

S1-A

FEPS European Young
Physiologists Symposium

S1-A1
Cell type-specific subcellular distribution of ion channels in the central nervous system

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Neuronal excitability is underlined by active conductances generated by voltage-gated ion channels. The functional impact of a voltage-gated ion channel is highly affected by its precise location in the axo-somato-dendritic domains of nerve cells.

In this presentation, I will demonstrate that the organizing rules governing the subcellular distribution of voltage-gated Na⁺ (Nav) and K⁺ (Kv) channels are more complicated than previously believed.

In the axon initial segment (AIS) of neurons, high density of Nav channels ensure low threshold for action potential initiation and Kv channels are key regulators of action potential repolarization, firing rate and pattern. Highly sensitive immunohistochemical methods allowed us to reveal heterogeneity in the density and distribution of distinct Nav and Kv channel subunits within the AIS of different types of nerve cells. This precise arrangement is likely to contribute to the diversity of firing properties observed among central neurons.

In dendrites, active invasion of action potentials generated in the axon is essential for associative synaptic plasticity and neuronal ensemble formation. In pyramidal cells this action potential backpropagation is supported by dendritic Nav channels. Employing the high resolution SDS-digested freeze-fracture replica-labelling method allowed us to reveal quantitative differences in the subcellular distribution of the predominant Nav channels in the hippocampal CA1 pyramidal cells.

Our results suggest that variability in the subcellular distribution of ion channels is a way of increasing neuronal diversity in the central nervous system.

S1-A2
Rapid generation of human neurons for modeling neuropsychiatric disorders

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Previous studies demonstrated the availability of various methods for differentiating human embryonic stem cells (ESCs) and induced pluripotent cells (iPSCs) into induced neurons (iN). Alternatively, human fibroblasts can also be directly converted into induced neuronal (iN) cells. These studies also described that with present techniques conversion is inefficient, synapse formation is limited, and only small amounts of neurons can be generated. However, with the advent of patient- derived, disease-specific iPSC technology, there is an enormous potential in iN cells for studying the pathogenesis of various, poorly understood neurological disorders, such as autism, schizophrenia or Alzheimer's disease. Furthermore, this technology also enables researchers to design drug screening systems, and to produce neurons for purposes of regenerative medicine. This requires the capability of large-scale production of human iN cells and with a high yield, and also necessitates the generation of iN cells that readily form synapses. Moreover, such goals could be facilitated by a high degree of reproducibility independent of the starting cell line and by production of a relatively homogeneous population of functional iN cells for experimental purposes.

We performed lentivirus-mediated exogenous over-expression of the transcription factor neurogenin 2 (Ngn2) in human ESCs and iPSCs to derive induced neurons. Potential differences in neuronal maturation, morphology, synapse formation and synaptic function was then quantified by various techniques including electrophysiology.

We show that human ESCs and iPSCs can be converted into functional iN cells with nearly 100% yield and purity in less than 2 weeks by forced expression of a single transcription factor. The resulting ES-iN or iPS-iN cells exhibit quantitatively reproducible properties independent of the cell line of origin, form mature pre- and postsynaptic specializations, and integrate into existing synaptic networks when transplanted into mouse brain.

Our approach enables large-scale studies of human neurons for goals such as analyses of human diseases, examination of human-specific genes, and drug screening.

S1-A3
Descending effect on spinal nociception by amygdaloid glutamate varies with the submodality of noxious test stimulation

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Amygdala has an important role in the processing of primary emotions, such as fear. Additionally, amygdala is involved in processing and modulation of pain. While the amygdala, particularly its central nucleus (CeA), has been shown to contribute to pain control, the descending pain regulation by the CeA is still only partly characterized. Here heat and mechanical nociception was tested in both hind limbs of healthy rats with a chronic guide cannula for microinjection of glutamate into the CeA of the left or right hemisphere.

