combination with TrkB-IgGs resulted in an increased number of proliferative cells.

Altogether, we could show that there are two substantially different neurogenic regions in the OHSC. Furthermore, our data indicate that amyloid-β has a proliferative function specifically in the DG. The differentiation and maturation of these newly generated cells is triggered by BDNF-signalling.
**P238**

**Reduced vitamin C uptake causes deficits during the regeneration of peripheral nerves after crush injury in SVCT2+/− mice**

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**Question:** Reduced vitamin C uptake affects myelination in the peripheral nervous system causing hypomyelination of axons accompanied by sensorimotor impairments. We propose that the underlying mechanism is a decreased expression of different collagens followed by a deficient structure of the extracellular matrix. Here we investigated if a reduced availability of vitamin C has negative effects on the regeneration of the sciatic nerve after crush injury.

**Methods:** The sciatic nerve of the right hind leg was compressed for 30 seconds (nerve crush) followed by weekly execution of functional tests like RotaRod, beambalance and gait testing, as well as nerve conduction studies. After 3, 7, 14, 21 and 28 days the animals were perfused and nerves were dissected. For G-Ratio measurements, axon counts and antibody staining (IHC) the nerves were embedded in solid epoxy resin or TissueTek®O.C.T.™. 3 mice of every genotype (SVCT2+/− and wild type) were used to examine the transcription ratio of collagens in quantitative Realtime PCRs (qRT-PCRs).

**Results:** In all functional tests we observed worse performance of SVCT2+/− animals compared to wild type mice after crush injury (p < 0.05). The RotaRod and gait testing showed very significant differences from day 7 after crush. G-Ratio values were significantly higher in SVCT2+/− mice (p < 0.05), indicating deficient remyelination. The total number of axons per mm2 was also diminished in SVCT2+/− mice, pointing towards an impairment in axonal regeneration. In IHC analysis the expression of collagen I and IV was decreased in SVCT2+/− mice (p < 0.05), indicating deficient remyelination. The total number of axons per mm2 was also diminished in SVCT2+/− mice, pointing towards an impairment in axonal regeneration. In IHC analysis the expression of collagen I and IV was decreased in SVCT2+/− mice (p < 0.05), indicating deficient remyelination. The total number of axons per mm2 was also diminished in SVCT2+/− mice, pointing towards an impairment in axonal regeneration.

**Conclusions:** Taken together, these data indicate that a reduced availability of vitamin C has negative effects on the regeneration of peripheral nerves. IHC, qRT-PCR and our preliminary Western Blot results show that the expression of collagens I and IV are affected by reduced vitamin C uptake. Preliminary cell culture experiments show a potential rescue of myelination by exogenous collagen coating. Further experiments are planned to clarify the molecular mechanism of vitamin C-dependent collagen expression and peripheral nerve regeneration.

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**P239**

**A novel model for neurogenesis studies**


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To study neurogenesis is time-consuming as it involves the painstaking counting of double labeled cells.

In order to speed up the assessment of efficacy of a given compound on neurogenesis we developed a fast and easy-to-use test system based on organotypic hippocampal slice cultures from F-344 rats transgenic for the fluorescent reporter protein "Discoma sp. reef coral red fluorescent protein"(DsRed). DsRed expression parallels the expression of doublecortine, a protein typical for neuronal progenitor cells. Concerning neurogenesis detection the fluorescence based method is faster than the commonly used immunohistochemical approaches and more sensitive than previous approaches utilizing PCR.

In parallel to neurogenesis both neuronal damage after ischecmic or toxic insults and cell proliferation can be studied in the same approach on tissue level. Application of the epidermal growth factor (EGF) resulted in an increased DsRed fluorescence, indicating an enhanced neurogenesis, in an intensified Bromodeoxyuridine (BrDU) uptake in the neurogenic zones, indicative for a boosted cell proliferation, but did not alter the propidium iodide (PI) uptake after oxygen-glucose deprivation OGD) and was thus not neuroprotective. The beta blocker Metoprolol neither altered the OGD-induced PI uptake nor did it affect the DsRed fluorescence, whereas the loop diuretic furosemide did not change PI fluorescence but reduced neurogenesis. The cardiac glycoside digoxin also dose-dependently reduced neurogenesis. In this study we present a newly developed medium throughput system that is suited for the pre-selection of candidate compounds from high throughput system for a subsequent laborious testing in vivo, as exemplified by using compounds from the groups of cardiac glycosides, loop diuretics, beta blockers and growth factors. The growth factor EGF was identified as suitable reference compound.

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**P240**

**Igsf9- and CX_CR1-GFP mouse lines for studying pruning of cerebellar climbing fibres**

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Pruning, the elimination of excess synapses, is a phenomenon of pivotal importance for a correct wiring of the central nervous system. Among the model systems that have been established for studying pruning, cerebellar climbing fibre (CF) to Purkinje cell (PC) connections represents a highly...
suitable model because i) pruning occurs during postnatal development, ii) “winner” and “loser” CFs are distinguishable using morphological and electrophysiological [1] parameters, iii) several mutants are available, in which pruning of CFs is deteriorated, and iv) PCs and CFs as well as neighbouring glia cells can be visualized using live-cell imaging [2].

We aim at analysing the cellular events underlying pruning of CFs in two mouse lines, the Igsf9-GFP line [3], in which a subset of CF is labelled by GFP, and the CX3CR1-GFP line [4], in which microglia express GFP.

We find that postnatal development is undisturbed in both lines. Specifically, GFP-labelled CFs show a normal maturation, i.e. they show the “creeper” and “nest” stage as well as pruning of all but one CF. Individual GFP-labelled CFs can easily be targeted with stimulation pipettes, allowing unequivocal identification of CFs independent of their electrophysiological behaviour [5]. Our preliminary data indicate that paired-pulse depression (PPD), the hallmark of CFs [5] is less pronounced in immature CFs, indicating that their release probability is lower than in mature CFs.

GFP-labelled microglia allow for studying the developmental profile of microglia positioning within the cerebellar cortex, for characterising their functional status (amoeboid, ramiﬁed, or activated) and for identifying putative targets of their branches. Analysing perfusion-fixed cerebellar slices, we find a developmentally-regulated distribution of microglia within the cerebellar cortex. Interestingly, microglia target the PC layer with their branches in the very time window of CF pruning, indicating that microglia may either remove cellular debris of pruned CFs or even play a more active role in pruning.


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P241
Energy-dependent functions in astrocytes and mitochondria are impaired in brain from adrenoleukodystrophy (X-ALD)-model animals which have excessive levels of very long-chain fatty acids

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Question: Clinical symptoms in the severe neurodegenerative disease X-ALD (X-linked adrenoleukodystrophy) are caused by defects in the peroxisomal ABCD1 transporter protein. ABCD1 mediates the uptake of free very-long-chain fatty acids (VLCFA) as well as their CoA-esters into peroxisomes. Thus, ABCD1 dysfunctions result in VLCFA accumulation throughout the body of X-ALD patients. VLCFA accumulation is considered as pathogenic X-ALD triggering factor. We have the aim to understand the underlying molecular mechanism of the X-ALD pathogenesis, which is still largely elusive.

Methods: A mouse model for X-ALD, the Abcd1-knockout mouse, developed independently in three laboratories displays biochemical abnormalities, like reduced VLCFA β-oxidation and accumulation of VLCFA (similar to the situation seen in patients). We thank Aurora Pujol (IDIBELL, Barcelona, Spain) for the Abcd1-/- animals and the cooperation. Abcd1-/- animals were used in our studies.

Results: Astrocytes from wild-type and Abcd1-knockout mice, exposed to supraphysiological concentrations of the VLCFA C22:0, C24:0 and C26:0 exhibit multiple impairments of energy metabolism. Thus, long-term exposure to VLCFA induces enhanced ROS generation, strong in situ depolarization of mitochondria. Moreover, the VLCFA-induced intracellular Ca²⁺ response is diminished in Abcd1-/- astrocytes. Finally, VLCFA promote the cell death in astrocytes. Isolated brain mitochondria from Abcd1-/- and wild-type mice respond similarly to VLCFA, indicated as increased ROS generation, impaired oxidative ATP synthesis and diminished Ca²⁺ uptake capacity.Remarkably, even in mitochondria from adult wild-type and one-year-old Abcd1-/- deficient mice, the response of ATP synthesis to VLCFA was comparable. Notable for a possible understanding of the X-ALD pathogenic mechanism is our particularly surprising finding that in Abcd1-/- astrocytes, the capability to revert oxidized pyridine nucleotides to NAD(P)H was severely diminished.

Conclusions: The defective ABCD1 exerts no adaptive pressure on mitochondrial functions. The differences in responses of mitochondria and astrocytes, which we observed for the hydrocarbon chain length of VLCFA suggest that detrimental activities of VLCFA in astrocytes involve defective cellular functions besides those in mitochondria. Astrocytes from Abcd1-/- mice respond more sensitively to VLCFA than those derived from wild-type mice. Finally, we demonstrate that VLCFA increase the vulnerability of Abcd1-/- astrocytes, and we suggest that their antioxidative defense is diminished in Abcd1-/- conditions.
**P242** Glycine uptake via sodium/neutral amino acid transporters activates a swelling-dependent anion conductance in microglial cells

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**Question:** Our previous phagocytosis study on murine brain macrophage-derived BV-2 microglial cells and primary microglial cells suggests that the formation of engulfment pseudopodia and pArticle uptake are Cl−-dependent, depend on cell volume regulatory mechanisms and are related to the activation of a swelling-dependent Cl− conductance (IClswell) [Harl et al., Cell Physiol Biochem, 2013. 31(2-3): p. 319-337]. The following study revealed that extracellular glycine (0.3-5 mM) Na+-dependently stimulates phagocytosis of microglial cells under iso- and hypertonic conditions, causes cell membrane potential depolarization and cell swelling; these could be attributed to glycine uptake via Na+/neutral amino acid transporters (SNAT) [Komm et al., Amino Acids, 2014. 46(8): p. 1907-17]. In the present study we investigated if the glycine-induced cell swelling is sufficient to activate IClswell.

**Methods:** IClswell was measured over time in BV-2 cells under isosmotic conditions in absence and presence of 5 mM extracellular glycine using the whole-cell perforated clamp technique.

**Results:** Cells were kept under isosmotic conditions for 5 minutes and then either superfused with isotonic bath solution containing 5 mM glycine, or further superfused with glycine-free extracellular solutions (control). Cl− currents measured at +100 and -100 mV before solution exchange were +78.5±10.0 and -56.2±4.1 pA (n=16) in cells to be exposed to glycine and +56.0±1.1 and -43.3±5.6 pA (n=6) in control cells (not significant). When superfusing cells with 5 mM glycine, Cl− current activation became evident after ~10 minutes and reached peak amplitudes after ~25 minutes; maximum current amplitudes at +100 and -100 mV were +268.0±74.9 and -104.0±17.5 pA (n=3). In control cells the respective currents were +81.6±3.0 and -47.3±8.3 pA (n=3). The glycine-induced current was significantly higher compared to currents measured before application of glycine as well as compared to currents measured in cells kept in the absence of glycine for the same time. Importantly, current activation showed the same time course as glycine-induced cell swelling. The glycine-induced current displayed characteristics of IClswell previously characterized in BV-2 cells; i.e., outward rectification, time-dependent inactivation at positive holding potentials and sensitivity to the Cl− channel blocker NPPB.

**Conclusions:** Our results show that glycine-induced swelling of BV-2 cells is paralleled by the activation of IClswell. Since this current is essential for phagocytosis, its activation under conditions associated with increased glycine concentration in brain tissue by release from dying cells following trauma or ischemia could be highly relevant for glycine-induced immunomodulation in brain macrophages.

**P243** Glycine modulates membrane potential, cell volume, and phagocytosis in murine microglia

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**Question:** Phagocytes form engulfment pseudopodia at the contact area with their target particle by a process resembling cell volume (CV) regulatory mechanisms. We evaluated whether the osmoregulatory active neutral amino acid glycine, which contributes to CV regulation via activation of sodium-dependent neutral amino acid transporters (SNATs) improves phagocytosis in isotonic and hypertonic conditions in the murine microglial cell line BV-2 and primary microglial cells (pMG).

**Methods:** Phagocytosis of polystyrene microspheres was visualized by scanning electron microscopy (SEM). Gene expression was analyzed by reverse-transcriptase (RT)-PCR. Cell membrane potentials (Vmem) and Na+- currents were measured using the perforated patch clamp technique. CV was measured by flow cytometry.

**Results:** In BV-2 cells and pMG, RT-PCR analysis revealed expression of SNATs (Slc38a1, Slc38a2), but not of GlyRs (Glra1-4). In BV-2 cells, glycine (1 and 5 mM) led to a rapid, Na+-dependent depolarization of Vmem (from -55.8±4.5 to -20.3±3.6 mV in presence of 5 mM glycine; n=14, p+ with choline, and under hypertonic conditions, but not by the GlyR antagonist strychnine or the GlyR agonist taurine. Interestingly, hypertonicity-induced suppression of phagocytosis was rescued by 1 mM glycine.

**Conclusions:** We show that the neutral amino acid glycine stimulates phagocytosis by microglial cells under iso- as well as hypertonic conditions by activation of Na+-coupled neutral amino acid transporters (SNATs).

**P244** “Store-operated” and P2X-induced Ca2+ entry are differentially modulated by the antibiotic minocycline in murine BV2 microglia cells


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Microglial activation is a hallmark of inflammatory, central nervous system diseases and sustains the inflammatory process. Microglia cells are mostly activated by ATP released from necrotic or damaged cells in the parenchyma, activating purinergic P2X receptors that can conduct Ca2+ ions. The P2X4 and P2X7 isotypes have differential sensitivity towards ATP, with P2X7 having a higher activation threshold. Antibiotics, such as minocycline, have been shown to block microglial activation but the precise mechanisms are not known, in particular with regard to cellular Ca2+ homeostasis. Here,
we applied 2-photon Fluo-4 Ca²⁺ fluorescence microscopy to cultured murine BV2 microglia cells to study Ca²⁺ influx in response to ATP, minocycline (MC) and the PLC inhibitor U731. We made the novel finding and present the first demonstration of, “store-operated” Ca²⁺ entry (SOCE) in BV2 microglia cells. Intra-cellular Ca²⁺ levels increased in a dose-dependent manner, with largest effects at 0.1 and 1 mM external ATP. Increases were strictly dependent on extracellular Ca²⁺ levels. At higher (1 mM) ATP levels, 0.1 mM MC specifically blunted ATP-induced Ca²⁺ entry, presumably via block of P2X7. This was not observed at lower ATP doses that seemingly activate P2X4. Phospholipase C inhibition via U731 had no effect on ATP-induced Ca²⁺ entry. Also, SOCE was not blocked by MC or U731. We suggest that minocycline specifically blocks activation of P2X7 and subsequent Ca²⁺ entry, and thus, may be a potent inhibitor of microglia activation. We also show here for the first time that MC does not block “store-operated” Ca²⁺ influx.

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**P245**

**Glycine and noradrenaline may modulate endocytosis in murine microglia**

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Microglia cells are the professional phagocytes of the central nervous system (CNS). They are activated in infected areas in the brain following traumatic insults or pathogen invasion- as well as in neurodegenerative diseases. The biogenic amine noradrenaline is an endogenous local immune-suppressive mediator in the CNS. Activation of beta-2 adrenoreceptor decreases inflammatory processes. Steininger and co-workers observed that the elevation of intracellular cAMP levels, via the beta-agonist, isoproterenol, suppresses phagocytosis in microglial cells (Brain Res. 2011;1407:1-12). Further, glycine enhances phagocytosis in microglial cells via a glycine-receptor-independent mechanism (Amino Acids. 2014; 46(8):1907-17). In the present study, we evaluated whether noradrenaline or glycine modulates endocytosis in microglial cells. Compared to glycine-containing media, glycine-free conditions increased by tendency endocytosis after ten minutes but attenuated endocytosis by tendency after sixty minutes. Endocytosis is sensitive to extracellular sodium ions and chloride. Noradrenaline at the tested concentrations showed only a minor modulatory impact of endocytosis.

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**P246**

**Astrocyte calcium signaling in the Pre-Bötzinger Complex**

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Astrocytes can modulate neuronal activity, however, whether astrocytes of the preBötzinger Complex exhibit respiratory rhythmic fluctuations of their intracellular calcium concentration is still controversial. Here we analyzed calcium-imaging experiments within preBötzinger Complex in rhythmically active medullary slices from TgN(hGFAP-EGFP) mice. A considerable number of astrocyte somata exhibited synchronized low-frequency (< 0.03 Hz) calcium oscillations. After band-pass filtering, signals that in some respiratory cycles preceded the calcium signal of EGFP-negative neurons were observed in 10.2% of astrocytes, indicating a functional coupling between astrocytes and neurons in pre-Bötzinger Complex. A model simulation confirmed that such preinspiratory astrocytic signals can arise from coupled neuronal and astrocytic oscillators, supporting a concept that slow oscillatory changes of astrocytic functions modulate neighboring neuronal activity to add variability in respiratory rhythm.
Energy homoastasis and redox regulation

P247
Astrocytic neuronal lactate shuttle: fuel substrate for maintenance of ionic homeostasis in rat hippocampus

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**Question:** Lactate can serve as a major energy source in state of glucose deprivation like ischemia. However, recent evidence claims that lactate could even be the preferred energy substrate over glucose and it was shown that lactate can maintain excitatory synaptic transmission. Its role as signaling molecule has also been established. The astrocytic neuronal lactate shuttle hypothesis postulates that lactate produced in astrocytes gets shuttled to neurons through monocarboxylate transporter 2 (MCT2) during high energy demand. In this study, we evaluated the effect of lactate shuttle on ionic homeostasis and energy metabolism using the MCT2 inhibitor, 4-α-cyano- hydroxycinnamate (4-CIN) in rat hippocampus.

**Methodology:** Stimulation induced DC coupled field potential and Na\(^+\), Ca\(^{2+}\) or K\(^+\) concentration transients were measured in CA3 pyramidal layer using ion sensitive microelectrodes during 4-CIN application, while oxygen consumption was measured with Clark-style oxygen sensor microelectrodes. NAD(P)H and FAD signals, which are indicators of cytosolic and mitochondrial energetic metabolism, were monitored with live fluorescence imaging.