The aim was to assess whether the descending pain regulatory effect by glutamate in the amygdala varies with the

submodality or the body side of nociceptive testing, brain hemisphere or the amygdaloid glutamate receptor. Motor performance was assessed with the Rotarod test. Amygdaloid glutamate, independent of the treated hemisphere, produced a dose-related heat and mechanical antinociception that varied with the submodality of testing. Heat antinociception was short lasting (minutes), bilateral and not reversed by blocking the amygdaloid NMDA receptor with MK-801. In contrast, mechanical antinociception lasted longer (>20 min), was predominantly contralateral and reversed by blocking the amygdaloid NMDA receptor. At an antinociceptive dose, amygdaloid glutamate failed to influence motor performance. The results indicate that independent of the brain hemisphere, the spatial extent and duration of the descending antinociceptive effect induced by amygdaloid glutamate varies with the amygdaloid glutamate receptor and the submodality of pain.

S1-A4

The effect of NOS inhibitors on radical signaling and antioxidant response in Wistar rats

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Despite of many experimental studies, the effect of NOS inhibition on activation of radical pathway (AT1R-NADPH oxidase-superoxide) is not yet sufficiently clarified. In our study, we used two inhibitors of NO-synthase, specific inhibitor of nNOS: 7-nitroindazole (7-NI) and nonspecific inhibitor of NOS: NG-nitro-L-arginine-methyl ester (L-NAME). The aim of our study was to determine changes in free radical signaling, antioxidant and detoxification response in left ventricular of heart (LV) and brainstem (BS) of young and adult Wistar rats (WR) during NO-deficiency.

Young (4 weeks) and adult (10 weeks) WR were treated with 7-NI (10 mg/kg/day), L-NAME (50 mg/kg/day) or drinking water (Control) during 6 weeks. Systolic blood pressure was measured by non-invasive plethysmography. Level of superoxide was determined by Lucigenin-enhanced chemiluminescence. Expression of genes (AT1R, p22phox subunit of NADPH oxidase, SOD and NOS isoforms, HO-1, MDR1a and housekeeper GAPDH) were identified by real-time PCR. Activity of NOS was detected by conversion of [3H]-L-arginine on [3H]-L-citrulline and activity of SOD was measured by UV VIS spectroscopy.

Blood pressure was increased after L-NAME treatment in young and adult WR. Activity of NOS was decreased only after L-NAME application (young and adult rats) without changes in eNOS and nNOS mRNA. Both inhibitors decreased superoxide level in LV of young animals without changes in AT1R or p22phox mRNA expression, while in adult rats only L-NAME affected AT1R and p22phox mRNA elevation in BS. Increased SOD activity positively correlated with SOD3 and HO-1 mRNA elevation only in this group, where we found also MDR1a mRNA elevation. Increase in MDR1a was observed also in young rats in different tissues

and different inhibitors: in LV of L-NAME group and BS of 7-NI group.

We can conclude that radical signaling and antioxidant response was mainly influenced in L-NAME group and this response was more pronounced in LV of young rats and in BS of adult WR rats.

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S1-A5

Quantitative pilomotor axon-reflex test to assess autonomic dysfunction in Parkinson's disease

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BACKGROUND: Early detection of Parkinson's disease (PD) contributes to treatment success but is limited by the lack of techniques to assess early PD symptoms such as autonomic neuropathy. We recently demonstrated that peripheral autonomic nerve function can be assessed through iontophoresis of phenylephrine to elicit axon-reflex mediated piloerection in an indirect skin region outside the region of iontophoretic stimulation using the quantitative pilomotor axon-reflex test (QPART). (1) AIM. This study aimed to assess the hypothesis that QPART is a valid measure of autonomic pilomotor nerve dysfunction in early PD.

METHODS: Six healthy subjects and six age matched PD patients (Hoehn and Yahr stages I-II) participated in a controlled study. Piloerection was stimulated by iontophoresis of 1% phenylephrine on the dorsal forearm. Silicone impressions of resulting goose bumps were digitally quantified by number and area. Comparative autonomic measures included heart rate variability (HRV)-analysis, cutaneous vasomotor function assessment using laser Doppler flowmetry, and cutaneous sudomotor function measurement using skin conductance responses.

RESULTS: In PD patients, the number of impressions in the indirect region was lower compared with healthy subjects (19.0±7.9 PD vs. 27.3±15.5 subjects, mean±SD, p <0.05). Sudomotor function was attenuated in PD patients compared with healthy controls (p <0.01) whereas we observed non-significant trends toward decreased HRV and vasomotor function in PD patients (p=n.s.).