**Results:** 4-CIN significantly decreased the orthorhomic population spike from 6.78 ± 0.78 to 5.02 ± 0.72 mV and the effect was reversible upon wash out (p<0.01). The antidromic population spike, which results from antidromic stimulation of pyramidal cells, was not altered indicating that similar numbers of axon fibers were recruited. Hence, there is less efficient synaptic transmission during MCT2 inhibition. It also led to prolongation of half decay times of stimulus induced K\(^+\) and Na\(^+\) transients in CA3 stratum pyramidale, indicating reduced transport of K\(^+\) and Na\(^+\). Furthermore, it increased baseline extracellular K\(^+\) concentration by 13.4 ± 1.85% (p<0.001). We did not observe any significant effect on Ca\(^{2+}\) transients in both stratum pyramidale and radiatum. Oxygen consumption also decreased by 20% during 4-CIN application; the drug quenched blue fluorescence rendering the NAD(P)H signal to be masked by an artifact. The sharp overshoot phase of FAD signal decreased but there is an overall baseline shift towards increased oxidation. 4-CIN had no effect on intracellular pH. With prolonged application of 4-CIN; metabolic breakdown was observed.

**Conclusion:** In conclusion, lactate shuttled to neurons partly meets the energy demand for excitatory synaptic transmission and ionic homeostasis. High energy consumption by the Na\(^+\)/K\(^+\) ATPase pump could be the contributing factor for preferential disturbance of Na\(^+\) and K\(^+\) homeostasis. The decreased oxygen consumption and altered FAD signal supports the role of lactate as energy substrate.

P248
The effects of Cyclophiline D knock-out on oxygen consumption of mice brain mitochondria

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Cyclophillin D (CypD) located at the matrix side of mitochondrial inner membrane is as a peptidyl-prolyl cis-trans isomerases involved in the regulation of permeability transition. Its enzymatic activity is controlled by in vivo modulators (Ca\(^{2+}\)) and by CsA. Genetic knock-out of CypD allows to study whether or not CypD or CsA act also on other components of mitochondria. For that purpose we investigated several bioenergetic features of brain mitochondria of CypD knock-out (Ppif\(^{-}\)) mice (KO) and compared the values with those from wild type mice (WT) under conditions of Ca\(^{2+}\) in the presence and absence of CsA.

**Methods:** Mixtures of non-synaptic and synaptic brain mitochondria from 12 weeks old mice were isolated by differential centrifugation. Complex-I and II oxygen consumption (OXPHOS) of mouse brain mitochondria (BM) were measured using a high resolution OROBOROS oxygraph containing a Clark electrode. Mitochondrial membrane potential and extramitochondrial free Ca\(^{2+}\)-accumulation were monitored by fluorescence spectrophotometry using the potential sensitive dye safranine or the Ca\(^{2+}\)-sensitive dye Calcium Green.

**Results:** We detected that under control conditions (un Elevated Ca\(^{2+}\)) missing CypD in BMKO caused significantly increased (i) rates of state 3glu/mal, (ii) RCIglu/mal, (iii) ratios of state 3glu/mal/state 3suc/rot, (iv) membrane potentials (v) rates of initial Ca\(^{2+}\) accumulation and (vi) Ca\(^{2+}\)-thresholds compared to BMWT. In the additional presence of 1 µM CsA all these parameters measured in BMWT and BMKO did not significantly differ from each other since CsA increased (i) only slightly and non-significantly the rate of state 3glu/mal, (ii) the mitochondrial potential, (iii) the rates of initial Ca\(^{2+}\) accumulation, and (iv) the Ca\(^{2+}\)-threshold as compared to control conditions but did not further improve the data of BMKO.

**Discussion:** These effects can be explained by a model in which at missing CypD opening of the PTP is at the lowest level. This level can also be reached in BMWT in the additional presence of CsA. Interestingly CsA reduces the state 3glu/mal of BMWT but not of BMWT pointing to additional unspecific effects of CsA and CypD on other components of probably complex I related components of OXPHOS.
P249
Mitochondria are a target of cardioprotection by ivabradine
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Background: Ivabradine is a bradycardic agent, which inhibits the I_{f}-current in the sinus node. In pigs, ivabradine reduced infarct size even when given only at reperfusion and in the absence of heart rate reduction. Ivabradine’s protective action at reperfusion, when a burst of reactive oxygen species (ROS) is released, suggests it might attenuate ROS formation. Mitochondria are a major source of ROS after ischemia/reperfusion (I/R). Thus, we wanted to analyze a potential effect of ivabradine on mitochondrial function during simulated I/R.

Methods: Mitochondria were isolated from C57Bl/6J mouse hearts and subjected to simulated I/R (6 min hypoxia/3 min reoxygenation). After simulated I/R, mitochondrial complex I activity, mitochondrial ROS concentration and ATP production were quantified.

Results: After simulated I/R, ivabradine did not affect mitochondrial complex I activity, but reduced the ROS concentration (144±12 vs. 122±12 nmol H_{2}O_{2}/100 µg protein; p=0.01), and increased the ATP concentration (148±20 vs. 181±21 µmol/l; p=0.01).

Conclusion: Mitochondria seem to be a target of protection induced by ivabradine by reducing mitochondrial ROS formation and increasing mitochondrial ATP production. Further studies are necessary to identify such target structure/s of ivabradine on mitochondria.

P250
Regulation of microRNA(miRNA) profile by AMPK-dependent kinase (AMPK): a potential link between metabolic disturbances and cardiovascular disease
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The AMPK is a key kinase involved in the regulation of cellular energy homeostasis. We recently demonstrated that the activation of the AMPKα2 (but not AMPKα1) subunit affects the post-transcriptional processing of miR-143/145 via phosphorylation of p53. Moreover, we identified the angiotensin-converting enzyme (ACE) as a target of miR-143/145 in endothelial cells. Since AMPKα2 plays an important role in regulation of renal and cardiac tissue homeostasis, we set out to characterize the specific miRNAs that were affected by AMPKα2 in vivo and to determine their implication in cardiovascular disease development and conditions associated with AMPK dysregulation i.e. diabetes. Thirty five differentially regulated miRNAs were identified using aortae from AMPKα2-/- mice and their wild-type (AMPKα2+/+) littermates. Interestingly, all of them were previously reported to be modified by p53 and 17/35 have also been reported to be regulated by the AMPK activator metformin, suggesting a regulation of the miRNAs by an AMPK-p53-miRNA axis. Since the AMPKα2 plays an important role in renal and cardiac homeostasis, we verified the miRNA profile in kidney and heart (left ventricle) of AMPKα2-/- vs. AMPKα2+/+ animals. Of the miRNAs of interest, 11 were significantly downregulated in both organs: the gene clusters miR-143/145 and miR-15/16, the let-7 family members let-7b-5p, let-7c-5p, let-7g-5p, as well as miR-146a-5p, miR-185-5p, miR-126-3p and miR-199-5p. To determine whether the AMPK-p53-miRNA axis is of pathophysiological relevance, we compared AMPK activation and miRNA levels in hearts and kidneys of streptozotocin(STZ)-induced diabetic mice. In wild-type mice, diabetes induction increased the phosphorylation of the AMPK substrates, p53 and acetyl-coenzyme A carboxylase, and increased the AMPK-regulated miRNAs. Furthermore, the AMPKα2 mediated increase in mature miR-143/145 levels resulted in a significant reduction in renal and cardiac ACE expression. Similar results were obtained in other diabetic mouse models, i.e. fat diet-induced diabetes or in mice carrying a mutation in the insulin 2 gene that causes misfolding of the insulin protein (Ins2Akita mice).

In conclusion, modulation of AMPKα2 activity alters the miRNA expression profile of vascular, cardiac and renal tissue leading to changes in protein (e.g. ACE) expression. This pathway represents a molecular basis for the direct link between metabolic disturbances and cardiovascular disorders.

P251
Detection of H_{2}O_{2} and superoxide using a horseradish peroxidase assay: an electron spin resonance approach
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The quantitative determination of hydrogen peroxide and its primary precursor, the superoxide radical, is a demanding task in studies of physiological systems. These reactive oxygen species (ROS) are usually formed in rather low concentration by several sources such as mitochondria, NADPH-oxidases and other oxidases. They are involved in immune response and signaling pathways but also can have deleterious effects on cell components. Because of their physiological importance, several techniques have been developed for monitoring ROS formation most of which are based on absorptive or fluorescent properties of dyes interacting with one or several different ROS species. Here, we present a method to measure formation of the superoxide radical (O_{2}^{-}) and H_{2}O_{2} simultaneously by electron spin resonance (ESR) technique exploiting its high selectivity for radical states with sufficient life time.

The primary superoxide is effectively detected by oxidaion of cyclic hydroxylamines as spin probes (Dikalov S.I., 2007). The electron transferred from the most efficient cyclic hydroxylamine CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) to O_{2}^{-} renders an ESR active
nitroxide (‘CM), as measure for superoxide production, and H$_2$O$_2$. Recently, we observed that CMH also serves as a substrate for horseradish peroxidase. Careful initial studies with H$_2$O$_2$ showed that two ‘CM radicals are produced per one consumed H$_2$O$_2$, a stoichiometry consistent with the HRP reaction mechanism, allowing to calibrate the setup. In a second step, the O$_2$ and H$_2$O$_2$ formation was monitored in a superoxide flux generating biochemical system (xanthine oxidase/xanthine) in presence or absence of superoxide dismutase or catalase. Finally, the kinetics of O$_2$ and H$_2$O$_2$ formation and their degradation could be determined in a cellular system (monocytes).

The proposed ESR-based method provides a valuable tool for quantitative measurement of O$_2$ and H$_2$O$_2$ either in a separate or simultaneous mode. As compared to other methods it provides an intrinsic amplification factor of three for a single O$_2$$^-$, and is also applicable to opaque cellular systems. It is compared to fluorescent based methods, and advantages and limitations are discussed.

P252
Characterization of NADPH oxidase-dependent ROS detection using triple Nox knockouts
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NADPH oxidase (Nox)-derived reactive oxygen species (ROS) are described to have a wide range of functions in the vasculature. Angiotensin II-mediated Nox2-dependent O$_2$$^-$ mediates endothelial dysfunction. H$_2$O$_2$ produced by Nox4 is protective role during ischemic and inflammatory stress. Determination of Nox-dependent ROS production in intact cells is, however, difficult and direct assays of enzyme activity have not been validated. To address this, we generated mice lacking all vascular NADPH oxidases (Nox1, Nox2 and Nox4) and subjected their organs to the current standard assays.

As expected, the oxidative burst of white blood cells was detected in blood using lucigenin, luminol and LO12 chemiluminescence and was completely absent in 3N$^{-/}$$. Amplex red based assay of kidney preparations demonstrated an attenuated H$_2$O$_2$ production in the triple KO mice (1.87 for WT and 1.4 μmol/mg tissue for 3N$^{-/}$). Moreover, stimulation of cultured WT murine podocytes with TGFβ (24 h) enhanced their peroxide formation by 2.8 fold and also this effect was absent in the triple knockout mice. In contrast, in isolated membrane preparations and homogenates stimulated with NADPH, no differences between tissue from wildtype and triple knockout mice were detected by any of the assays used.

In conclusion, our findings reveal that current assays of Nox activity in membrane fractions do not recapitulate ROS production of intact cells. It is unlikely that the signal detected in these assays reflect Nox activity.

P253
Systemic radical scavenger treatment of a mouse model of Rett syndrome
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Rett syndrome (RTT) is a neurodevelopmental disorder which affects almost exclusively girls. Female Rett patients are born after a normal pregnancy and show an apparently normal development for the first 6-18 months of life. Subsequently, they develop complex symptoms including pronounced cognitive impairment, stereotypic hand movements, loss of already learned skills, epilepsy, and severe breathing disturbances with intermittent systemic hypoxia. Mouse models of Rett syndrome (MeCP2-deficient mice) replicate several of these symptoms. Rating mitochondrial function in male Rett mice (Mecp2$^{-/-}$) revealed intensified mitochondrial respiration, an oxidative burden, and a more vulnerable cellular redox homeostasis. Since these alterations become evident already at presymptomatic stages, they may contribute to the manifestation of typical RTT symptoms and facilitate disease progression. In previous in vitro tests, we confirmed that the free radical scavenger Trolox dampens neuronal hyperexcitability, reinstates synaptic plasticity, ameliorates cellular redox balance, and improves hypoxia tolerance in Mecp2$^{-/-}$ hippocampus. Therefore, we now performed a placebo-controlled blinded systemic treatment of Mecp2$^{-/-}$ mice with Trolox, starting at presymptomatic stages. Trolox-treated Mecp2$^{-/-}$ mice showed a normalization of blood glucose levels. Furthermore, low doses of Trolox improved the hypoxia-susceptibility of Mecp2$^{-/-}$ hippocampus. Yet, against expectation, in vivo treatment did not improve body weight and/or size, motor function, behavior and learning, regularity of breathing or neuronal network function. Rather, the frequent animal handling dampened the phenotypic differences among WT and Mecp2$^{-/-}$ mice, which may have masked potential merits of Trolox. Taken together, these findings demonstrate that radical scavengers may be promising for the treatment of various aspects in Rett syndrome. However, the route of drug administration and the frequency of animal handling are critical parameters to be optimized.

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* Supported by the Cluster of Excellence and DFG Research Center Nanomicroscopy and Molecular Physiology of the Brain (CNMPB), and the International Rett Syndrome foundation (IRSF).
P254
Intact cells vs. membrane: NADPH derived ROS detection in transfected HEK293 cells
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NADPH oxidases (NOX) are important sources of cellular reactive oxygen species (ROS). As NADPH oxidases are membrane-bound complexes, ROS production of particulate fractions in vitro is usually attributed to their activity. However, methods to quantify ROS formation in vitro have not been validated. To do so different ROS-producing enzymes were overexpressed in HEK293 cells. ROS production was subsequently determined in intact cells and in NADPH-stimulated cellular extracts using standard chemiluminescence technique and luminol/HRP, L-O12 and lucigenin, respectively as enhancers.

In intact HEK293 cells transfected with Nox4, as compared to control cells, a massively increased H₂O₂-production was detected by luminol/HRP chemiluminescence. However, NADPH-stimulated luminol/HRP chemiluminescence in membranes from both cells types was identical. Similarly, O₂⁻ production was detected by cells transfected with Nox1 or Nox5 utilizing lucigenin chemiluminescence, whereas in membrane fractions again no difference was detected in the Lucigenin signal. In contrast, transfection with a plasmid coding for endothelial NO synthase increase the Lucigenin chemiluminescence in intact cells and membrane fractions. Our results suggest that NADPH oxidases activity cannot be measured in particulate fractions by the methods currently available for ROS detection. Signal differences in this assay are likely a consequence of activity of other enzymes like eNOS.

P255
Anti-oxidative role of cytoglobin in podocytes: potential association with chronic kidney disease?
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Cytoglobin (CYGB) is a recently discovered member of the mammalian globin family, in addition to hemoglobin and myoglobin. Despite extensive research efforts, its physiological role remains unknown, but possible functions include reactive oxygen species (ROS) detoxification and signaling. Accumulating evidence suggests that ROS play a crucial role in podocyte detachment and/or apoptosis during diabetic nephropathy. To assess the putative anti-oxidative function of CYGB in podocytes, we are using the human podocyte cell line ABB/13, which expresses high endogenous CYGB levels. We generated stable CYGB knock-down and overexpressing cell models and we are currently studying CYGB-dependent gene expression, cell viability and oxidative stress response. CYGB deficient cells showed an increase in cell death and up-regulation of genes involved in apoptosis and redox balance. A ROS sensing dye (H₂-DCF-DA) and redox-sensitive GFP probes were used to demonstrate that CYGB knock-down cells are more sensitive to oxidative stress compared to CYGB overexpressing podocytes. Interestingly, gene array expression analysis of biopsies from CKD patients showed a pronounced CYGB induction in diabetic nephropathy, validated by RT-qPCR in independent nephropathy samples. Moreover, genome-wide association studies (GWAS) revealed that CYGB is potentially implicated in chronic kidney disease (CKD).

In conclusion, data of our study demonstrate for the first time that CYGB (i) is expressed in a human podocyte cell line, (ii) protects podocytes from oxidative stress and cell death, and (iii) may be involved in CKD, particularly in diabetic nephropathy. In parallel to validating our findings in an independent podocyte model, we will study CYGB-dependent transcriptome changes and differentiation processes to gain further functional insight in the molecular mechanism of CYGB in podocytes.

P256
Redox-indicator mice stably expressing genetically-encoded roGFP
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Reactive oxygen species (ROS) and the associated cellular redox alterations contribute to cellular signaling but are also associated with neurodegeneration and neuropathology. For long, ROS/redox imaging was hampered by the lack of reliable and reversibly-responding optical probes. The development of genetically-encoded optical redox sensors deriving from fluorescent proteins has overcome this gap and revolutionized redox imaging on the cellular and subcellular level. A demanding issue of these sensors is, however, the successful delivery of coding DNA to the tissue of interest. This either requires transfection/transduction of cultured preparations or surgical procedures with viral injections into the desired organs of each individual animal. To overcome the limitations of cultured preparations, to circumvent surgical interventions, and to extend redox imaging to more complex and adult preparations, we now established transgenic redox indicator mice. These mouse lines were generated by pronuclear DNA microinjections and stably express the redox sensor roGFP1 (reduction oxidation sensitive green fluorescent protein 1) under the Thy1 promoter in either the neuronal or the muscle line, (ii) protects podocytes from oxidative stress and cell death, and (iii) may be involved in CKD, particularly in diabetic nephropathy. In parallel to validating our findings in an independent podocyte model, we will study CYGB-dependent transcriptome changes and differentiation processes to gain further functional insight in the molecular mechanism of CYGB in podocytes.

roGFP

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Reactive oxygen species (ROS) and the associated cellular redox alterations contribute to cellular signaling but are also associated with neurodegeneration and neuropathology. For long, ROS/redox imaging was hampered by the lack of reliable and reversibly-responding optical probes. The development of genetically-encoded optical redox sensors deriving from fluorescent proteins has overcome this gap and revolutionized redox imaging on the cellular and subcellular level. A demanding issue of these sensors is, however, the successful delivery of coding DNA to the tissue of interest. This either requires transfection/transduction of cultured preparations or surgical procedures with viral injections into the desired organs of each individual animal. To overcome the limitations of cultured preparations, to circumvent surgical interventions, and to extend redox imaging to more complex and adult preparations, we now established transgenic redox indicator mice. These mouse lines were generated by pronuclear DNA microinjections and stably express the redox sensor roGFP1 (reduction oxidation sensitive green fluorescent protein 1) under the Thy1 promoter in either the neuronal or the muscle line, (ii) protects podocytes from oxidative stress and cell death, and (iii) may be involved in CKD, particularly in diabetic nephropathy. In parallel to validating our findings in an independent podocyte model, we will study CYGB-dependent transcriptome changes and differentiation processes to gain further functional insight in the molecular mechanism of CYGB in podocytes.
As H$_2$O$_2$ can act as endothelium-mediated vasodilator we produces predominately H$_2$O$_2$ and as it has constitutive activity. Nox4 is different to those Nox enzymes at it pro-

by producing NO-scavenging superoxide, promote athero-

sis in ApoE knockout mice

P257

**NADPH Oxidase 4 attenuates the development of atheroscle-

rosis in ApoE knockout mice**

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Increased formation of reactive oxygen species (ROS) is thought to contribute to arteriosclerosis development. NADPH oxidases of the Nox family are important sources of ROS. It was previously reported that the Nox1 and Nox2, by producing NO-scavenging superoxide, promote athero-

sclerosis. Nox4 is different to those Nox enzymes at it pro-

duces predominately H$_2$O$_2$ and as it has constitutive activity. As H$_2$O$_2$ can act as endothelium-mediated vasodilator we hypothesized that Nox4 may delay arteriosclerosis develop-

ment.

Spontaneous atherosclerosis-development was determined in tamoxifen-inducible Nox4 conditional knockout mice crossed into ApoE -/- mice under normal chow. Accelerated atherosclerosis was determined in the same line in the partial carotid artery ligation model during high fat Western diet treatment. In the partial ligated carotid artery model, micro-

CT revealed a more prominent lumen loss in Nox4 KO mice as compared to Cre negative control animals (p<0.05). By histology, an increased plaque burden was observed in Nox4 KO animals. Similarly, in the long term study of spontaneous atherosclerosis-development, planimetry revealed a signifi-
cant higher aortic plaque burden. Moreover, plaque collagen content was increased after Nox4 knockout. Mechanistical-

ly, deletion of Nox4 induced endothelial cell activation. This resulted in an increase in molecule expression as observed in lung endothelial cells from Nox4 -/- mice. Ac-
corodingly, monocyte adhesion to endothelial cells of Nox4 -/- mice was increased as compared to wild type controls. Thus, the H$_2$O$_2$, producing NADPH oxidase Nox4 is an en-
dogenous anti-atherosclerotic enzyme. Inhibition of Nox4 in humans may accelerate atherosclerosis development.

P258

**Glutathione and mitochondria determine acute defense re-

sponses and adaptation to cadmium-induced oxidative stress and toxicity of the kidney proximal tubule in vitro and in vivo**

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Cadmium (Cd$^{2+}$) induces oxidative stress that ultimately defines cell fate and pathology. Mitochondria are the main energy-producing organelles in mammalian cells, but they also have a central role in formation of reactive oxygen spe-
cies, cell injury and death signaling. As the kidney proximal tubule (PT) is the major target in Cd$^{2+}$ toxicity, the roles of the oxidative signature and mitochondrial function and bio-
genesis in Cd$^{2+}$-related stress outcomes were investigated in vitro in cultured rat kidney proximal tubule cells (PTCs) (WKPT-0293 Cl.2) for acute Cd$^{2+}$ toxicity (1-30µM, 24h) and in vivo in Fischer 344 rats for sub-chronic Cd$^{2+}$ toxicity (1 mg/kg CdCl$_2$ subcutaneously, 13 days). Whereas 30 µM Cd$^{2+}$ caused ~50% decrease in cell viability, apoptosis peaked at 10 µM Cd$^{2+}$ in PTCs. A steep dose-dependent decline in reduced glutathione (GSH) content and an increase of the oxidized glutathione (GSSG)/GSH ratio occurred after acute exposure. Quantitative PCR analyses evidenced increased antioxidative enzymes (Sod1, Gclc, Gclm), proapoptotic Bax, metallothioneins 1A/2A, and decreased antiapoptotic proteins (Bcl-xL, Bcl-w). The positive regulator of mito-

chondrial biogenesis Pparg and mitochondrial DNA were increased and cellular ATP remained unaffected with Cd$^{2+}$ (1-10 µM). In vivo, active caspase-3, and hence apoptosis, was detected in the kidney cortex of Cd$^{2+}$-treated rats af-
ter FLIVO injection together with an increase in Bax mRNA. However, antiapoptotic genes (Bcl-2, Bcl-xL, Bcl-w) were also upregulated. Both GSSG and GSH increased with sub-

chronic Cd$^{2+}$ exposure with no change in GSSG/GSH ratio and augmented expression of antioxidative enzymes (Gpx4, Prdx2). Mitochondrial DNA, mitofusin 2 and Ppara were aug-

mented indicating enhanced mitochondrial biogenesis and fusion. Hence these results demonstrate a clear involvement of mitochondrial biogenesis and function in acute defense against oxidative stress induced by Cd$^{2+}$ in renal PTCs as well as in adaptive processes associated with chronic renal Cd$^{2+}$ toxicity.
Among the classic NADPH oxidases, Nox4 is unique as it is not localized on the plasma membrane, has constitutive activity and predominantly generates H₂O₂. We hypothesise that these features and specific signaling properties of Nox4 are consequences of so far unidentified Nox4-interacting proteins. For identification of Nox4-containing macromolecular complexes, we used proteomic approaches based on labeled (SILAC-Coimmunoprecipitation) or label-free (complexome profiling) quantification. The latter combining blue native electrophoresis (BNE) and quantitative mass spectrometry. Experiments were performed from subcellular fractions after variable solubilisation techniques with mild, non-ionic detergents. After solubilisation with the mild detergents Digitonin, Nox4 was identified in complexes of sizes more than one megadalton. These protein complexes could be found in HEK293 cells overexpressing human Nox4 as well as in mouse kidney, which has high endogenous Nox4 level. Less mild detergents like Triton X-100 or Dodecyl-β-d-maltoside disrupted these complexes, explaining why Nox4-interacting proteins cannot be recovered well by immunoprecipitations from such extracts. By SILAC-based CoIP, from Digitonin-soluble cell fraction or membranes, multiple Nox4-interacting candidates could be observed. Among them the ER-resident Chaperon Calnexin was recovered. Calnexin is involved in many important physiological functions like protein quality control along the secretory pathway, ER stress response and Calcium dynamics within the cell. The Calnexin-Nox4 interaction could be confirmed by reverse CoIP, proximity ligation assay and complexome profiling. Our results suggest that endogenous Nox4 forms macromolecular complexes with Calnexin which may locally control the redox-dependent protein folding.

**P259**

**NADPH oxidase Nox4 forms intracellular macromolecular complexes with Calnexin**

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Among the classic NADPH oxidases, Nox4 is unique as it is not localized on the plasma membrane, has constitutive activity and predominantly generates H₂O₂. We hypothesise that these features and specific signaling properties of Nox4 are consequences of so far unidentified Nox4-interacting proteins.

For identification of Nox4-containing macromolecular complexes, we used proteomic approaches based on labeled (SILAC-Coimmunoprecipitation) or label-free (complexome profiling) quantification. The latter combining blue native electrophoresis (BNE) and quantitative mass spectrometry. Experiments were performed from subcellular fractions after variable solubilisation techniques with mild, non-ionic detergents.

After solubilisation with the mild detergents Digitonin, Nox4 was identified in complexes of sizes more than one megadalton. These protein complexes could be found in HEK293 cells overexpressing human Nox4 as well as in mouse kidney, which has high endogenous Nox4 level. Less mild detergents like Triton X-100 or Dodecyl-β-d-maltoside disrupted these complexes, explaining why Nox4-interacting proteins cannot be recovered well by immunoprecipitations from such extracts. By SILAC-based CoIP, from Digitonin-soluble cell fraction or membranes, multiple Nox4-interacting candidates could be observed. Among them the ER-resident Chaperon Calnexin was recovered. Calnexin is involved in many important physiological functions like protein quality control along the secretory pathway, ER stress response and Calcium dynamics within the cell. The Calnexin-Nox4 interaction could be confirmed by reverse CoIP, proximity ligation assay and complexome profiling. Our results suggest that endogenous Nox4 forms macromolecular complexes with Calnexin which may locally control the redox-dependent protein folding.

**P260**

**Maintenance of endothelial NO release after inhibition of NADPH oxidase 4**

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The NADPH oxidase isoform NOX4 mainly produces H₂O₂. Previously we could show, that NOX4 is the major endothelial NOX isoform and constitutively active. Regulation of NOX4 and formation of reactive oxygen species are involved in NO release. The transcription factor NRF2 is a key mediator of cellular adaptation to redox stress. Therefore, regulation of NOX4 on the transcriptional level by NRF2 might be directly linked to NO release and endothelial function. Besides endothelial nitric oxide synthase (eNOS), a role of neuronal nitric oxide synthase (nNOS) in endothelial NO and H₂O₂ release has been proposed. In this study, endothelial cells (HUVEC) were constantly exposed to high laminar shear stress (24 h, 30 dyn/cm²). Application of shear stress stimulated NO formation and induced elongation of endothelial cells in the direction of flow. Lentiviral overexpression of NOX4 strongly increased endothelial H₂O₂ release, while downregulation using shNOX4 decreased H₂O₂ release. Furthermore, application of shear stress caused downregulation of NOX4 as well as upregulation of eNOS and antioxidative response via NRF2 and its target genes NQO-1 and HMOX-1. H₂O₂ can affect NO release by eNOS. We could show that downregulation of NOX4 leads to upregulation of eNOS mRNA and protein expression accompanied by attenuation of NRF2 pathway under flow conditions. Determination of NO release confirmed these results. This supports a compensatory mechanism maintaining a stable NO release after NOX4 inhibition in response to flow. Attenuation of NRF2 by shNRF2 inhibited shear stress-dependent induction of Nrf2 mRNA and protein expression and its target genes. In addition, shNRF2 enhanced the shear stress-dependent downregulation of NOX4. Finally, we detected a stable mRNA and protein expression of nNOS in endothelial cells, which was unaffected by flow. Interestingly, downregulation of NOX4 resulted in induction of nNOS expression.

In conclusion, our data suggest an important role of H₂O₂ production by NADPH oxidase 4 in maintaining NO release and endothelial function in response to flow. Inactivation of NADPH oxidase 4 attenuates NRF2 pathway and increases neuronal nitric oxide synthase as compensatory mechanisms.

**P261**

**Expression of the NADPH oxidase Nox4 during stem cell differentiation**

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Reactive oxygen species (ROS) contribute to cellular signaling and cell fate decision. ROS production is mediated by mitochondria and NADPH oxidases of the Nox family. Given that stem cells reside in a low ROS state, we hypothesize that Nox expression is altered in the course of stem cell differentiation. As a model system, murine induced pluripotent stem cells (mPSC) were produced by lentiviral transduction of murine embryonic fibroblasts (MEF) with the Yamanaka factors. Cells were subsequently clonally expanded and differentiated. Nox expression was studied at different stages by qRT-PCR. Induction of stem cell properties was associated with a strong downregulation of the NADPH oxidase Nox4 whereas Nox2 was upregulated. Nox1 expression was not detectable in any of the experiments. During spontaneous differentiation, the redox-dependent adaption of stem cells, the expression of NADPH oxidases was downregulated.
differentiation of miPSC by withdrawal of leukemia inhibiting factor (LIF) and feeder cells, Nox4 expression increased together with differentiation markers for all three germ lines. Nox2 expression levels decreased very early during differentiation and remained low throughout the whole process. Subsequently, miPSC were differentiated into endothelial cells by applying BMP-4 and VEGF. Also during this differentiation protocol Nox4 was induced and in parallel the expression of endothelial differentiation markers such as eNOS and VEGFR2. Nox2 however, was down-regulated from the beginning of the differentiation process and returned to the initial value towards the end of the differentiation in conclusion, Nox4 is a marker for mesenchymal differentiation. Future studies will have to show whether Nox4-derived ROS also contribute to miPSC differentiation.

P262
Age related changes in the contribution of eNOS, nNOS and reactive oxygen species (ROS) to regulation of vascular tone of murine basilar arteries

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Question: The basilar artery (BA) supplies the brain stem and, hence, its occlusion or rupture carry a high risk of severe disability and mortality. Although less studied than the arteries that supply the neocortex, evidence from larger animals is emerging that stimulation of perivascular neurons release vasoactive transmitters including NO. Here, we investigated how old age affects NO mediated regulation of murine BA tone with particular focus on nNOS.

Methods: BA from 8-10 week (j-BA) and >21 month old (o-BA) mice were isometrically mounted in a wire myograph. Media thickness was assessed by staining for F-actin and expression of eNOS and nNOS by quantitative RT-PCR. Electric field stimulation (EFS, 0.1 ms pulses, 10 Hz) was performed with two platinum electrodes placed in parallel to the vessels.

Results: Passive length tension relations did not differ between o-BA and j-BA. Endothelial denudation (ED) increased basal tone (BT) in j-BA and o-BA by respectively 5% and 28% of maximal force, F_max. The pan-NOS inhibitor, L-NAME (100 µM) increased BT by 10% (j-BA) and 23% (o-BA) whereas inhibition of nNOS (1 µM L-NPA) increased BT by 10% in both ages. Coincident with these different mechanical responses, eNOS expression was 2.5-fold higher in o-BA whereas nNOS expression was similar in both ages. The increased BT in o-BA was associated with a higher expression of calcium sensing receptors. CaSR improve cardiac performance. Similar to activation of ET(A) receptors in co-presence of PD142893, down-regulation of CaSR by siRNA reduced the contractile responsiveness within 24 h. Moreover, ET caused a concentration-dependent increase in cell width that is typically found for pro-hypertrophic stimuli. Cell shortening was not modified.

P263
The role of cardiac PD142893-sensitive ET(B1) receptors in cardiac adaptation to high endothelin levels

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Endothelin (ET) triggers cardiac hypertrophy together with the sympathetic nervous system and the renin angiotensin system. The peptide hormone increases load free cell shortening in an acute way but does not induce a loss of function in chronic conditions. This study tries to clarify how endothelin is inducing cardiac hypertrophy (increasing cell sizes) but also maintaining cellular function. Isolated and cultured adult rat ventricular cardiomyocytes were used and exposed to endothelin (1–100 nmol/l) for 24 h. Cell shortening, cell size, and expression of calcium sensing receptors were analyzed thereafter. Endothelin caused a concentration-dependent increase in cell width that is typically found for pro-hypertrophic stimuli. Cell shortening was not modified. However, co-inhibition of ET(B1)-receptors by PD142893 caused a concentration-dependent loss of function. The data suggests that ET(B1)-receptor activation counterbalances the negative effect of cardiac hypertrophy caused by activation of ET(A) receptors. ET increased the expression of calcium-sensing receptor (CaSR). CaSR improve cardiac performance. Similar to activation of ET(A) receptors in co-presence of PD142893, down-regulation of CaSR by siRNA reduced the contractile responsiveness within 24 h. Moreover, ET caused a concentration-dependent up-regulation of CaSR (RNA and protein) and this was finally blocked by PD142893. Finally, pharmacological inhibition of
CaSR caused a negative effect of ET on cell shortening. In conclusion, the data suggest that ET causes a ET(A)-driven mal-adaptive cardiac hypertrophy that can be converted into an adaptive type of hypertrophy by activation of ET(B1) receptors. The conclusion is based on the findings that inhibition of ET(B1) receptors and of CaSR induced a negative contractile responsiveness of cardiac myocytes to electric stimulation. Furthermore, it is based on findings that an inhibition of CaSR reduces cell shortening.

P264
The role of the atypical protein Kinase C iota for cardiac function: an in vivo study using cardiac specific gene transfer
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In contrast to the conventional and novel Protein Kinase C subfamilies, whose role in cardiac function is rather well established, very little is known about the function of aPKCs in the heart. We therefore set out to study the role of PKC(iota) (also referred to as PKC(lambda) in humans) in the heart. Because a specific pharmacology for PKC(iota) is virtually absent, we employed the approach of expressing constitutively active (CA) and dominant negative (DN) mutants of PKC(iota) in the heart. They were fused to a red fluorescence protein (TagRFPT) to simultaneously visualize expression and sole TagRFPT expression served as an internal control. We use an adeno-associated virus system with cardiac specificity for transfection (Serotype 9 and cardiac specific promotor). Injection was performed into the left jugular vein at day 21 after birth. Expression and subcellular distribution was probed 7 weeks after injection by direct fluorescent or using TagRFPT-specific primary antibodies in immunofluorescence experiments and Western-blots. The effect of expression was investigated employing echocardiography 2, 4 and 7 weeks after injection. To additionally reveal the putative role of PKC(iota) in cardiac hypertrophy we performed transaortic constriction (TAC) experiments in mice injected with the three virus variants (PKC(iota)-DN, PKC(iota)-CA and TagRFPT). TAC was imposed 5 weeks after virus injection (8 weeks after birth) and the effect of expression was studied in echocardiography experiments just prior to TAC and 2 weeks after TAC. Western-blot data obtained from hearts 7 weeks after injection revealed that overall, PKC(iota)-mutant expression equaled the expression of endogenous PKC(iota), thus we only imposed very mild PKC(iota) overexpression. Investigation of the transduction efficiency revealed that by our injection regime more than 75% of all myocytes (ventricular and atrial) displayed readily detectable, PKC(iota)-mutant specific fluorescent. Employing echocardiography, we found a significant increase in left ventricular weight in the DN-PKC(iota)-mice, also after normalizing to the body weight. In addition, functional parameters were also significantly changed in the DN group, particularly deceleration time (increased) and deceleration speed (decreased), as well as an abnormal filling pattern of the mitral valve. Underlined by single cell experiments using patch-clamp-technique, we suppose that PKC(iota) plays an crucial role in cardiac myocyte function and malfunction, such as cardiac hypertrophy.