CONCLUSIONS: This study demonstrates that axon-reflex pilomotor responses are impaired in early PD stages compared with healthy controls, consistent with impaired sudomotor function, suggesting that QPART is a valid test to detect early autonomic nerve dysfunction in PD. (1) Siepmann T, Gibbons CH, Illigens BM, et al. Quantitative pilomotor axon reflex test: a novel test of pilomotor function. Arch Neurol. 2012;69(11):1488-92.

S1-A6

Reprogramming of liver metabolism by alternative p38-mediated modulation of neutrophil migration

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Nonalcoholic fatty liver disease (NAFLD) is a major health problem and the main cause of liver disease in Western countries. Although NAFLD is strongly associated with obesity and insulin resistance, its pathogenesis remains poorly understood. The disease begins with an excessive accumulation of triglycerides in the liver, which stimulates an inflammatory response. Alternative p38 mitogen-activated kinases (p38 γ and p38 δ), have been shown to contribute to inflammation in different diseases. Here we demonstrate that p38 δ is elevated in livers of patients with NAFLD and that mice lacking p38 γ/δ in myeloid cells are resistant to diet-induced fatty liver, hepatic triglyceride accumulation and glucose intolerance. This protective effect is due to defective migration of p38 γ/δ -deficient neutrophils to the damaged liver. We further show that neutrophil infiltration in wild-type mice contributes to steatosis development by means of inflammation and liver metabolic changes. Therefore, p38 γ and p38 δ in myeloid cells provide a potential therapeutic target for NAFLD treatment.

S1-A7

A hypermuscular mouse model to study SOCE and muscle fatigue

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In our mouse model, a naturally occurring 12-bp deletion in the myostatin gene is considered responsible for the compact phenotype (Mstn^{Cmpt-dl1Abc}) labeled by a tremendous increase in body weight along with signs of muscle weakness and easier fatiguability. To characterize and better understand the mechanism underlying these alterations, we monitored store-operated Ca²⁺-entry (SOCE) and also measured the cytosolic [Ca²⁺] (rhod2) using whole cell voltage clamp technique.

Since STIM1 and Orai1 endogenous protein levels in the mutant flexor digitorum brevis (FDB) muscles were reduced by ~30% we hypothesized that SOCE, a potential candidate to assist in the longer-term maintenance of EC coupling may be consequently altered. Enzymatically isolated fluo-8 AM loaded FDB fibers were used. To elicit a massive SR Ca²⁺-release a RyR1 agonist (4-chloro-meta-cresol, 4-CmC) was applied in a Ca²⁺ free medium and in the presence of the SR Ca²⁺ pump inhibitor (thapsigargin, TG). The above cocktail triggered a deep transient store depletion which in turn switched on SOCE. The peak of this slow Ca²⁺ transient was normalized to the peak of the SR Ca²⁺ release transient (0.63 $\pm$ 0.09, n=18 vs 0.32 $\pm$ 0.08*, n=11, p $\leq$ 0.02).

The voltage dependence of the normalized fluorescence of the calcium transients in response to 100 ms long membrane depolarizations ranging between -60 and +30 mV, with 10 mV increments were not statistically different and were well fitted with a Boltzmann distribution (V0.5: -23.22 $\pm$ 1.35 mV vs -24.15 $\pm$ 0.77 mV with respective k values of 6.14 $\pm$ 1.15 vs 6.93 $\pm$ 0.65). To both elicit the release response and substantially deplete the SR of calcium and induce muscle fatigue, the evolution of [Ca²⁺]_i during a train of eight long-lasting depolarizations (200 ms) to a maximally activating voltage (+30 mV) were monitored. The release flux parameters and the amount of Ca²⁺ released during these pulses were decreased in the mutant muscles.

Though myostatin deficiency is an unwanted condition by itself, understanding the underlying mechanisms may help developing new safe strategies to cure muscle wasting diseases and alleviate symptoms of muscle weakness.