P265
Cytosolic renin exerts anti-necrotic effects under ischemic conditions in an angiotensin-independent manner
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Objective: The classical renin-angiotensin system (RAS) increases oxidative stress and exerts pro-inflammatory effects through angiotensin generation by secretory renin. However, after myocardial infarction an alternative renin transcript is expressed that encodes for a non-secretory cytosolic renin with presumably protective functions. To demonstrate the existence of an endogenous protective renin system we investigated these protective functions after cyto-renin overexpression, renin downregulation and renin activity inhibition under glucose depletion. Additionally, we analysed the effects with regard to the expression of the renin binding protein (RnBP) as potential interaction partner of cytosolic renin.

Methods: H9c2 rat cardiomyoblast control cells (non-transfected or empty pRES vector-transfected), cells overexpressing cytosolic renin [E(2-9)renin cells] and cells after renin- or RnBP downregulation were cultured under control conditions as well as under glucose depletion for 24 hours in the presence or absence of the rat renin inhibitor CH732 (10^{-4} M). Transcript levels of secretory renin [E(1-9)renin], cytosolic renin [E(1A-9)renin] and RnBP were quantified by real-time PCR and western blot. Functionally, we examined necrotic events [LDH ratio (release/content)]. Interaction of Renin and RnBP was analyzed by immunofluorescence.

Results: Control cell lines exposed to glucose depletion exhibited a marked increase of cytosolic renin transcript (4–5fold, p<0.05) and renin protein levels (2fold, p<0.05) as well as RnBP transcript levels (1.4fold, p<0.05). Furthermore, the necrosis rate was significantly increased after glucose depletion (1.8fold, p<0.05), renin downregulation (1.8fold, p<0.05) or strong 75% RnBP downregulation (2fold p<0.05). In contrast, cytosolic renin overexpression or mild 30% RnBP downregulation protected the cells from necrosis after glucose depletion. These protective effects of cytosolic renin overexpression were not affected by renin inhibition by CH732.

Conclusions: Cytosolic renin is cardioprotective in vitro by reducing the rate of necrosis under ischemia-related conditions such as glucose depletion. The effects are independent on angiotensin generation but in contrast dependent on RnBP-expression and co-localization. The cyto-renin is possibly a RnBP inhibitor.
Reduced maximal oxygen consumption and diminished left ventricular wall thickness in aquaporin-1-deficient mice  
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Aquaporin-1 (AQP1), a membrane channel for water and CO₂, is strongly expressed in cardiac tissue. We wanted to study the consequences of a lack of AQP1 for maximal systemic O₂ consumption and for the properties of the heart. First, we studied maximal O₂ consumption (V_o2,max) of aquaporin-1-deficient (AQP1-ko) and WT mice. V_o2 was measured in an open system and was maximized by using a helox inspiratory gas mixture at 4°C to induce heat loss in the animals. Under normoxia, AQP1-ko mice exhibited a V_o2,max of 0.14 ml O₂/g/min (SD 0.05) compared to 0.17 (SD 0.02) in WT mice (P=0.002). The latter value represents ~ 6 times the basal V_o2. Under hypoxia (11% O₂ in the inspiratory gas), V_o2,max decreased in both mouse strains, but the difference between AQP1-ko and WT remained. In an attempt to understand the cause of the reduced V_o2,max, we studied morphological parameters of the hearts of both strains. The left ventricular (LV) tissue mass was significantly lower in AQP1-ko vs. WT mice (4.4 mg / mm tibia length vs. 3.8 mg/mm, P=0.02). This was due to a diminished LV wall thickness in AQP1-ko vs. WT (1.22 mm vs. 1.37 mm, P<0.0001). At the cellular level, we found that in AQP1-ko LV myocytes are smaller, with cross-sectional areas of 277 µm² for ko and 325 µm² for WT (P<0.0001). At the same time, capillary density is significantly reduced with values of 2830 mm⁻² for AQP1-ko and 3100 mm⁻² for WT animals (P=0.0001). Because AQP1 in several tissues has been shown to be one determinant of capillary growth, we speculate that impaired capillary growth may be the primary event during heart development leading to reduced fiber thickness, thinner LV walls and a reduction of LV mass. We conclude that the maximal stroke work of the AQP1-ko hearts should be markedly impaired. Thus, the properties of these hearts may be responsible for the reduced V_o2,max of AQP1-ko animals.

Conditional, cardiomyocyte-specific knock out mice for Akt1, but not Akt2, are protected from angiotensin II induced cardiac hypertrophy  
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We analysed the isoform-specific role of Akt1 and Akt2 kinases in the development of Angiotensin II (AngII) induced pathological cardiac hypertrophy. We generated Tamoxifen-inducible, cardiomyocyte-specific Akt1 (iCM-Akt1KO) and Akt2 (iCM-Akt2KO) knockout mice. Osmotic mini-pumps with PBS or 1.5 mg/kg/day AngII were implanted for 14 days in transgenic and wildtypic (wt) male mice aged 19-28 weeks. Echocardiographic measurements were performed on day 0, 7 and 14, followed by histology analysis. Just after 7 days of AngII treatment wt and iCM-Akt2KO mice, but not iCM-Akt1KO, exhibit a significant increase of left ventricular mass (wt: 0d/136mg, 7d/174mg; iCM-Akt1KO: 0d/107mg, 7d/119mg; iCM-Akt2KO: 0d/133mg, 7d/168mg) and wall thickness (wt: 0d/0.86 mm, 7d/1.29 mm; iCM-Akt1KO: 0d/0.8 mm, 7d/0.82 mm; iCM-Akt2KO: 0d/0.82 mm, 7d/1.35 mm) measured by echocardiography, which are sustained over 14 days. This is underlined by weighted values after 14 days, heart weight/body weight ratio is significantly raised by AngII treatment, but iCM-Akt1KO did not respond to AngII (wt: PBS/4.1 mg*g⁻¹, AngII/5.2 mg*g⁻¹; iCM-Akt1KO: PBS /4.1 mg*g⁻¹, AngII/4.1 mg*g⁻¹; iCM-Akt2KO: PBS /4.2 mg*g⁻¹, AngII /4.9 mg*g⁻¹). Stroke volume and cardiac output were clearly reduced only in wt and iCM-Akt2KO mice. Histological studies reveal an increase of cardiomyocyte cross sectional areas and a decrease of cardiomyocyte amounts for wt and iCM-Akt2KO mice. Histological studies reveal an increase of cardiomyocyte cross sectional areas and a decrease of cardiomyocyte amounts for wt and iCM-Akt2KO mice. Histological studies reveal an increase of cardiomyocyte cross sectional areas and a decrease of cardiomyocyte amounts for wt and iCM-Akt2KO mice. Histological studies reveal an increase of cardiomyocyte cross sectional areas and a decrease of cardiomyocyte amounts for wt and iCM-Akt2KO mice. Histological studies reveal an increase of cardiomyocyte cross sectional areas and a decrease of cardiomyocyte amounts for wt and iCM-Akt2KO mice.

The epidermal growth factor receptor (EGFR) is a ubiquitously expressed receptor tyrosine kinase, which activates various intracellular signaling pathways upon ligand binding or trans-activation. Therefore, the EGFR regulates proliferation, migration, differentiation and matrix homeostasis and is implicated in the development and maintenance of multiple organs and their function. In the cardiovascular system, EGFR is associated with parainflammatory processes and impaired cardiac and vascular function. In this context, the regulatory effect of EGFR on miRNA expression is of special interest, since dysregulated miRs have been associated with pathological cardiac and vascular phenotypes. For our studies, mice with a strong reduction of EGFR (KO) in cardiomyocytes (CM) were used. Those animals showed a strong reduction in life span and suffered from a dramatic eccentric cardiac hypertrophy with increased stroke volume and left ventricular wall and lumen thickness. To investigate potential miRNAs influenced by EGFR, miRNA-qPCR-Arrays from whole heart tissue were performed and possible candidate miRNAs were confirmed by TaqMan®PCR. In the heart miR-221 and -222, which possess a common primary-miR (pri-miR), were upregulated in KO animals compared to...
wildtype. Because the heart consists of different cell types, miRNA expression was investigated in isolated CMs and cardiac fibroblasts (CFBs). Our results demonstrate an up-regulation of miR-221/-222 in CMs of KO, but not in CFBs of KO animals. To evaluate the underlying mechanism of EGFR-mediated changes in miR-221/-222 expression in the heart, the level of pri-miR-221/-222 was quantified by TaqMan®PCR. The pri-miR-221/-222 was increased in KO animals, suggesting that the clustered miR-221/-222s are regulated on a transcriptional level. To investigate the capability of a functional impact of miR-221/-222, we correlated the absolute copy numbers for miR-221/-222 and CDKN1B (p27) mRNA, a validated target of miR-221. Although no differences between wildtype and KO animals in p27 mRNA expression was observed, a decrease of p27 protein in the heart of KO animals could be detected. In contrast to the results in the heart, the miR-221/-222 promoter activity indicated a positive regulation by EGF activated EGFR. In conclusion, the EGFR KO leads to heart hypertrophy and miR-221/-222 increase. This increase in miRs may be an anti-hypertrophic mechanism triggered by structural changes caused by EGFR KO.

**P269**

The adiponectin paralog CTRP9 but not CTRP7 mediates anti-hypertrophic effects in adult rat cardiomyocytes through an AMPK, adiponectin receptor and calreticulin dependent mechanism

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**Background:** Adiponectin has direct beneficial effects on cardiomyocytes in several pathological heart conditions including ischemia-reperfusion injury or cardiac hypertrophy. Recently, a family of structural and functional adiponectin paralogs, comprising 15 members so far, was discovered and designated as C1q/tumor necrosis factor-alpha-related proteins (CTRPs). While the anti-hypertrophic and anti-apoptotic actions of adiponectin are well recognized, questions remain unanswered concerning the role of the CTRPs in these processes.

**Methods and Results:** Among all adiponectin paralogs, CTRP7 and CTRP9 are induced in failing human hearts. CTRP9, which is expressed in adult human cardiomyocytes, inhibits cardiomyocyte hypertrophy induced by phenylephrine, angiotensin II or endothelin. CTRP7, which is mainly derived from cardiac fibroblasts, does not mediate anti-hypertrophic effects and cannot compensate for a CTRP9 loss. CTRP9 induces an AMPK- and SIRT3-dependent transcriptional activation of Trx1 and SOD2, which results in a reduction of cell death and hypertrophy in response to 

**Conclusion:** These results demonstrate for the first time that CTRP9 inhibits hypertrophy induced by various pro-hypertrophic agents via AMPK-mediated activation of antioxidative enzymes in an adiponectin receptor and calreticulin-dependent fashion.

**P270**

A genetic variant prevents CVB3-induced cardiovascular effects in vitro

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In humans as well as in mouse models in infection with enteroviruses, particularly coxsackieviruses of type B (CVB), are associated with ventricular arrhythmias and sudden cardiac death. For better understanding, we investigated the mechanisms underlying the development of virus-induced, life-threatening arrhythmias. The proarrhythmic potential was predicted to be increased by viral proteins modulating K$_{7.1}$/KCNE1 channels. Aim of this study was to find parameters which affect virus-mediated effects on K$_{7.1}$/KCNE1 channels. Here, we show that AG7088, an anti-viral compound already used in clinical trials, is not able to prevent the viral effects on these ion channels. However, in contrast to the pharmacological compound we demonstrate that in presence of the common Asian polymorphism K$_{7.1}$-P448R the virus-induced ion channel modulations can be prevented in vitro. Therefore, it can be assumed that new pharmacological agents mimicking this polymorphism can reduce the proarrhythmic risk associated with CVB3 infections and may help to understand the CVB3-mediated arrhythmias.
Besides providing mechanical stability, fibroblasts are also thought to modulate the electric properties of cardiomyocytes. After myocardial infarction or during heart failure, fibroblasts proliferate and convert to myofibroblasts. These cells have a depolarized membrane potential, express Connexin 43 and can electrically couple to cardiomyocytes in vitro. The resulting depolarization of cardiomyocytes could lead to slowing of conduction and enhanced spontaneous activity and both of these could promote arrhythmias.

To analyze the electric interaction between cardiomyocytes and fibroblasts we used optogenetic methods enabling selective manipulation of the membrane potential in fibroblasts. To this aim transgenic NIH3T3 fibroblasts cell lines were generated stably expressing the light-dependent proton pump Archaerhodopsin (ArchT3.0) for hyperpolarization and the light-sensitive ion channel Channelrhodopsin2 (ChR2) for depolarization. Patch-clamp analysis showed light-induced (530 nm, 14.1 mW/mm²) outward currents with maximal current density of 1.83 ± 0.27 pA/pF (n=15) in ArchT3.0 expressing fibroblasts leading to hyperpolarization of 40.6 ± 8.1 mV (n=15). Fibroblasts form the ChR2 cell lines showed light-induced (470 nm, 8.0 mW/mm²) inward currents of 1.02 ± 0.12 pA/pF (n=8) and a depolarization of 15.1 ± 9.6 mV (n=5).

The effect of fibroblast membrane potential on cardiomyocyte function was tested using co-cultures of transgenic NIH3T3 fibroblasts with purified human iPS cell-derived cardiomyocytes. Conduction velocity and spontaneous activity were analyzed by recording field potentials on micro-electrode arrays. In preliminary experiments we found that light-induced hyperpolarization of ArchT-fibroblasts reduced conduction velocity to 31.1% and spontaneous beating frequency to 35.4% of controls. Illumination of ChR2-fibroblasts led to acceleration of beating frequency up to 122.5% and a slight reduction of conduction velocity to 96.1%. Thus both, de- and hyperpolarization of fibroblasts slowed conduction between cardiomyocytes, furthermore depolarization increased whereas hyperpolarization decreased spontaneous activity.

In conclusion optogenetic methods enable the selective modulation of membrane potential in fibroblasts. With this method their electrical interaction with cardiomyocytes can be studied in co-cultures in vitro. These methods will allow the investigation of functional electrical coupling between cardiomyocytes and fibroblasts and also the effect on cardiac arrhythmias in vivo.

In vivo and in vitro studies of Gaq / Gq11 double knockout (dko) mouse hearts, such as echocardiography, electrophysiology of isolated heart muscle cells and fluorometry (calcium handling) show substantial alterations. Therefore we employed transcriptome-analysis (“deep sequencing”) of isolated heart muscle cells to reveal differential expression patterns of wild-type (wt) and double knockout (dko) cardiomyocytes to understand the genetic basis of these changes. Following new generation sequencing (NGS) and bioinformatics analysis we identified 3,100 genes with expression levels that were significantly different between wild-type (wt) and dko cells. By using quantitative real-time PCR (qPCR), we verified these results and attempted to further identify important pathways that are affected by Gaq / Gq11 knockout. These detailed analysis should allow us a better understand the role of these pathways in physiological processes, but also in pathological changes in the course of heart diseases.

Total RNA was isolated from cardiomyocytes of dko and wt mouse hearts. After transcription into cDNA, the expression of identified candidate genes from the NGS was revisited by qPCR. Subsequently, the results of both methods (NGS and qPCR) were compared.

Following bioinformatics analysis a total of 89 pathways with altered expression were identified. Initially, genes from the extracellular matrix and cardiac muscle contraction pathways were selected and their expression profile was investigated by qPCR. Although, differential expression between dko and wt mice could not be confirmed for all chosen genes, a large number of genes were verified by detailed qPCR employing specific marker genes.

Functional studies on dko animals highlighted numerous phenotypic alterations in the behavior of the organ and of isolated cardiac myocytes that motivated NGS to reveal differentially expressed genes. Out of the vast number of differentially expressed genes specific candidates from the initial NGS-analysis we could extract robust genes by marker-gene driven detailed qPCR investigation. These data will foster further investigation on the protein and protein function level in order to foster our detailed understanding of basal Gq/G11-signalling in cardiac myocytes.

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P273
Knock out of soluble Guanylyl Cyclase affects myocardial titin phosphorylation and myofilament stiffness

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**Question:** Soluble guanylyl cyclase (sGC) has a crucial role in smooth muscle but also in heart muscle. Soluble GC regulates activity of PKG which has been shown to phosphorylate titin and thereby affects the elasticity of the heart. Here we analyzed how heart- specific and global depletion of the sGC alters titin phosphorylation and titin based passive myofilament stiffness. We further hypothesized that due to potential crosstalk with the PKG signalling pathways other kinases that phosphorylate titin (PKA, PKC) could be affected by the sGC depletion.

**Methods:** Titin isoform composition, phosphorylation and titin-based passive stiffness were analyzed in mouse hearts from global soluble guanylyl cyclase KO (sGCKO) and cardiac specific KO (MHC-sGCKO). Titin isoform composition was tested by agarose-strengthened 2.1% SDS-PAGE. Titin phosphorylation was analyzed by Western blot analysis using phospho-specific antibodies targeting titin’s N2B or PEVK domain. Kinase activity was estimated by using phospho-specific antibodies targeting the activated kinases itself or kinase substrates. Passive stiffness was measured using isolated skinned cardiac myocytes.

**Results:** Titin isoform composition was unchanged in both analyzed groups. sGCKO hearts showed reduced relative N2-Bus phosphorylation at S4010 (PKA-dependent) and S4099 (PKG-dependent). MHC-sGCKO showed reduced S4010 phosphorylation, whereas S4099 phosphorylation was unchanged. PEVK phosphorylation at S11878 and S12022 (PKCα- and CaM-Kinase2δ-dependent) was unchanged in sGCKO, but reduced in MHC-sGCKO. PKG and PKA activity, assumed from Troponin I phosphorylation, were strongly reduced in sGCKO and unaltered in MHC-sGCKO. PKCα activity was slightly reduced in sGCKO and unchanged in MHC-sGCKO. Instead of an increase in titin based passive stiffness, which could be expected from the observed changes in titin domain phosphorylation we determined a mild but significant decrease in sGCKO hearts compared to wild-type controls. In MHC-sGCKO hearts passive stiffness was unchanged.

**Conclusion:** Our data demonstrate that sGC depletion impairs not only PKG- but also PKA-dependent titin phosphorylation. The unchanged PKG dependent titin phosphorylation in MHC-sGCKO is unexpected and raises the question of a putative intercellular cross-talk or intracellular mechanism compensating for sGC depletion.

P274
Effects of cytosolic renin on apoptosis in cardiac cells. Results of over expression, knock down and inhibition of activity

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**Objective:** Secretory renin exerts pro-apoptotic, pro-necrotic and pro-fibrotic effects through angiotensin generation from it’s only known substrate, angiotensinogen. After myocardial infarction the expression of a renin transcript is induced in the rat that encodes for a non-secretory renin isoform. This renin (cyto-renin) remains in the cytosol or is imported into mitochondria. Aim of the present study was to investigate the role of cyto-renin for apoptosis under ischemia related conditions, such as glucose depletion. Also we tested the hypothesis that effects of cyto-renin are independent of angiotensin generation.

**Methods:** To this end we investigated the consequences of overexpression as well as of down regulation of cyto-renin on apoptosis in the presence and absence of the renin inhibitor CH732 under basal conditions and under glucose depletion. H9c2 rat cardiomyoblast control cells (non-transfected or empty pIRES vector-transfected), cells overexpressing cytosolic renin [E(2-9)renin cells] and cells after siRNA renin knock down were used. Transcript levels of secretory renin and cyto-renin were quantified by RTPCR and western blot. Functionally, we examined apoptosis (caspase activity, annexin V labelling).

**Results:** After glucose depletion control cells show increased renin transcript (4–5fold, p≤0.05) and protein levels (2fold, p≤0.05). Cyto-renin overexpression in E(2-9)renin cells increased the apoptosis rate under baseline conditions [E(2-9)renin: 7.4±0.5% vs. control cells: 15.1±1.5%, p≤0.05]. Glucose depletion increased the rate of apoptosis significantly in control cells (7.4±0.5 vs. 20.5±1.3) but not in E(2-9)renin(15.1±1.5 vs. 10.3±0.8%). Cyto-renin knock down resulted in an increased apoptosis rate (6.3±0.4% vs. 17.9±2.0%, p<0.05) under baseline conditions. These protective effects of cyto-renin were still seen under renin activity inhibition by CH732.

**Conclusions:** One of the mechanisms that leads to the cardioprotective effects of cyto-renin is the reduction of apoptosis under ischemia-related conditions such as glucose depletion. The effects are independent of angiotensin generation.
Calcium reuptake into the sarcoplasmic reticulum via the sarcoendoplasmatic reticulum calcium ATPase (SERCA) is mainly regulated by phosphorylamban (PLN). When phosphorylated at one or both of its regulatory sites Ser 16 and Thr 17, it dissociates from SERCA which results in increased activity of the pump. Recently, our group showed that in microvascular smooth muscle, the energy-sensing enzyme AMP-activated protein kinase (AMPK) is able to phosphorylate PLN at least at one of its regulatory sites, namely Thr 17, thereby decreasing intracellular calcium concentration and inducing vasodilation. In this study, we elucidated whether AMPK could phosphorylate myocardial PLN and induce a lusitropic effect. Using the specific AMPK-activator A-769662 in organ bath experiments on freshly isolated mouse heart slices (DMSO: n=6, 30 µM A76: n=9), we could not only confirm the already known positive inotropic effect of AMPK activation after exposure to A-769662. We also observed a concentration-dependent (10 µM–100 µM) acceleration of diastolic relaxation by about 218% (positive lusitropic effect). Western Blots showed an AMPK-dependent phosphorylation of PLN Thr 17. The isoproterenol-induced phosphorylation of PLN at both Ser 16 and Thr 17 could be further enhanced by A-769662 (30 µM). In summary, our results suggest a regulatory role of AMPK in the control of myocardial SERCA activity via phosphorylation of its regulator PLN. AMPK activation may augment b, mediated lusitropy.

**Methods and results:** We explored inflammatory endothelial activation and its effects on oxidative stress, NO bioavailability and cGMP-PKG signalling in myocardial biopsies of HFpEF patients and provided proof-of-concept in obese ZSF-1 rats. In myocardium of HFpEF patients and obese ZSF-1 rats, E-selectin and ICAM-1 were upregulated. NOX2 expression was raised in macrophages and endothelial cells, but not in cardiomyocytes. Nitrotyrosine expression was also limited to endothelial cells. Myocardial nitrite/nitrate concentration, cGMP content and PKG-activity were drastically reduced. Our previous studies demonstrated that decreased cGMP concentration and PKG activity lead to increased myocardial passive stiffness through hypophosphorylation of the giant cytoskeletal protein titin (Hamdani, et al. Circ HF 2013).

**Conclusion:** Metabolic risk therefore drives HFpEF development through inflammation of the myocardial microvasculature, which alters signalling from endothelial cells to cardiomyocytes.

**P277**
**Myosin mutations in familial hypertrophic cardiomyopathy: functional imbalance among individual cardiomyocytes as trigger for development of myocyte disarray and fibrosis**

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Familial hypertrophic cardiomyopathy (FHC), a frequent autosomal dominant cardiac disorder, is mostly caused by mutations in sarcomeric proteins. More than 1000 mutations in at least 20 different proteins have been found, most are heterozygous. Yet, the FHC-pathomechanisms leading to cardiomyocyte hypertrophy, cellular disarray, and interstitial fibrosis are unclear. Based on our previous work on mutations in the ventricular b-myosin heavy chain (b-MyHC) we hypothesize that unequal expression of the mutated protein and resulting functional imbalance among individual cardiomyocytes initiate development of FHC at least for b-MyHC mutations.

To test this hypothesis, we measured force generation at different [Ca2+]c, cross-bridge turnover and passive properties of individual, chemically permeabilized cardiomyocytes from septum of FHC-patients and donor hearts as controls. To minimize unequal phosphorylation of sarcomeric proteins at sites for protein kinase A (PKA), all myocytes were treated with PKA. We also determined the relative abundance of mutated and wildtype b-MyHC-mRNA in individual cardiomyocytes of the same cardiac samples with a restriction digest approach.

b-MyHC-mutations R723G and A200V both cause reduced calcium sensitivity (pCa50) and a reduction of Fmax of the cardiomyocytes by at least 30%. In contrast, cardiomyocytes from a patient with missense mutation R589H in cardiac myosin-binding protein C (cMyBPC) and a mutation in AMPK (T98I) were very similar to donor myocytes in all biomechanical parameters studied. Surprisingly, individual
cardiomyocytes with myosin-mutations R723G or A200V, showed a much larger variance in pCa50 compared to donor cells, ranging from significantly reduced to almost normal. In contrast, the cMyBPC- and AMPK-mutations had no effect on pCa50 and no other effect on sarcomere contraction. Single-cell mRNA-quantification for the β-MyHC and cMyBPC mutations revealed a large variation in the relative expression of the respective mutant β-MyHC- or cMyBPC-mRNA from cardiomyocyte to cardiomyocyte, ranging from almost pure mutant to almost pure wildtype mRNA in individual cells. Most likely the large variance in pCa50 of individual cardiomyocytes with mutation R723G or A200V is due to the unequal expression of mutated and wildtype β-MyHC-mRNA in the cells. For cardiomyocytes with the cMyBPC-mutation, no effect of unequal expression on the variance of pCa50 is consistent with the lack of any effect of this mutation on pCa50, while other factors may well be functionally affected. We conclude that unequal abundance of mutated sarcomeric proteins with altered contractile function will induce functional imbalances among neighboring cardiomyocytes, due to large variation at partial activation caused by the variance in pCa50 in case of mutations R723G and A200V, and in other, as yet to be identified parameters for the cMyBPC- and AMPK-mutations. Such functional imbalance may well trigger development of cardiomyocyte disarray and fibrosis in FHC.

Question: The picrosirius red staining is a standard method for the detection of fibrotic tissue and scar formation after myocardial infarction. However, the use of this staining method is limited on frozen cardiac tissue sections due to a low color contrast. In addition, co-immunostaining with antibodies, which allow co-localization studies or the analysis of several cell compartments in one approach, is not possible in picrosirius red stained tissue sections. Recently, we observed that wheat germ agglutinin (WGA) might also label fibrotic tissue in cardiac sections after myocardial infarction. Therefore, the aim of the present study was to investigate WGAs potential for the staining and quantification of post myocardial infarction (MI) scar formation.

Methods: Animal experiments were performed in male C57Bl6 mice (12 week). All animals underwent 45 minutes of regional myocardial ischemia induced by an occlusion of the left anterior descending coronary artery. After myocardial infarction and four weeks of reperfusion animals were sacrificed and hearts were snap frozen. Picrosirius red (SR) staining or WGA-FITC labelling was performed on 8µm cryosections. Infarct sizes were analyzed using a planimetric approach in both SR- and WGA labelled heart sections. Furthermore, to test whether myocardial fibrosis can be analysed on WGA-FITC labelled cryosections using a computer based quantification approach, we performed a histogram-based quantification approach on WGA-FITC labelled cryosections, and the results were compared to the infarct sizes obtained by planimetry. In addition, co-immunostaining of WGA-labelled heart sections with collagen I antibody was performed. Results: The staining pattern of WGA-FITC stained heart sections was very similar compared to SR-stained heart sections. Regression analysis of infarct sizes determined by planimetry showed a strong correlation of R²=0.96 (p=0.0039) between both staining/labelling methods. In addition, the comparison of planimetric and histogram-based analysis in WGA-FITC stained cryosections showed a determination coefficient of R²=0.83 (p=0.0301). Furthermore, co-immunostaining of WGA-FITC labelled cryosections with an additional antibody was applicable. The WGA / collagen I co-staining showed comparable distribution of scar formation after myocardial infarction.

Conclusion: Our results clearly demonstrate that WGA-FITC is a suitable marker for cardiac fibrosis in frozen tissue sections. In combination with the histogram-based analysis, this new quantification approach is 1) easy and fast to perform 2) suitable for raw frozen tissue sections, and 3) allows the use of additional antibodies in co-immunostaining.

P279
JDP2- or YB-1 overexpression impairs contractile function of cardiomyocytes
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The transcription factors AP-1 and SMADs are mediators of hypertrophy or apoptosis. Both processes contribute to cardiac remodeling and heart failure progression. This implies that inhibition of AP-1 or SMADs may improve heart function. However, inhibition of AP-1 in transgenic JDP2-overexpressing mice reduced contractile function of cardiomyocytes (Hill et al., 2013). Due to the lifelong JDP2-overexpression, secondary effects on contractile function could not be excluded. We, therefore, investigated, if short-term overexpression of JDP2 in transgenic mice also influences contractile function. In another approach, adenoviral overexpression of the SMAD-inhibitor YB-1 in cardiomyocytes was chosen. Cardiac-specific JDP2-overexpression under control of the α-MHC-promoter was repressed by doxycycline feeding. JDP2 overexpression was started one week prior to isolation of ventricular cardiomyocytes. Cardiomyocyte contraction...
was analysed under electrical stimulation at 2 Hz. In this situation JDP2-cardiomyocytes revealed reduced cell shortening (10.2 ± 3.2 %dL/L) compared to WT (11.9 ± 3.6 %dL/L) (p<0.05, n=2 independent preparations with 54 cells analysed). However, responses to beta-adrenergic stimulation by isoprenaline (ISO, 10 nM) were similar in JDP2- and WT-cardiomyocytes (13.9 ± 2.9 %dL/L in JDP2-mice vs. 13.6 ± 3.7 %dL/L in WT). For the overexpression of the SMAD-inhibitor YB-1, ventricular cardiomyocytes of rats were infected with Ad-YB-1 for 24 or 48 h. Also here, impairment of contraction under electrical stimulation at 2 Hz was found. In non-transfected cardiomyocytes cell shortening was 11.7 ± 2.5 %dL/L. Already 24 h after YB-1 overexpression, cell shortening was reduced to 10.8 ± 2.8 %dL/L, and remained impaired after 48 h (10.8 ± 3.1 %dL/L) (p<0.05, n=4 independent cell preparations) Responsiveness to ISO was still present and cell shortening was the same as in control cells (14.0 ± 2.9 %dL/L in Ad-YB1-cells vs. 14.7 ± 3.1 %dL/L in controls).

In conclusion, short-term expression of AP-1 or SMAD-inhibitors in transgenic mice or adult cardiomyocytes of rats leads to impairment of contractile function. While electrically stimulated basal contraction is reduced, beta-adrenergic responsiveness is preserved. This is in contrast to long-term effects of JDP2-overexpression which was found to also impair ISO-responses. Therefore, short-term interventions on AP-1/SMAD signaling may be an option to reduce processes of hypertrophy and apoptosis in cardiac remodeling taking into account reduction in basal contractile function.

**P280**

**Imaging based assay for screening of cell cycle modifying substances in postnatal cardiomyocytes**

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Cardiovascular diseases are a major cause of death in the Western world. Especially large myocardial infarctions and the potentially resulting left ventricular dysfunction are chronic diseases with a poor prognosis. Due to limited endogenous cardiac regenerative capacity, we are exploring the regulation of the cell cycle in cardiomyocytes (CMs) as a potential treatment strategy. Within a few days after birth, CMs switch from hyperplastic to hypertrophic growth accompanied by acutokinetic mitosis (mitosis without cytokinesis), resulting in binucleated CMs, and endoreplication (mitosis without cytokinesis and karyokinesis), leading to polyploid CMs. The mechanisms and factors regulating these cell cycle variations remain largely unknown. We are using CMs from double transgenic CM-HC-H2B-mCherry/CAG-eGFP-anillin mice for the analysis of postnatal CM development. This double transgenic system enables us to unequivocally identify CM nuclei as well as cell cycle variations (Hesse et al., 2012). For a better understanding of the processes that cause the postnatal exit of the cell cycle we are currently testing knockdowns of genes by RNAi that influence cell division, endoreplication and acutokinetic mitosis. We found that knockdown of the cyclin dependent kinase inhibitors p21 and p27 led to increased cell cycle activity in CMs as visualized by eGFP-anillin fluorescence. As a long term goal we aim to identify substances that are able to bring adult CMs back into the cell cycle and to promote cell division.

**P281**

**Age-dependent upregulation of NOS1 triggers induction of NADPH oxidases and impairment of contractile function in murine ventricular myocytes**

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**Question:** Nitric oxide (NO) modulates calcium transients and contraction of cardiac myocytes. However, the contribution of NO-dependent mechanisms to the impaired contractile function of aged myocardium is unknown. We aimed to investigate age-dependent changes in the sources of cardiac NO and the contribution of NO to the age-related impairment of cardiac myocyte contraction.

**Methods:** We used C57BL/6 mice, NOS1−/− mice and mice with cardiomyocyte-specific nitric oxide synthase 1 (NOS1) overexpression. We analyzed sarcomere shortening, calcium transients (Indo-1 fluorescence), acto-myosin ATPase activity (malachite green assay) and NADPH oxidase activity (lucigenin chemoluminescence) of isolated ventricular myocytes. Furthermore, cardiac gene expression was quantified (Western blots, qPCR).

**Results:** NOS1 was upregulated in hearts of aged C57BL/6 mice. In isolated myocytes, NOS1 expression was reduced by forskolin-induced cAMP signaling and induced by inhibition of cAMP signaling using acetylcholine. Cardiac expression of the β1-adrenergic receptor was upregulated whereas expression of cardiac adenylate cyclase isoforms 5 & 6 were downregulated in aged C57BL/6 mice. Pharmacological inhibition of NOS1/NO using L-NVIIO or PTIO normalized the decelerated contraction kinetics of aged myocytes. NOS1−/− mice displayed no age-related changes in contraction, calcium transients, acto-myosin ATPase activity and cardiac expression of NADPH oxidase subunits. Cardiac expression of NOX2, NOX4, p47phox and p22phox was induced in NOS1-overexpressing mice.

**Conclusions:** Reduced efficacy of β-adrenergic signaling in the aged myocardium promotes upregulation of NOS1. NOS1-derived NO mediates deceleration of contraction kinetics in aged myocytes and NOS1 deficiency protects from these age-related alterations. NOS1 upregulation induces cardiac expression of NADPH oxidase components, favoring a pro-oxidative remodeling. Nitrosative and oxidative stress act synergistically to impair contraction of aged cardiac myocytes.
**P282**

**Time course of QT prolongation induced by mechanical unloading in a rodent model of heterotopic heart transplantation**

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**Question:** Support of failing hearts with ventricular assist devices (VADs) is associated with a high incidence of newly occurring severe ventricular arrhythmias. The mechanisms underlying this major complication are unresolved but likely include surgical trauma, perioperative recovery and mechanical irritations. Furthermore, data obtained in patients following VAD implantation suggest a direct influence of unloading on cellular electrophysiology. However, due to the underlying heart disease which also affects ventricular repolarization, these studies are difficult to interpret. We, therefore, investigated the hypothesis that ventricular unloading in the absence of underlying heart disease directly impairs repolarization in vivo.

**Methods:** Left ventricular unloading was induced by heterotopic cardiac transplantation of nonfailing hearts in syngeneic male Lewis rats (n=10, 269±16 g). During the same procedure, telemetric ECG devices (D.S.I.) were implanted to record the ECG either of the control orthotopic (n=5) or unloaded heart (n=5) for 56 days. ECG analysis was performed using ECG auto v.2.5 (EMKA) with animal specific waveform libraries. Statistical analysis was conducted using Prism v.5 (Graphpad), all data are given as mean±SEM.

**Results:** Unloading induced pronounced cardiac atrophy (left ventricular weight after 56 days: control, 748±24 mg, n=10; unloaded, 345±33 mg, n=10; P<0.0001). In control as well as unloaded hearts, the heart rate (HR) regulation normalized within the first four days after surgery and remained stable over the following seven weeks (control, 339±7 bpm, n=5; unloaded, 346±6 bpm, n=5; P=0.55). In contrast, ventricular repolarization was severely impaired in the unloaded hearts. The QT interval was prolonged intraoperatively, but normalized within 24 hours in control as well as in unloaded hearts. Between day 2 and 5, the QT interval in unloaded hearts progressively increased by 60% and remained elevated at this level over the remaining observation period (mean QT interval over the remaining period: control, 55±1 ms, n=5; unloaded, 91±4 ms, n=5; P<0.0001).

**Conclusion:** This study demonstrates that even in the absence of underlying heart disease and ventricular trauma, left ventricular unloading per se markedly impairs repolarization in the presence of a preserved heart rate regulation, most likely via a molecular remodeling. This implicaes that a reduction in left ventricular workload could indeed be causally linked with the occurrence of ventricular arrhythmias in patients with VAD support.

**P283**

**Reduced cardiac EGF receptor expression interferes with the contractile function of cardiomyocytes – gender-specific effects**

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**Question:** Beside activation by its classical ligands, the EGF receptor (EGFR) can also be transactivated by other hormones. Although this receptor appears to act as an integrator of a variety of extracellular signals, little is known about its cardiac function.