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S1-A8

MiRNA-24 antagonism prevents renal ischemia reperfusion injury

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Ischemia-reperfusion (I/R) injury of the kidney is one of the major causes of acute kidney injury. MicroRNAs are powerful regulators of various diseases. We investigated the role of apoptosis-associated miR-24 in renal I/R-injury. MiR-24 is up-regulated in the kidney following I/R-injury of mice (4.7 fold, p <0.05) and in patients after kidney transplantation (2.1 fold, p <0.01). Cell sorting experiments revealed a specific miR-24 enrichment in renal endothelial (1.7 fold, p=0.08) and tubular epithelial cells (2.0 fold, p <0.01) after I/R-induction.

In vitro, anoxia/hypoxia induced an enrichment of miR-24 in endothelial (2.3 fold, p <0.001) and tubular epithelial cells (1.3 fold, p <0.05). Enrichment of miR-24 induced apoptosis, whereas it's silencing ameliorated apoptotic responses. MiR-24 effects were mediated through regulation of H2A histone family, member X as well as heme oxygenase 1, which were experimentally validated as direct miR-24 targets through luciferase reporter assays. In vivo, silencing of miR-24 in mice following I/R-injury resulted in a significant improvement of survival (p <0.05) and kidney function (p <0.01), a reduction of apoptosis (p <0.05), improved histological tubular epithelial injury (p <0.01) and less infiltration of inflammatory cells (p <0.01). In vitro, adenoviral overexpression of miR-24 targets lacking miR-24 binding sites along with miR-24 precursors rescued various functional parameters in endothelial and tubular

epithelial cells. HO-1 and H2A.X were also found to be regulated by miR-24 in vivo ($p < 0.01$ and $p = 0.08$, respectively). Therefore, miR-24 inhibition is a promising future therapeutic option in the treatment of patients with ischemic acute kidney injury.

S1-A9

Elevated adipose and liver IRAP/oxytocinase activity may account for increased oxytocin degradation in obesity. Effect of IRAP blockade on obese phenotype

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Insulin regulated aminopeptidase (IRAP) has been shown to be identical molecule to oxytocinase, an enzyme playing a pivotal role in oxytocin degradation during pregnancy. The oxytocinase regulates circulating oxytocin levels and its biological activity in tissues by hydrolysis of the peptide. Since oxytocin treatment in rodents exerts anti-obesity effects, we aimed to determine whether obesity itself is accompanied by changes in plasma oxytocin and if this change is a result of altered oxytocin production or degradation.

Zucker fatty rats represent a well established model of obesity and insulin resistance. Obesity was accompanied by marked reduction in plasma oxytocin levels in these rats. Hypothalamic expression of oxytocin and genes involved in its regulation and secretion were not affected by the obese phenotype. To the contrary, we noticed elevated liver and adipose tissue IRAP/oxytocinase activity in Zucker fatty rats. These results indicate increased oxytocin degradation rather than altered oxytocin production in obesity. In addition, chronic IRAP/oxytocinase inhibition by benzopyrane derivate HFI-419 via osmotic minipumps improved glucose tolerance in obese Zucker rats without affecting body weight or adipose tissue mass.

In conclusion, our study highlights the importance of IRAP/oxytocinase in pathogenesis of obesity and suggests IRAP/oxytocinase inhibition as candidate approach in the therapy of metabolic disease.

S1-A10

The A+-helix of PCI, which is removed by testisin cleavage, is a cell penetrating peptide and responsible for internalization of PCI by Jurkat cells

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Background: Human protein C Inhibitor (hPCI) is a multi-specific serpin, which is expressed in various tissues and present in many body fluids. In contrast, PCI expression in rodents is restricted to the reproductive tract. Both, human and mouse PCI (mPCI) can permeate through cell membranes and translocate to the nucleus. In testis PCI and the membrane anchored serine protease testisin are localized to similar sites. Male PCI-/- mice exhibit a disrupted blood-testis barrier, produce malformed sperm, and are infertile. Since deficiency of testisin impairs sperm maturation in epididymis and fertilizing ability, we investigated the interaction of PCI with testisin.