**Methods:** We analyzed mice with a targeted deletion of EGFR in VSMC and a strong reduction of EGFR in cardiomyocytes (referred to as EGFR KO; Schreier et al. Hypertension 2013). The hearts of these transgenic mice were analyzed concerning gene expression (real-time PCR) and contraction (sarcomere shortening).

**Results:** Hearts of EGFR KO mice were characterized by hypertrophy. This cardiac hypertrophy was significantly more pronounced in male mice. Cardiac mRNA expression of key calcium handling proteins (DHP receptor, RyR2, SERCA2, NCX) was not different between EGFR KO mice and control littersmates. However, sarcomere shortening and relengthening were decelerated in cardiomyocytes of EGFR KO mice, whereas fractional shortening was unchanged. Additionally, we detected reduced mRNA expression of β-adrenergic signaling components (β1-adrenergic receptor, Gs, AC5, AC6) and increased mRNA expression of NOS1 and NOX4 in cardiomyocytes of EGFR KO mice. Interestingly, these alterations in contraction and cardiac gene expression only occurred in male EGFR KO mice.

**Conclusions:** Previously we showed that cAMP concentration regulates NOS1 expression in isolated cardiomyocytes and upregulated NOS1 induces NADPH oxidase. Therefore, we suggest, that in male EGFR KO mice reduced efficacy of β-adrenergic signaling contributes to cardiac expression of NOS1 and subsequently NOX4. NOS1 and NADPH oxidase were previously shown to synergistically decelerate contraction of cardiomyocytes, providing a possible explanation for slowed shortening/relengthening of cardiomyocytes from male EGFR KO mice.

**P284**

**Optogenetic stimulation of the G_s-signaling cascade accelerates beating of the intact heart**

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Activation of Gs-signaling in cardiomyocytes results in positive chronotropy and inotropy but chronic Gs-stimulation can also induce cardiac hypertrophy and enhance the propensity...
for arrhythmia. Stimulation of the Gs-signaling cascade in vivo by perfusion of pharmacological agonists lacks organ specificity and has a low spatio-temporal accuracy. Here we report the alternative use of JellyOp, a directly Gs-coupled, light-sensitive receptor (Bailes et al. PloS One, 2012) for optogenetic control of the Gs-signaling cascade.

G4 embryonic stem (ES) cells were genetically engineered to express JellyOp and GFP under control of the chicken β-actin promoter. In vitro differentiation was used to obtain JellyOp-expressing cardiomyocytes and their spontaneous beating accelerated while illumination with blue light (470 nm). The light-induced increase in frequency was similar to pharmacological Gs-activation with isoprenaline and showed a sigmoid dependence on light-intensity with a half maximal light intensity (Eli50) of 12 nW/mm2.

JellyOp ES cell clones with intact number of chromosomes were used for diploid complementation to generate a transgenic mouse line. In these mice whole hearts showed GFP signals and JellyOp expression was confirmed in α-actinin positive cardiomyocytes by immunohistochemical staining. Overexpression of JellyOp (per se) did not induce cardiac hypertrophy because heart weight normalized to femur length of > 6 month old JellyOp mice was similar to wildtype controls (p=0.906, n=5). Illumination (5 min, 470 nm, 1.34 mW/mm2) of isolated atrial tissue elevated cAMP levels to 309.7±44.1% (n=7) of baseline, which was similar to pharmacological Gs-activation with isoprenaline (370.9±27.7%, n=5). Light-induced chronotropic response was analyzed in isolated hearts during Langendorff perfusion. Brief illumination of JellyOp (per se) did not induce cardiac hypertrophy because heart weight normalized to femur length of > 6 month old JellyOp mice was similar to wildtype controls (p=0.906, n=5). Illumination (5 min, 470 nm, 1.34 mW/mm2) of isolated atrial tissue elevated cAMP levels to 309.7±44.1% (n=7) of baseline, which was similar to pharmacological Gs-activation with isoprenaline (370.9±27.7%, n=5). Light-induced chronotropic response was analyzed in isolated hearts during Langendorff perfusion. Brief illumination (100 ms, 537 µW/mm², 470 nm) of the right atrium led to an almost instantaneous (delay ~300 ms) acceleration of the sinus rhythm. Frequency increase depended on the applied light intensity with an Eli50 of 12 nW/mm² (100 ms illumination, n=3). Interestingly, in preliminary experiments we found that illumination of the left atrium at the region of pulmonary vein insertion led to supraventricular extrabas with altered p-wave morphology indicating local Gs-induced pacemaker activity.

In summary optogenetic JellyOp activation in cardiomyocytes enables confined stimulation of the Gs-signaling pathway in the intact heart with high spatio-temporal precision.

P285

**Fibroblasts from diaphragms of dystrophin-deficient mdx but not wild-type mice up-regulate LOX-1 expression by endothelin-1**

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**Background:** Lectin-like oxidised LDL receptor (LOX)-1 expression is up-regulated by a variety of stimuli such as endothelin (ET)-1 and angiotensin II. LOX-1 overexpression in transfected cardiac fibroblasts has been implicated in increased collagen formation. The mdx mouse, a model for Duchenne muscular dystrophy, shows skeletal muscle fibrosis with excessive collagen accumulation in the diaphragm. As ET-1 levels are increased in cardiac fibrosis, we tested the hypothesis that LOX-1 expression is up-regulated by ET-1 in cultivated fibroblasts from murine diaphragms.

**Methods:** Fibroblasts were isolated from diaphragms of 100 days-old male mdx (C57Bl/10ScSn-Dmd<sup>mdx</sup>/J, n=4) and wildtype (WT) mice (C57Bl/10Sc, n=4). Cells were exposed to ET-1 concentrations from 10^-9 up to 10^-7 mol/l. To block ET receptors, cells were pre-incubated with BQ-788 or BQ-123 (10^-6 mol/l) for 60 minutes. Cells were harvested after four hours and total RNA was isolated by standard procedures. LOX-1 expression was quantified by reverse transcription PCR and corrected for porphobilinogen deaminase expression. Data are 2^-ΔΔCt and presented as mean ± standard error. ET-1-mediated effects were analysed by Kruskal-Wallis ANOVA by ranks followed by Dunnnett’s test.

**Results:** Basal LOX-1 mRNA expression in fibroblasts from diaphragms of mdx mice (0.13±0.01) was not different to the expression in cells from WT mice (0.09±0.04). ET-1 treatment resulted in significant up-regulation of LOX-1 mRNA expression (10^-9 mol/l: 0.31±0.07; 10^-8 mol/l: 0.24±0.05; 10^-7 mol/l: 0.30±0.06). ET-1-mediated effects (10^-7 mol/l) were reversed by BQ-788 (0.16±0.06), an inhibitor of the ET<sub>B</sub> receptor, but not BQ-123 (0.38±0.12). In contrast, LOX-1 expression was not significantly altered by ET-1 in fibroblasts from diaphragms of WT mice (10^-9 mol/l: 0.15±0.07; 10^-8 mol/l: 0.09±0.03; 10^-7 mol/l: 0.10±0.03).

**Conclusions:** ET-1 increased LOX-1 expression in fibroblasts from diaphragms of mdx but not WT mice. Our data indicate that ET-1 signalling via the ET<sub>B</sub> receptor is established in proliferating fibroblasts from dystrophic muscle. This may contribute to the development and progression of fibrotic remodelling of mdx muscles. Further experiments will be needed to elucidate the underlying mechanisms.

P286

**Contractile properties of myofibrillar bundles isolated from plectin-deficient mouse psoas muscle**

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**Background:** Plectin, expressed in different isoforms, links actin microfilaments, microtubules and intermediate filaments in an isoform specific manner. Mutations in the human plectin gene cause a variety of diseases, affecting primarily muscles. Ple1d, a Z-disc specific isoform, is the only isoform of plectin in tissues exposed to mechanical stress such as skin and muscle. Ple1d, a Z-disc specific isoform, is the only isoform of plectin that is specifically expressed in skeletal and cardiac muscle. To imitate the clinical features of plectin-associated muscle weakness, a Ple1d-/- mouse model has been generated that shows the typical histological features of human myofibrillar myopathy, i.e. aggregation of desmin-IFs and mitochondria. However, the effect of Ple1d on biomechanical properties of skeletal myofibrils is unknown.

**Methods:** We analysed the kinetics of contraction-relaxation cycles using subcellular myofibril bundles (MF) from Ple1d-/- (Ple1d-KO) and wildtype (WT) mice psoas muscle as in Telley et al., (2006). During active contractions at maximal pCa, MF were stretched by 15% of their initial length to mimic eccentric contractions.
Results: Compared to myofibrils from WT, the Ple1d-KO exhibited neither significant differences in maximum active or passive tension nor in the kinetics of Ca\textsuperscript{2+}-induced (k\textsubscript{ACT}) or mechanically-induced (k\textsubscript{REL}) force development. Upon rapid removal of Ca\textsuperscript{2+}, myofibrillar force decays biphasically, starting with a slow linear phase (rate constant k\textsubscript{LIN}, duration t\textsubscript{LIN}) followed by a rapid exponential phase (rate constant k\textsubscript{REL}). Before stress WT and Ple1d-KO MF exhibited no significant differences in any of the three relaxation parameters: KO MF (n=19 MF from 4 mice): k\textsubscript{LIN} = 4.61 ± 0.21 s\textsuperscript{-1}, t\textsubscript{LIN} = 0.046 ± 0.002 s, k\textsubscript{REL} = 45.55 ± 2.55 s\textsuperscript{-1}; WT MF (n=13 MF from 5 mice): k\textsubscript{LIN} = 4.75 ± 0.53 s\textsuperscript{-1}, t\textsubscript{LIN} = 0.043 ± 0.003 s, k\textsubscript{REL} = 49.50 ± 4.57 s\textsuperscript{-1}. However, the after the MF were mechanically stressed by the eccentric contractions, the duration of the initial slow phase (t\textsubscript{LIN}) and the rate constant of the rapid exponential phase (k\textsubscript{REL}) became significantly slower in Ple1d KO compared to WT: KO MF: k\textsubscript{LIN} = 3.75 ± 0.21 s\textsuperscript{-1}, t\textsubscript{LIN} = 0.073 ± 0.005 s, k\textsubscript{REL} = 21.13 ± 1.24 s\textsuperscript{-1}; WT MF: k\textsubscript{LIN} = 3.55 ± 0.35 s\textsuperscript{-1}, t\textsubscript{LIN} = 0.056 ± 0.005 s, k\textsubscript{REL} = 26.23 ± 2.07 s\textsuperscript{-1}. Therefore, presence of plectin-1d reduces stress-induced deceleration of relaxation.

Conclusion: The kinetics of force relaxation appears to be sensitively influenced by the high mechanical stress. Since the relaxation kinetics is determined by organized sarcomere dynamics, stress-induced slower down of relaxation could be a sign for sarcomere inhomogeneity.

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Structure-related force deficit predicted by quantitative multiphoton microscopy of single skeletal muscle fibers from an animal model of human desminopathy
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Question: Inherited degenerative muscle diseases, such as myofibrillar myopathies, often result in progressive weakness, impaired quality of life and eventually, premature death. We examined the ultrastructural basis of muscular malfunction of one of the most common myofibrillar myopathies caused by the human R350P desmin mutation. Mutant desmin filaments are thought to build pathological protein aggregates, which may remodel the 3D arrangement of myofilaments within single myofibers. The time course, in particular the onset of the phenotype, is not well characterized. Therefore, we introduce a novel approach to provide, for the first time, quantitative data on changes in the myofibrillar ultrastructure in desminopathy muscles from young animals.

Methods: We analyzed wildtype littermates of heterozygous and homozygous mice with the R349P desmin mutation that corresponds to the human R350P desminopathy. Label-free Second Harmonic Generation microscopy was used to image the myosin architecture of single muscle fibers in 3D and was complemented by desmin immunofluorescence imaging. Ultrastructural changes were quantified by two parameters. One descriptor of myofibrillar alignment is called ‘cosine angle sum’ (CAS) and one measure for sarcomere lattice disruptions is the ‘vernier density’ (VD). Single muscle fibers of M. soleus, M. extensor digitorum longus and Mm. interosseus plantae were analyzed.

Results: Homozygous R349P muscle fibers showed de-arranged myofibrillar lattice geometry which was reflected by significantly larger VD values compared to wildtype and heterozygous mice. The myofibrillar misalignment of muscle fibers from homozygous mice resulted in significantly lower CAS values compared to wildtype and heterozygous mice. These ultrastructural changes could be quantified in each of the analyzed muscles to a different extent.

Conclusions: Remodeling of the 3D myofibrillar architecture in R349P mice could be a first structural indicator of muscle weakness in human R350P desminopathy patients in addition to the occurrence of protein aggregates. Therefore, the early changes in myofibrillar architecture in homozygous R349P mice might be a prime determinant for the ongoing progression of the disease which we will address in upcoming studies on aged mice.

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P288
Gene targeting of CALD1 which encodes caldesmon: effect on murine development and smooth muscle contractility
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Background: Caldesmon (CaD), which binds to actin, myosin, calmodulin and tropomyosin, is expressed in two isoforms generated by alternative splicing: h-CaD present in skeletal muscle, and I-CaD present in smooth muscle and a wide variety of non-muscle cells. Based on biochemical evidence, CaD was proposed to inhibit force production and to contribute to the latch state during tonic contractions. Recently it was reported that homozygous h-CaD deficient mice are viable and exhibit a but small slowing of relaxation of vascular SM ex vivo (Guo et al., 2013). However, the results were confounded by upregulation of non-muscle I-CaD in SM. Here, we investigated whether ablation of all CaD splice variants has a more pronounced effect on SM contractility and whether ablation of the N-terminal myosin binding domain interferes with force maintenance.

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Methods: We generated two mouse models with targeted deletion of (i) exon2 encoding the myosin binding domain resulting in expression of truncated CaDs lacking myosin binding in both isoforms (∆exon2), and (ii) deletion of all splice variants of the complete protein (CaD-KO). Expression of truncated h-CaD and l-CaD in ∆exon2 and complete loss of protein in CaD-KO mice was verified by Western Blotting.

Results: ∆exon2 mice have a normal life span and no obvious phenotype but the litter size is smaller. Expression of the truncated CaD was reduced by ~50% whereas that of other contractile proteins was not different. Tension maintenance and dose response relations to carbachol of longitudinal ileal SM strips and to noradrenaline of tail arteries were not significantly different but relaxation from maximal Ca2+-activated contractile proteins was not different. Tension maintenance of the physiological umbilical hernia whereby the size of the abdominal wall defect was variable. Otherwise no other gross macroscopic or microscopic pathology was evident. Bladder contractility of hets was not significantly affected.

Conclusion: Reduction of CaD expression by ~50% and loss of myosin binding has but a small effect on contractility. However, CaD is essential during development for abdominal wall closure which likely can be ascribed to the tropomyosin and / or actin binding domains of l-CaD.

Results: Angiotensin II induced an extensive cardiac dilatation associated with repressed contractile function already after two days of AngII treatment. A similar phenotype was found in iCMp38KO mice. The cardiac depression was associated with a severe weight loss (WT 26.9 g basal; 25.5 g AngII; KO 26.8 g basal; 23.0 g AngII) in p38 KO. Microarray analysis revealed that AngII treatment induced a major alteration of the cardiac gene expression profiles in WT and SM22α-p38 KO hearts. Moreover, comparison of gene expression in WT and p38KO hearts after AngII revealed major differences which may be due to cardiac dilatation. Surprisingly, the M. plantaris of Ang II treated Sm22α-p38 KO mice showed approximately 1300 differentially expressed genes (> 3-fold) when compared to AngII treated controls. They had started already a muscle wasting related gene expression program, indicated by up-regulation of a plethora of atrogenes (e.g. FoxO1, FoxO3, Trim63 (MuRF1), Fbox3 (Atrogin1)) which are associated with skeletal muscle protein degradation. Furthermore, we identified several upregulated cytokines (IL-6, IL1β, a.o.) in the heart, which might play an important role in the cross talk between heart and skeletal muscle.

Conclusions: The high number of deregulated genes in the skeletal muscle of SM22α- p38KO mice after two days of AngII treatment are most likely triggered by the cardiac dilatation as a primary event. IL-6 and IL1β are interesting candidates released by the heart which may induce the wasting associated upregulation of atrogenes in skeletal muscle.

**P290**

Effects of soft diet on central nuclei, fibre diameters and collagen content in masticatory muscles of dystrophin-deficient mdx mice

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Question: Duchenne muscular dystrophy (DMD) patients experience often extreme variations of dentofacial traits and some orofacial functional limitations probably due to masticatory muscle weakness. There is little information about effects of mechanical load on masticatory muscles in DMD patients. To determine possible effects of exercise in terms of reduced masticatory load we investigated the influence of soft diet on masseter and temporal muscles in the mdx mouse, an animal model for DMD. To this end we performed histological analyses of masseter, temporal and, as a control, soleus muscles of mdx and wildtype (WT) mice.

Methods: Muscle sections from superficial and deep masseter anterior and posterior, temporalis and soleus muscles of 100-days old mdx and wildtype mice (n=4) fed hard and soft diet were histologically analyzed. The sections were stained with hematoxylin and eosin (H&E) according to standard protocols. Fibres containing central nuclei were counted and fibre diameters determined. Sirius red stain was performed to detect collagen content (connective tissue).
The area covered by connective tissue was quantified using BZ-II software (Keyence, Osaka, Japan). Body weight of the animals and muscle weights were taken immediately after sacrificing.

**Results:** The mean body weights of mdx mice fed hard and soft diet did not differ. The same observation was made for the two groups of WT mice. However, the mean body weight of mdx mice fed soft diet was significantly increased compared to WT mice fed soft diet (mdx vs. control; 30.6±1.0 g vs. 27.3±1.9 g, n=4, both, p < 0.05). Similarly, mdx mice fed hard diet had higher body weight values compared to WT mice fed hard diet. Masticatory muscles of mdx mice fed soft and hard diet contained 75–88% and 78–84% fibres with centralised nuclei, respectively. Mean fibre diameter of muscles from mdx mice fed hard diet did not differ from age-matched mdx muscles in the soft diet group. The same result was obtained for muscles of corresponding WT mice. Sirius red staining performed on muscle sections demonstrated the expected increased collagen content in muscle tissue of mdx mice. The area covered with connective tissue (Sirius red positive) was between 11 and 13% for all tested mdx muscles and between 6 and 7% for the corresponding WT muscles.