Methods: Western blotting and Edman degradation were used to detect and to identify the testisin cleavage sites of the PCI molecule. Recombinant wt mPCI and a truncated mPCI (lacking the first 18 amino acids, $\Delta R1-A18$ mPCI), purified from E. coli system, were applied to investigate the effect of testisin-cleavage on the internalization of PCI by cells. Cell membrane penetration of peptides corresponding to the peptides released by testisin cleavage from the N-terminus of human and mouse PCI was studied.

Results: Both, human and mouse PCI were cleaved by testisin at the reactive site and at an additional site close to the N-terminus. This cleavage resulted in the release of a peptide rich in basic amino acids. Recombinant wt mPCI was internalized by Jurkat T cells and detected in nuclear fractions. Testisin-cleaved mPCI and $\Delta R1-A18$ mPCI were not internalized. We could also demonstrate internalization of synthetic peptides corresponding to the N-terminal peptides of human and mouse PCI released by testisin cleavage.

Conclusion: Our data suggest that the A+-helix of PCI is a cell penetrating peptide, which mediates the internalization of PCI through cell membranes. Testisin or other proteases, which release this N-terminal peptide, may thereby regulate PCI internalization.

S1-A11

In vitro studies on the mechanisms involved in chemoprevention using *Calluna vulgaris* on vascular endothelial cells exposed to UVB

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Aim and objectives: Skin exposure to ultraviolet radiation B (UVB) induces changes in the vascular endothelial cells from the dermis, changes that are important for the homeostasis of the cutaneous circulatory and immune systems. Most of the photochemopreventive agents that have been studied up to now focused on protecting the keratinocytes and fibroblasts without taking into account the role of the chromophores, found in the blood vessels, in mediating the photoinduced inflammation and neoangiogenesis. This study aims to investigate the mechanisms involved in the in vitro effect of

UVB on endothelial vascular cells (HUVECs) pretreated with the *Calluna vulgaris* (Cv) extract.

Materials and methods: Two concentrations of Cv, below the limit of cytotoxicity IC₅₀, were used (2,5 and 7,5 mEq/ml) and two doses of UVB (50 and 100 mJ/cm²). The generation of reactive oxygen species at 24 h after irradiation was determined by quantifying the malondialdehyde (MDA), the ratio between reduced and oxidised glutathione (GSH/GSSG) and by measuring the activity of glutathione peroxidase (GPx). The apoptosis was evaluated using flowcitometry, confocal microscopy (with annexin V and PI) and by determining the activity of caspase-3 (ELISA). The level of the transcription factor NF-κB and γ-H2AX was determined using western blot, and its intracellular localisation by confocal microscopy.

Results: UVB exposure led to the formation of lipid peroxides in a dose dependent manner (p <0.001), induced apoptosis, increased the γ-H2AX levels and the activation of NF-κB. Pretreatment with 2.5 μg GAE/ml Cv improved the antioxidant defense, protected against DNA lesions and was able to decrease cellular death at low dose of irradiation. The dose of 7.5 μg GAE/ml Cv was prooxidant, favored the formation of DNA lesions, amplified the NF-κB activation UVB-induced (p <0.01) and led to high levels of cellular death. Both doses of Cv inhibited caspase-3 activation. The modulatory effect of Cv extract on endothelial cells exposed to UVB depend on the concentration of Cv used.

This study provides insides into the mechanisms triggered by UVB and antioxidants on skin endothelial cells.

hypertensive factor which he named renin in 1898 in the international congress organized in Moscow. The renin studies started to ramify only three decades later. At present it is one of the current hot areas of circulation research from genes and peptides to antihypertensive drugs. In his days Robert Tigerstedt became a leading international figure of physiology. He participated in the First International Congress of Physiology in 1898 in Basel, Switzerland and was elected to serve as President of the next international one, which was never held due to the First World War. Robert Tigerstedt was invited in 1901 back to his home country and became professor of physiology in at the University of Helsinki. He started several areas of research in circulation, nutrition and medicine. He is thus also called the father of clinical research in Finland. He was the author of several books in physiology, handbooks included which were widely used in several countries. He also published the first textbook of physiology in Finnish. It was one of the texts of basic medicine in Finnish language. Robert Tigerstedt was a close friend of Alfred Nobel. After the death of Alfred Nobel, Tigerstedt served in the group to realize Nobel's will i.e. the creation the Nobel foundation and formulated the regulations of the greatest annual scientific prizes in several fields of science, Physiology or Medicine included. The first Nobel Prize in Physiology was given to Ivan Pavlov in 1904. This structure of Prizes has lasted now more than a century and expanded to new areas, like Peace. Alfred Nobel's own innovations covered explosives, dynamite and like and an immense fortune was collected by the Nobel Foundation and now annually used to promote human benefits. Ragnar Granit, also a Finn, active within one of Robert Tigerstedts' own research areas, electrophysiology, has obtained the Nobel Prize in Physiology for work on color vision.