**Conclusions:** Decreased masticatory load through soft diet does not seem to attenuate pathological changes in mdx masticatory muscles regarding the amount of centralised nuclei, mean fibre diameter and fibrosis. A comparison of the muscle fibre types regarding their distribution and mean fibre diameter is in progress.

**P291**

**Exercise intensity and volume influences regulation of focal adhesion constituents in human skeletal muscle**

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**Introduction:** Within the context of our investigations, we considered a regulation of structural and functional muscular adaption processes mediated through focal adhesion components (FAs). FAs have been identified as mechanical sensors, able to transduce mechanical stimuli into biochemical signals, which might be critical for human skeletal muscle adaptation. It has been shown that integrin-linked kinase (Ilk), as a central structural protein of FAs, regulates skeletal muscle integrity in mice under sedentary conditions. However, it remains unknown whether FAs are regulated by physiological loading, e.g. exercise, in human muscle. Therefore, we aimed here to study Ilk and its related partners vinculin, α/β-parvin (Parva/Parvb) and Pinch-1/-2 as well as related signaling pathways after exercise conditions in response to different kind of exercise modes in human skeletal muscle.

**Methods:** 2 cycling intervention groups: 1. Moderate (Mod, 9 sessions at 45% of VO2max, 16.5h total exercise time) and intensive interval (Int, 9 sessions, 95% of VO2max, 3.71h total exercise time); 6 biopsies from M. Vastus lateralis in each subject (pre, 4h/24h post 3rd session, 4h/24h/72h post 9th session). FA components as well as myosin heavy chains and related signaling pathways were analyzed by western blot (WB).

**Results:** According to our data, we were able to show that the central FA components Ilk, vinculin and parvas are regulated by physical loading, while the intensity and duration of exercise (Mod vs. Int) obviously determines critical differences in FA constituents’ pattern. We found decreased Ilk levels in Mod group throughout all post exercise time points, comparable in Int group compared to pre training. Vinculin showed increased values compared to pre in both groups after exercise. MyHC1 protein was decreased from 24h post 3rd session through 72h post 9th session compared to pre and 4h post 3rd session in Mod group, while the pattern was fluctuating with a peak at 24h post 3rd session in the Int group (details will be presented).

**Discussion:** Our results imply that FA components function as mechanically sensitive motors and induce divergent muscular adaption processes in human muscle depending on exercise intensity and volume. Therefore, FAs seem to be a molecular sensor that critically determines human skeletal muscle adaptational spectrum. Our present target is to determine changes in downstream located signalling molecules, such as pAktS473 and pFakT397, indicating that the protein turnover in FAs leads to altered signalling properties in human skeletal muscle tissue.
**Conclusion:** Voluntary running reduced body weight and amount of white adipose tissue in LDLR-/- mice. Endothelial dysfunction in LDLR-/- mice could not be improved by voluntary running. In a clinical context, physical exercise alone might not have an influence on functional parameters and LDL-C levels in patients with familial hypercholesterolemia. However, physical activity in these patients may be in general beneficial and should be performed.

**P293**

Computer laboratories for life-science education: the Virtual Physiology series

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First versions of the Virtual Physiology teaching series were developed in the mid-nineties of last century. Since then, these computer simulations of classical experiments in neurophysiology (e.g. SimNerv), cardiac physiology (SimHeart) or the physiology of skeletal (SimMuscle) and smooth muscle tissue (SimVessel) have been used to supplement or replace experiments with animal tissue at hundreds of universities and schools worldwide. An overhaul suitable for use on current operating systems is overdue.

The Marburg Neurodynamics group, using the advice of experts from other research groups and with the help of external companies, has overtaken the reprogramming of the Virtual Physiology series as resolution independent versions for use on current operating systems that can now downloaded for free from www.virtual-physiology.com as fully functioning demo versions.

Virtual Physiology II currently comprises five updated computer laboratories (see also Figure): SimHeart and SimVessel feature physiological and pharmacological experiments with the isolated, perfused heart in the Langendorff-setup and with isolated smooth muscle strips of blood vessels (aorta) and the stomach (antrum) to examine the muscle contractions after application of physiological transmitters (Acetylcholine), hormones (Adrenaline) and diverse drugs like the competitive receptor antagonists (Atropine, Phentolamine, Propranolol) as non-competitive modulators of Ca²⁺-currents and Ca²⁺-concentrations (Verapamil, g-Strophantin). An additional “drug laboratory” can be used practising the correct preparation of the requested dilutions.

SimMuscle and SimNerv offer highly realistic experimental set-ups on the computer screen to record contractions of the frog’s gastrocnemius muscle (e.g. single twitches vs. tetanic contractions, muscle fatigue, curves of isometric and isotonic maxima) or compound action potentials from the frog’s sciatic nerve (dependence on electrode positions, refractory period, anode break potentials, etc.), respectively. The fifth teaching tool, SimNeuron, additionally allows performing widely used voltage- and current-clamp experiments in an easy to overlook lab design to examine, for example, the threshold of action potential generation and its dependencies on stimulus strength and duration. It is also possible to measure the current-voltage curves to determine physiologically relevant parameters like maximum conductances, reversal potentials etc. and to examine the effect of a blockade of Na⁺- or K⁺- currents by TTX or TEA.

Renal functions

**P294**

Ectopic hyperplastic renin expressing cells can transform into erythropoietin expressing cells at any time

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Based on previous observations that renal juxtaglomerular cells -deficient for the von Hippel-Lindau (Vhl) protein- undergo a functional transformation from a renin expressing to an erythropoietin (EPO) expressing cell type, we wanted to find out, if hyperplastic/ectopic renin expressing cells that develop as a consequence of a defective renin-angiotensin-aldosterone-system are also capable to produce EPO. Therefore, we have investigated kidneys of aldosterone synthase knockout (Aldo-KO) mice with a renin cell specific 1) conditional Vhl deletion occurring already during development (Aldo-Vhl-KO-RenCre mice) and a 2) Vhl deletion induced in adult kidneys (Aldo-Vhl-KO-Cx40Cre mice).

We found out, that the typical ectopic renin expressing cell-cuffs forming multilayers around afferent arterioles in
Aldo-KO kidneys did not develop in the case of a conditional renin cell specific Vhl deletion. The loss of renin expressing cells was paralleled by a decrease of renin mRNA expression level of 1.0 ± 0.13 in Aldo-KO kidneys to 0.10 ± 0.02 in Aldo-Vhl-KO-RenCre kidneys. Similarly, plasma renin concentrations (PRC) were reduced from 3172.7 ± 538.2 to a level of 123.7 ± 28.4, which are typical values for wildtype mice. However, a closer look to the histological stainings of Aldo-Vhl-KO-Cx40iCre kidneys revealed, that there were still multilayers surrounding the afferent arterioles, but they were thin probably due to the loss of renin expression/renin storage vesicles. Vhl deletion induced in adult kidneys of Aldo-Vhl-KO-Cx40iCre mice also resulted in an absence of ectopic renin expressing cells with similar renin mRNA expression levels and PRC values as shown for the conditional Aldo-Vhl-KO-RenCre mice. However, histological investigations of Aldo-Vhl-KO-Cx40iCre kidneys displayed a considerable difference to the conditional knockout mice: the afferent arterioles were still surrounded from huge ectopic cell cuffs. Vhl deletion in both knockout mouse models resulted in a much stronger elevation of renal EPO mRNA expression levels (about 11-fold vs. Aldo-KO) and of plasma EPO concentrations (20 to 30-fold vs. Aldo-KO) than compared to wildtype kidneys with a renin cell specific Vhl deletion, indicating that the ectopic cells express EPO now instead of renin. These findings indicate, that also ectopic renin expressing cells are very plastic: the functional transformation occurs relatively quickly, whereas the morphological transformation needs more time.

**Question:** Rapidly developing arterial hypertension is a serious side effect of the treatment with vascular endothelial growth factor receptor (VEGFR) inhibitors such as sunitinib. We have shown that early sunitinib-induced hypertension is associated with increased renal vascular resistance, decreased fractional Na+ excretion and unaltered fractional Li+ excretion suggesting that sunitinib enhances renal Na+ reabsorption in the distal nephron or collecting duct (CD). We tested the hypotheses that VEGF down regulates the epithelial sodium channel (ENaC) in CD cells and that the ENaC inhibitor amiloride decreases arterial pressure rise within 4 days. Amiloride reduced arterial pressure by 10 mmHg within 4 days in sunitinib-treated but not in control rats (p < 0.05). The acute natriuretic responses to amiloride were similar in both groups while plasma K+ concentrations were significantly higher in sunitinib-treated than in control rats after 4 days of amiloride treatment (5.3 ± 0.2 vs. 4.3 ± 0.2 mmol/l, p < 0.001).

**Conclusions:** Our data indicate that VEGF down regulates ENaC in renal CD cells and suggest that increased ENaC activity contributes to sunitinib-induced hypertension. While an increased natriuretic response to amiloride was not readily discernable, our finding of an elevated plasma K+ concentration points towards an increased ENaC activity in sunitinib-treated rats.

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**P295**

**Vascular endothelial growth factor down regulates the renal epithelial sodium channel**

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**Question:** According to the vascular and tubular effects of the natriuretic peptides ANP and BNP, their common receptor guanylyl cyclase A (GC-A) is expressed in renal vessels and tubular cells. Additionally GC-A is expressed in podocytes, with unknown functions. In order to clarify the role of GC-A in podocytes we generated mice with podocyte specific deletion of GC-A (Podo/GC-A KO).

Podocytes of control mice (Podo/GC-A CTR) had higher GC-A mRNA expression than all other glomerular and tubular cells. GC-A mRNA in podocytes of Podo/GC-A KO was reduced to 1% of CTR. Despite of this effective deletion, Podo/GC-A KO and CTR did not differ regarding their blood pressure, glomerular filtration rate, albuminuria, salt and water excretion under control conditions. Moreover, infusion of ANP and BNP resulted in similar increases in the GFR and in the renal perfusion in KO and CTR.

Since natriuretic peptides are renoprotective in several disease models, we next investigated whether these protective properties are related to GC-A activation in podocytes. Application of the mineralocorticoid DOCA + a high salt intake for 5 weeks induced similar slight increases in the blood pressure in KO and CTR. However, compared with CTR, Podo/GC-A KO developed massive albuminuria (CTR: 15-fold vs. control; KO: 300-fold vs. control), a reduction of GFR and marked glomerular damage including podocyte foot process effacement, mesangial expansion and glomerulosclerosis.

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**P296**

**Natriuretic peptides exert marked renoprotective effects via activation of the natriuretic peptide receptor GC-A in podocytes**

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According to the vascular and tubular effects of the natriuretic peptides ANP and BNP, their common receptor guanylyl cyclase A (GC-A) is expressed in renal vessels and tubular cells. Additionally GC-A is expressed in podocytes, with unknown functions. In order to clarify the role of GC-A in podocytes we generated mice with podocyte specific deletion of GC-A (Podo/GC-A KO).

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P297
Systemic hypoxia affects attenuated expression of erythropoietin in old age

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Introduction: The exposure to hypoxia in both altitude and simulated conditions are valid methods used to improve physical performance at sea-level or altitude. This is affected by a hypoxia-induced increase of erythropoiesis which leads to an increased oxygen uptake. These mechanisms are only investigated for young people, yet. It is promising to use the hypoxia-induced adaptions also to improve the physical performance in old age. However, due to physiological changes in old age, findings of young people might not be transferable. The purpose of this study was to evaluate the possible different points of time as well as the rates of EPO-expression during a predefined systemic hypoxia in old as compared to young cohorts to identify the required, probably age-dependent, dose of hypoxia to increase erythropoiesis.

Method: Thirty-five old (60 to 70 years) as well as twenty-nine young people (18 to 30 years) were each randomly assigned to hypoxia groups (HG-old; HG-young) or control groups (CG-old; CG-young). The control groups received ambient air (fractional inspired concentration of oxygen=FiO2: approximately 20.9%) and the hypoxia groups received normobaric hypoxia. The FiO2 was individually adjusted to 80% to reach a sO2 of 80% in the hypoxia groups, the FiO2 differed significantly (main group effect: F1,27=1.981, p=.049, n2=0.136) between old (11.7±1.6%) and young people (10.6±1.1%). In HG-old and HG-young, EPO levels after 180 minutes (old: +14.11±16.54%, time effect: p=.000; young: +44.60±41.89%, time effect: p=.007) and 30 minutes after the intervention (old: +27.45±23.73%, time effect: p=.026; young: +78.50±48.70%, time effect: p=.004) significantly increased, where the improvement were significantly greater in the young cohort (group effects: at 180 minutes: p=.023; 30 minutes after the intervention: p=.000).

Discussion: We observed that old people require a higher FiO2 to reach a predefined systemic hypoxia as compared to young people suggesting the existence of attenuated compensatory mechanisms (e.g. due to an age-dependent decrease in sensitivity of chemoreceptors the compensation of the respiratory and/or the cardiovascular system might be reduced). Furthermore, we found a significant increase of EPO levels after 180 minutes independent of age, where the accumulation of EPO may already occurs between 90 and 180 minutes. However, the EPO expression rate in old age was attenuated, which may be caused by the age-dependent decrease in activity of the hypoxia-induced factor (HIF) and the reduced renal functional capacity. For the first time it was shown that there might be an age-dependent differentiation in the dose of hypoxia.

P298
Reverse effect on renin expression by inhibition of nitric oxide and cyclooxygenase-2 in aldosterone synthase deficient and wild type mice

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Question: Chronic challenges of the renin-angiotensin-aldosterone system (RAAS), such as a decrease of extracellular volume due to restricted NaCl intake or a loss of blood cause recruitment of renin-producing cells in the kidney cortex. In mice with pharmacologic inhibition of the RAAS this reversible adaptation of renin cells comprises two distinct components, namely a transformation of vascular smooth muscle cells in the afferent arteriole into renin producers on the one hand and an induction of renin expression in perivascular cells of the juxtaglomerular apparatus, which is commonly referred to as JGA hypertrophy on the other hand. Considering the regulating factors it has been reported that the recruitment of renin cells in the afferent arteriole is likely dependent on the NO system, via its second messenger cGMP, which does not affect renin expression in the perivascular juxtaglomerular area. The latter process is supposed to involve signaling via COX-2 derived prostanoids in the induction of JGA hypertrophy. Since elevated renin levels are usually accompanied by an upregulated COX-2 expression one could ask, whether this is causality or epiphenomenon. In our current study we assessed the effect of NO and COX-2 in states of permanent renin overexpression.

Methods: We investigated mice lacking aldosterone synthase (AS/-) which exhibit a drastically increased number of renin cells, that are partly located in the media layer of preglomerular arterioles and more prominent in cellular multilayers around afferent arterioles. The mice were subjected to either NO-synthase blockade by L-Nitroarginine methyl ester (L-NAME) or a COX-2-blockade using the selective COX-2 inhibitor SC-236 for two weeks.

Results: In contrast to our expectations we found that NO and COX-2 prostanoids regulate the renin expression in AS/- mice in a reversed fashion compared to mice in which the number of renin cells is elevated due to pharmacologic interventions, such as enalapril treatment. In AS/- mice COX-2 inhibition reduced renin expression to 25% of the initial value, whereas NO-blockade did not alter the number

In conclusion, ANP/GC-A/cGMP signaling in podocytes does not appear to be involved in the acute regulation of kidney function. However it has marked protective effects on podocyte integrity attenuating massive albuminuria and renal failure in response to kidney damage.
of renin cells. In low salt/enalapril treated wild type mice we made the contrary observation. SC-236 had no effect on renin expression, while L-NAME administration led to a decrease to 40%. These changes were paralleled by plasma renin concentration values.

**Conclusion:** We conclude that the regulative mechanisms of renin-producing cells in mice with pharmacological inhibition of the RAAS in which a reversible transformation occurs, differ from the mechanisms of genetic loss-of-function models, in which renin cells exist prior to treatment.

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**P299**

**Nephrotoxic adverse reactions to new peptide ACE-Inhibitors in comparison to traditional ACE-Inhibitors**

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**Question:** ACE-Inhibitors (ACEi) are first line treatment for arterial hypertension in humans. However, they are contra-indicated during pregnancy since there is a severe adverse reaction in the fetal kidney. This is in line with the observation that inactivating mutations in the Renin-Angiotensin-System (RAS) lead to a fatal kidney phenotype in man and in mice. Recently, peptides in common food products such as sour milk have been identified to inhibit ACE thus representing an alternative for the classical pharmacological ACEi. Therefore we aimed to investigate whether the application of the dipeptide ACEi Isoleucin-Tryptophan (IW) in pregnant mice differs from the mechanisms of genetic loss-of-function models of renin cells. In low salt/enalapril treated wild type mice we made the contrary observation. SC-236 had no effect on renin expression, while L-NAME administration led to a decrease to 40%. These changes were paralleled by plasma renin concentration values.

**Conclusion:** We conclude that the regulative mechanisms of renin-producing cells in mice with pharmacological inhibition of the RAAS in which a reversible transformation occurs, differ from the mechanisms of genetic loss-of-function models, in which renin cells exist prior to treatment.

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**P300**

**[Ca\textsuperscript{2+}] oscillations and IL-6 release induced by \(\alpha\)-haemolysin from *Escherichia coli* require P2 receptor activation in renal epithelia**


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Urinary tract infections are commonly caused by \(\alpha\)-haemolysin (HlyA)-producing *Escherichia coli*, which by pore-formation in cell membranes renders cells permeable to ions and water. In erythrocytes, the effect of HlyA is strongly amplified by P2X receptors activated by ATP most likely passing through the HlyA pore itself. In renal epithelia, HlyA induces [Ca\textsuperscript{2+}]\textsuperscript{2+}, oscillations and subsequent interleukin-6 (IL-6) and IL-8 release. We speculate that this effect is caused by HlyA-induced ATP release and subsequent P2 receptor activation.