S1-B

History of European Physiological Sciences, Physiology - Origin of Prizes

S1-B1

Robert Tigerstedt, Alfred Nobel and The Prizes

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As one the first professors of the Academy of Turku represented medicine the medical education started in Finland 1640. Only some 200 years later and after the transfer of the University to new capital Helsinki, also the experimental medical research started. Robert Tigerstedt (1852-1923) was one of those to open this path. After his dissertation on muscle function he had to move to Stockholm for better circumstances. There he stayed two decades (1881-1900), and became professor of physiology. There he also did one of his major findings i.e. on the humoral factor present in kidneys and the regulation of blood pressure. He reported this renal

S1-B2

From Ivan Pavlov to space Physiology

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Brain and Space. These two phenomena are associated in the history of Russian science by physiologist Ivan Pavlov (1849-1936) and first human ever been to space Yuri Gagarin (1934-1968). Ivan Pavlov was awarded the Nobel Prize for the year 1904 in Physiology or Medicine "in recognition of his work on the physiology of digestion...". However, I. Pavlov is famous and admired mostly for inventing his pioneering approach to study the central nervous system – the "conditioned reflexes" (CR, also known as "Pavlovian" or "classic" CR). This approach became an extremely popular tool due to its simplicity and reproducibility. Eventually, Pavlovian reflexes were accomplished by the instrumental CR. The dog drooling saliva ("Pavlovian dog") by ring became a powerful iconic cultural appearance of science, like Schrodinger's cat or Darwin's evolution. Pavlov's 30-year profound studies of higher nervous activities and enormous organizing activity motivated strong interest to neurophysiology in the Soviet Union for many years ahead. This, in turn, put firm basis to diverse applied studies in

physiology the Soviet Union, including space physiology set by Pavlov's disciples in 1940-1950s (Leon Orbeli, 1882-1958) and further by Vasilii Parin (1903-1971) and Oleg Gazenko (1918-2007). Space at that time was a novel and enigmatic habitat for the man. Pavlov's experiments on dogs allowed steady progress in training first dogs for space flight (Laika, Belka, Strelka, Veterok, Ugolyok), and, further to physiologically and psychologically prepare humans for microgravity. The Conditioned Reflexes are still in use in learning and memory studies. Due to high scientific standards set by Ivan Pavlov the Russian space biomedical program has ever been and still is successful. Thus, one can imagine a timely bridge between Pavlovian dog to cosmonaut dogs, between Pavlov and Gagarin, though they never met.

S1-B3

Female healers in early modern England: medical care for man and beast?

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Women played a major role in the provision of medical care in early modern England. They were generally the first, and often the only person, to provide medical care for humans. The good 'huswife' was also responsible for ensuring that her family enjoyed what we now call a 'healthy lifestyle'. It has long been thought, however, that females only looked after the health of very young or small animals such as poultry. While women might have been expected to care for cows in their dairy, the primary responsibility for horses and larger, more important animals lay with men.

As with most types of lay-healers, women would have obtained their medical knowledge both from the oral and the print tradition. In most cases, this probably included watching and working alongside their mother, or senior female in their household. It seems likely that both female and male laypeople utilized the same types of medical publications that practitioners did, in addition to acquiring information orally or through manuscript sources including recipe or household books or correspondence. This paper will discuss both the types of 'healing' roles women played in England during the sixteenth – eighteenth centuries, as well as the ways in which they learned the 'art of physick' (i.e. the contemporary term for medicine).

S1-B4

History of Dutch-Hungarian research platforms emerged from physiological sciences

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In the sixties of last century based on two university institutions aroused a notable Hungarian-Dutch scientific connection and cooperation within Europe. These institutes (may be honoured to name also as Schools) were the Physiological Institute in Pécs and the Rudolf Magnus Institute (RMI) in Utrecht headed by Kálmán Lissák and David deWied respectively. Around a dozen of young Hungarian physiologists spent academic research periods in RMI starting already in the seventies and joined the neuropeptide research projects which facilitated cooperation between brain physiology and neuropharmacology. A Hungarian scientist Béla Bohus should be recalled here as one of the tutors catalysing also this development. Next to neuropharmacology research on brain and behaviour and neuroendocrinology also developed and reached scientific accolade. From the early eighties the University of Groningen on one side and the Medical Universities in Szeged and Budapest (named after Albert Szent-Györgyi and Semmelweis respectively) joined in order to extend the scientific cooperations between the two countries. Areas of research activities in the last 30 years included physiology of learning and memory, the function of cholinergic brain, neurodegeneration and neuroprotection, brain development and aging, nutrition and brain metabolism just to name some of the most important fields may be recalled here. Based on brain research profiles a number of Hungarian PhD students finished their studies in Groningen and for years regular undergraduate courses are supported by joined efforts. For the future respectable jointed research areas have been emerged, mainly to pursue studies on healthy brain aging and on the pharmacological and non-pharmacological therapeutic aids supporting this research goal.

S1-B5

Hungarian heritage in physiological sciences

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Major historical milestones of teaching and research in physiological sciences will be highlighted, with emphasis on human physiology and pathophysiology in Hungary. Notable leaders of the field include among others Sámuel Rác (1744-1807; author of the first physiology textbook in Hungarian), Mihály Lenhossék (1773-1840), Kálmán Balogh (1835-1888), Jenő Jendrassik (1824-1891), Endre Hőgyes (1847-1906), Ernő Jendrassik (1858-1921), Aladár Beznák (1901-1959), István Went (1899-1963), Frigyes Verzár (1886-1979), Kálmán Lissák (1908-1982), Albert Szent-Györgyi (1893-1986; Nobel Prize 1937), László Hársing (1920-1995), Arisztid GB Kovách (1920-1996), Péter Bálint (1911-1998), Szilárd Donhoffér (1902-1999), Sándor Juhász-Nagy (1924-2007). To them we owe achievements that inspire generations to develop and enrich further their legacy. These achievements encompass influential scientific schools, renowned institutions of basic medicine, the first association of Hungarian physiologists as early as in 1891, then the Hungarian Physiological Society in 1931 (Albert Szent-Györgyi as the first secretary), foundation of Acta Physiologica Hungarica in

1950, and espousing an integrative approach in education of medical physiology and pathophysiology. These fathers of the Hungarian heritage also established the close nexus between teaching and scientific research, and between progressive theories and practical training. They assured the availability and use of the best domestic and foreign textbooks, and forged extensive international relationships and cooperation. Recent testimony to this heritage is afforded by hosting in Budapest the 28th World Congress of International Union of Physiological Sciences (IUPS-1980), the 4th World Congress of International Society for Pathophysiology (ISP-2002), and the present Joint Meeting of the Federation of European Physiological Societies and the Hungarian Physiological Society (FEPS-2014).

S1-C

FEPS Teaching Physiology Symposium Teaching Physiology in the Medical Curriculum: Traditional vs. Problem-based

S1-C1 Traditional medical curriculum in Semmelweis University

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Since reforms initiated by Flexner's report in 1910 the so-called traditional undergraduate curriculum implies that students should first acquire an understanding of the structure and function of the body before they would consider pathology and clinical medicine. The traditional curriculum for medical doctors at the Semmelweis University involves a 12-semester-long training period that covers at least 6,000 hours of teaching and it is divided into two parts. The first part consists of a two-year preclinical study period dealing with the basic or foundational sciences while the second part is focused on clinical studies, and lasts for four years. The internship period takes place during the 11th and 12th semesters, and is generally spent at University clinics or hospitals. Upon completion of the six-year programme students must submit and defend their written thesis and take a final written test and oral exam before an examination board. Having successfully passed all examinations, the student is granted the diploma and title M.D. (Medical Doctor). Since 1910 several challenges and considerable amount of criticism have arisen against this information-oriented,

traditional approach of teaching. These include the need for early clinical experience of the students, the problem of exploding scientific knowledge leading to information overload and the possibilities provided by new learning technologies such as e-learning, among others. It is important to realize that some of these problems are affecting not only the traditional teaching systems but medical education as a whole.