Here, we show that HlyA initiate marked, reversible [Ca\textsuperscript{2+}]\textsuperscript{2+} oscillations in renal epithelia. HlyA keenly triggered ATP release from MDCK cells and accordingly the HlyA-induced [Ca\textsuperscript{2+}]\textsuperscript{2+} oscillations were completely prevented by ATP-scavenging. These findings were confirmed with ATP-biosensor cells. In native 132-1N1 astrocytoma cells that do not express any P2 receptors, HlyA barely caused any changes in [Ca\textsuperscript{2+}]. Transfection with the hP2Y\textsubscript{2} receptor resulted in an extensive increase in HlyA-induced [Ca\textsuperscript{2+}]\textsuperscript{2+} oscillations. Moreover, the HlyA-induced [Ca\textsuperscript{2+}]\textsuperscript{2+} oscillations were markedly reduced in medullary thick ascending limb isolated from P2Y\textsubscript{2} receptor knockout mice compared to wild type. Interestingly, the HlyA-induced IL-6 release observed in wild type was absent in the knockout. These results suggest that HlyA triggers ATP release from renal epithelia, which via P2Y receptor activation is responsible for the HlyA-induced [Ca\textsuperscript{2+}]\textsuperscript{2+} oscillations and the following IL-6 release. Moreover, the current data are consistent with ATP as the mediator of HlyA-induced IL-6 release and thus, potentially essential to immune system-mediated eradication of the infection.
P301

AVP-mediated regulation of the paracellular permeability in the thick ascending limb (TAL)

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Question: The thick ascending limb of Henle’s loop (TAL) drives an important part of the reabsorption of divergent cations. This occurs through the cation selective paracellular pathway. Arginine vasopressin (AVP) stimulates the trans-epithelial transport in the TAL. We wanted to study how AVP stimulation affects paracellular permeability in the TAL.

Methods: TALs were mechanically dissected for microperfusion from 8–10 weeks old mice that were kept for 4 days on water-restricted (WR) (0.26ml/gBM/d) or water-loaded (WL) (0.78ml/gBM/d) conditions, and from 5–10 week old mice to be stimulated acutely with AVP. We measured trans-epithelial resistance and voltage (Rte, Vte), and calculated the equivalent short circuit current (Isc). From diffusion voltages, in presence of furosemide, we calculated the paracellular permeability (P) for Na+, Cl-, Mg2+ and Ca2+. Measurements were performed at 37°C with continuous perfusion at a constant pressure.

Results: Freshly isolated TAL from the WR group (n=20) were transporting more actively compared to the WL group tubules (n=15), presenting a higher Vte and Isc, while Rte was lower. Diffusion voltages were also increased in the WR group, indicating a higher PNa/PCl, and a higher PNa compared to WL. Acute TAL stimulation ex-vivo for 12 minutes with AVP (n=6), in paired experiments, increased trans-epithelial transport. Isc and Vte increased when stimulated, while in the time control group Isc and Vte decreased instead. At the same time, AVP induced more pronounced diffusion voltages compared to control, with an increase in PNa/PCI.

Conclusion: Water restriction and AVP increase paracellular selectivity and permeability for cations in the TAL.

P302

Structure and function of the podocyte slit diaphragm

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Question: The slit diaphragm is a multiprotein complex and constitutes the final component of the glomerular filtration barrier. Despite intense research for more than one decade its molecular ultrastructure and function of individual components have remained mostly elusive.

Methods: We established a complimentary set of conditional and constitutive Neph1, Neph2, Neph3 and Nphs1 knock-out mice. To compare SDs across the animal kingdom, chicken and mice kidneys were used. Detailed analysis was performed with functional assays, WB, light microscopy, IF, STORM-Microscopy, TEM, Immuno-EM, SEM, Helium-Ion-Microscopy, as well as Cryo-Electrontomography combined with 3D reconstruction.

Results: As expected NPHRIN and NEPH1 are essential to build a proper slit diaphragm. Yet in both types of constitutive knock-out animals rudimentary SDs based on the remaining Super-IgG molecule could be detected. Interestingly, Neph2 and Neph3 constitutive KO mice, while being postulated to be expressed in podocytes, did not show any obvious phenotype over a 2 year observational period. Within the native cryopreserved SD ultrastructural analysis revealed that in contrast to previous reports the SD is a multi-layered cell-cell contact that does not overlap in the midline, but rather forms multi-layered junctions. Individual components such as from cell membrane to cell membrane. Immuno-EM localized NPH1 to the basal and narrower aspects of this junction, while NPHRIN molecules form the top layer facing Bowman’s space. Despite NPHRIN’s fundamental role in mammals, reptiles and birds live healthily without NPHRIN and present with narrower and leakier NEPH1 based slit diaphragms.

Conclusion: This unique comparative approach revealed fundamentally new insights into the composition of the slit diaphragm. While NPHRIN is essential for the formation of the slit diaphragm in mammals, it is dispensable in birds. NEPH1 seems to form the basal aspects of this junction in mammals and is the main component in birds. This ultrastructural analysis challenges old views from the SD as a thin grey line linking two adjacent footprocesses but rather points towards a multi-layered cell-cell contact.

P303

Checkpoint kinase Chk2 controls renal Cyp27b1 expression, calcitriol formation, and calcium-phosphate metabolism


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Question: Checkpoint kinase 2 (Chk2) is the main effector kinase of ataxia telangiectasia mutated (ATM) and responsible for cell cycle regulation. ATM signaling has been shown to up-regulate interferon-regulating factor-1 (IRF-1), a transcription factor also expressed in the kidney. Calcitriol (1,25(OH)2D3), a major regulator of mineral metabolism, is generated by 25-hydroxyvitamin D 1-hydroxylase in the kidney. Calcitriol formation, and calcium-phosphate metabolism, while NEPHRIN and NEPH1 are essential to build a proper slit diaphragm. Yet in both types of constitutive knock-out animals rudimentary SDs based on the remaining Super-IgG molecule could be detected. Interestingly, Neph2 and Neph3 constitutive KO mice, while being postulated to be expressed in podocytes, did not show any obvious phenotype over a 2 year observational period. Within the native cryopreserved SD ultrastructural analysis revealed that in contrast to previous reports the SD is a multi-layered cell-cell contact that does not overlap in the midline, but rather forms multi-layered junctions. Individual components such as from cell membrane to cell membrane. Immuno-EM localized NPH1 to the basal and narrower aspects of this junction, while NPHRIN molecules form the top layer facing Bowman’s space. Despite NPHRIN’s fundamental role in mammals, reptiles and birds live healthily without NPHRIN and present with narrower and leakier NEPH1 based slit diaphragms.

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Methods: Chk2-deficient mice (chk2−/−) were compared to wild type mice (chk2+/+). Transcript levels of renal 25-hydroxyvitamin D 1α-hydroxylase, Chk2, and IRF-1 were determined by RT-PCR, Klotho expression by Western blotting, bone density by µCT analysis, serum or plasma 1,25(OH)2D3, PTH, and C-terminal FGF23 concentrations by immunoassays and serum, fecal, and urinary calcium and phosphate concentrations by photometry.

Results: The renal expression of IRF-1 and 25-hydroxyvitamin D 1α-hydroxylase as well as serum 1,25(OH)2D3 and FGF23 levels were significantly lower in chk2−/− mice compared to chk2+/+ mice. Plasma PTH was not different between the genotypes. Renal calcium and phosphate excretion were significantly higher in chk2−/− mice than in chk2+/+ mice despite hypophosphatemia and normocalcemia. Bone density was not different between the genotypes.

Conclusions: We conclude that Chk2 regulates renal 25-hydroxyvitamin D 1α-hydroxylase expression thereby impacting on calcium and phosphate metabolism.

P304
Influence of different diuretic states on the paracellular pathway of inner medullary collecting ducts and thin limbs
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Questions: In mammals different states of diuresis require perfectly balanced mechanisms of urinary concentration. However the role of paracellular transports in these concentrating mechanisms is not fully understood. The loop of Henle, collecting ducts and vasa recta generate an osmolality gradient from the isoosmotic cortex to the hyperosmotic papilla. To adapt the gradient to different states of water homeostasis transcellular and paracellular transport mechanisms must be strictly regulated. We investigated inner medullary collecting ducts (IMCDs), descending thin limbs (IMdTLs) and ascending thin limbs (IMaTLs) under different states of diuresis.

Methods: Three experimental groups of 4–6 week old Sprague Dawley rats were kept for three days under three different regimes of water intake: water restriction (0,18 ml / g BW / d), free access to water at furosemide (0,55 ml / g BW / d) . The paracellular properties of the isolated perfused IMCDs, IMdTLs and IMaTLs were investigated by detection of transepithelial resistance Rte and diffusion potentials DP. DPs were measured by 50 mM NaCl on the basolateral side and 245 mM NaCl on the luminal side. Experimental solutions were adjusted to an osmolality of 600 mosm / kg H2O to mimic inner medullary conditions.

Results: Treatments led to different urinary production and urinary osmolality. The water restriction group had an urinary production of 0,054 ± 0,005 ml / g BW / d and an osmolality of 2253 ± 191 mosm / kg H2O, the water diuresis reached values of 0,305 ± 0,010 ml / g BW / d and 364 ± 12 mosm / kg H2O, the group with diuresis influenced by furosemide was at 0,178 ± 0,013 ml / g BW / d and 859 ± 51 mosmol / kg H2O. The measurements of paracellular properties showed no differences in IMCDs and IMdTLs whereas differences could be observed in IMaTLs. Rte was similar in the three groups: water restriction: 15,0 ± 4,0 W cm², furosemide induced diuresis: 14,4 ± 2,4 W cm² and water diuresis: 14,0 ± 2,4 W cm². The DP of the water restriction group was at -0,27 ± 0,40 mV and significantly different to the DP of the water load group at -4,66 ± 1,77 mV. The DP of the furosemide treated rats was at -1,99 ± 1,72 mV. The calculated permeability ratio of sodium over chloride (P Na/Cl) was 0,99 ± 0,02 under water restriction, 1,22 ± 0,18 under furosemide induced diuresis and 1,45 ± 0,19 under water diuresis.

Conclusion: Under different states of diuresis the paracellular properties of IMCDs and IMdTLs do not change. However high water load does influence the paracellular tight junction properties of IMaTLs to higher cation selectivity.

P305
Fura-2 calcium imaging in renin producing cells in situ
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Question: The protease renin is produced and stored in lysosome related organelles in juxtaglomerular cells of the kidney. Despite the lack of knowledge about the membrane events mediating the release of stored renin into the extracellular space, there is agreement that cAMP and calcium are the cardinal intracellular signals controlling renin release. Calcium appears to play a rather unusual role in the control of renin secretion as it is thought to inhibit renin secretion i.e. exocytosis in juxtaglomerular cells. In order to obtain more direct insight into the regulation of intracellular calcium concentration in renin secreting cells, we have established an experimental model that allows to trace the intracellular concentration of calcium in renin secreting cells using the fluorescent calcium indicator fura-2.

Method: We isolated glomeruli with attached afferent arterioles from kidneys of mice harbouring a BAC chromosome with GFP under the control of the Ren-1 gene promoter. Thus GFP fluorescence identifies all cells actively expressing the renin gene. In pilot experiments with adult kindneys we detected GFP fluorescence in juxtaglomerulars only. Glomeruli with GFP fluorescence were selected by hand and transferred into a perfusion bath, where they were loaded with fura-2 AM. Fura-2 fluorescence could be well separated from endogenous GFP fluorescence. After washing, we conducted experiments with ringer-solution containing 100 nM angiotensin II and 100 µM ATP. Additional experiments were performed with either 1 nM endothelin-1 or 1 µM phenylephrine.

Results: We found that angiotensin II (100 nM) and ATP (100 µM) elicited calcium transients followed by sustained elevations of the intracellular calcium concentration in GFP-positive renin producing cells in all experiments. Notably other glomerular cells, which could not be distinguished between capillary endothelial cells, podocytes or mesangial cells frequently responded to ATP but not to angiotensin II. Experiments with endothelin 1 (1 nM) and phenylephrine
(1 µM) produced variable results. In some but not all experiments endothelin 1 and phenylephrine increased calcium concentration in renin producing cells.

**Conclusion:** We conclude that angiotensin II increased the intracellular calcium concentration via the AT1-receptor which is expressed in renin producing cells. ATP also increased the intracellular calcium concentration, presumably via purinergic P2Y-receptors expressed in these cells. Our experiments with Endothelin-1 and phenylephrine produced variable results. In several experiments we observed no reaction of glomerular cells on administration of endothelin and phenylephrine, whereas in other experiments only renin producing cells responded with a significant increase of intracellular calcium concentration. We conclude that ET(A) receptors and α-adrenoreceptors are not necessarily expressed in renin producing cells.

**P306**

**Kidney phenotype of mice with induced deletion of renin in juxtaglomerular cells**

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**Question:** Inactivating mutations of renin gene lead to identical deleterious kidney phenotype in humans and mice resulting in renal insufficiency and premature death. Such genomic changes affect almost 10% of all renal cells since renin is largely expressed throughout the kidney during embryonic development. However, in the adult kidney the renin-positive juxtaglomerular (JG) cells account for less than 0.1%. Therefore it is still unknown if the renin deficiency in JG cells is detrimental for kidney function and morphology. To fill this gap of knowledge we generated triple transgenic mice in which renin was inducibly deleted in JG cells when nephrogenesis was already accomplished.

**Methods:** Generation of transgenic mice through tet-on and cre-lox systems, kidney function phenotyping, Glomerular filtration rate (GFR) and blood pressure measurement, immuno-histochemistry.

**Results:** Doxycycline (Dox) was used to induce renin knockout in JG cells of mature male mice (3.5 months ± 2 weeks old, JG-RenKO). Dox-treated littermates with wildtype renin alleles were used as control (JG-RenWT). JG-RenWT and JG-RenKO were followed for six months after induction. Dox efficiently reduced the renin production in JG cells of JG-RenKO mice. However, renin expression recruited to the upstream parts of the afferent arterioles of the knockout animals. Correspondingly the arterial blood pressure in the JG-RenKO group was lower after the induction but normalized at the end of the observation period. The JG-RenKO mice developed glomerular hyperfiltration accompanied by diuresis, increased excretion of osmolytes and mild albuminuria. At the end of the experiment the kidneys of the JG-RenKO animals displayed mildly increased mesangial matrix deposition without further apparent structural aberrations.

**Conclusions:** Renin in the JG cells is dispensable for the maintenance of the normal kidney morphology in adult mice. The renin deficiency in JG cells leads to compensatory recruitment of renin production upstream in the afferent arterioles and increased GFR. Therefore the described transgenic mice represent a useful model to delineate the effect of glomerular hyperfiltration from further pathophysiological cues in chronic kidney diseases such as diabetic nephropathy.

**P307**

**Effects of sexual steroid synthesis in podocytes?**

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One of the most frequently occurring nephrotic syndrome in children is the minimal change nephrotic syndrome (MCNS). A remarkable feature of this syndrome is the persistence in only 20% of the patients while 70-80% show a spontaneous recovery during puberty, thus recovery is paralleled by increasing levels of sexual hormones. These findings point to an effect of sex hormones on the functional structure of podocytes. The podocytes form the glomerular filtration barrier with their foot-like processes, which is essential for the clearance of primary urine and retention of defined proteins in the blood. Dysfunction of this barrier results in foot-process effacement of podocytes and proteinuria, which both are common features of glomerulonephritis.

In this study, we found that primary rat podocytes express aromatase, the final enzyme of the steroid synthesis. Accordingly, we measured considerable amounts of 17β-estradiol in the supernatant of these primary cells by using a radio-immuno-assay (RIA). Phosphorylation of aromatase, which inhibits aromatase activity, in podocytes results in reduced amounts of 17β-estradiol in the medium, as previously shown in neurons and breast cancer cells. Furthermore, we found that estrogen receptors are expressed in rat podocytes. Most importantly, the expression is differentially regulated before and after puberty in a sex-dependent manner. Responsiveness of podocytes to estradiol together with estradiol synthesis in these cells offers the possibility of an autocrine in addition to an endocrine manner of regulation of podocyte function by estradiol. Our data suggest that sexual steroid synthesis in podocytes could underlie sex differences in the prevalence of MCNS.
Prorenin derived from renal tubules contributes to high circulating prorenin levels in diabetes mellitus

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Besides of its central importance in volume regulation the renin angiotensin system has a critical role in cardiovascular and renal diseases. Renin is mainly produced in and secreted from the granular juxtaglomerular cells of renal afferent arterioles. Additionally, renal tubules can express renin under pathophysiological conditions. Especially in the collecting duct renin expression has been found in angiotensin II dependent forms of arterial hypertension and in diabetes mellitus. In the plasma of humans and animals not only the enzymatically active renin is present but also high concentrations of its precursor prorenin are detectable. Interestingly, the plasma prorenin concentration is elevated in diabetic patients and higher prorenin levels are associated with diabetic nephropathy. In order to test whether prorenin from renal tubules contributes to the elevated prorenin levels in diabetest we generated mice with an inducible and specific deletion of the renin gene in all renal tubular segments using the Cre-lox system (tubule-renin KO). Plasma renin and prorenin concentrations, blood pressure and salt and water excretion were not different between tubule-renin KO and WT mice under control conditions. Mild diabetes mellitus was induced by low dose streptozotocin resulting in a 2.5-fold increase in fasting blood glucose in both genotypes. Hyperglycemia was accompanied by a 2-fold elevation of 24 hour urine excretion in tubule-renin KO and WT. The plasma concentrations of active renin were not significantly elevated 10 weeks after induction of diabetes compared to baseline in tubule-renin KO or wildtype mice. In contrast, diabetes resulted in a significant increase in the plasma concentration of prorenin in tubule-renin WT mice (baseline: 25+9 ng/ml; 10 weeks after induction of diabetes: 63+3 ng/ml; n=8, p < 0.01), whereas prorenin levels were not significantly elevated in tubule-renin KO mice (baseline: 28+9 ng/ml; 10 weeks after induction of diabetes: 43+4 ng/ml; n=8, p = 0.1), so that the plasma prorenin concentration was significantly higher in tubule-renin WT compared to tubule-renin KO. 10 weeks after induction of diabetes no increase in urinary albumin excretion was observed in either genotype. Taken together, the data indicate that prorenin from renal tubules contribute to the high circulating prorenin levels in diabetic mice. Whether the differences in plasma prorenin levels between tubule-renin WT and KO mice result in according differences in diabetic nephropathy is currently investigated in long term experiments.
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