These challenges necessitate improvements and while the curriculum remains traditional in the sense of its structure, careful steps are being taken to answer the abovementioned pressing challenges in order to improve the quality of medical doctors graduating at the Semmelweis University.

S1-C2 Problem-based learning in the medical curriculum at Maastricht University

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From its start in 1974, problem-based learning (PBL) was the hallmark of the new medical curriculum at Maastricht University. PBL is characterized by active, student-centered learning in a multidisciplinary setting. Real-life patient problems are the core of the curriculum, from the beginning to the end. Through PBL, findings of cognitive psychology are applied to educational practice. PBL stimulates activation of prior knowledge, to which new information can be linked; this way, it drives constructive learning and encourages critical thinking and understanding, rather than memorization. In addition, it is assumed to increase retention of knowledge, improve the students' general problem-solving skills, foster the development of self-directed learning skills, make them effective collaborators and strengthen their intrinsic motivation to learn.

Many studies have been performed to investigate whether PBL really pays off in terms of learning outcomes. Overall, comparisons of the knowledge levels of Maastricht students showed at least similar results. The noncognitive, generic skills of our students appear to be relatively well-developed. The small scale character of the curriculum is probably one of the most important factors in this respect. Students have to work together in small groups, in which communication skills and professional behavior are evaluated continuously. In addition, students appear to become independent learners with problem-solving skills. At the same time, other factors can have negative effects on knowledge and skills acquisition in a PBL curriculum. The relevance of basic science knowledge for clinical reasoning needs to be stressed and protected by careful integration of clinical and basic knowledge throughout the curriculum. Also, the way in which knowledge and skills are assessed clearly influences the effectiveness of a PBL curriculum. A well-designed assessment program is needed to stimulate longitudinal development of all relevant competences.

In the presentation, the current Maastricht medical curriculum with its underlying concepts will be presented, and both positive and negative experiences will be addressed.

S1-C3

Physiology in the medical curriculum, is it really necessary?

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Traditionally many of the medical curricula start with courses focused on anatomy, physiology, biochemistry etc etc. Students spent many hours studying basic knowledge in these fields. This anatomical knowledge is considered to be important in the surgical professions whereas basic physiological or biochemistry knowledge is thought to be indispensable in for example internal medicine. However in modern medicine many doctors work according to professional protocols based on scientific research and the best available evidence. The question is raised whether every doctor still benefits from basic knowledge of the forementioned basic subjects or should the medical curricula be changed to more practical medicine and epidemiology. This presentation addresses this question and will discuss the pro's and con's of incorporating physiology in the medical curriculum.

S1-C4

Physiology teaching in the traditional curriculum of Semmelweis University

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The main goal of teaching physiology traditionally as a separate course in the medical curriculum is to give medical students a firm basic comprehensive knowledge to the understanding of pathological disorders and pharmacology before the introduction of clinical courses.

The traditional physiology course in Hungary has a two-semester schedule and follows a topic by topic approach where the students firstly learn the normal functioning of the different organ systems from the subcellular level to their complex operation and regulation. As a next step, special care is taken to highlight how the different basic physiological processes integrate into the complex functioning of the human organism.

Structurally, the course consists of lectures, seminars and lab practices. The lectures are aimed to give an up-to-date overview on the theoretical background by an expert of the topic. The lecturer guides the students through the topic by synthesizing and supplementing the available textbooks, emphasizing the essence and setting the physiological facts into a clinical context. Seminars are given to smaller groups (45-50 students) where the teachers conduct an interactive discussion on the acquired material. The fundamental purpose of lab practices is to deepen the theoretical knowledge using a practical approach in small (15-18) groups. Besides, lab experiments are designed to develop several skills including cooperativity, manual skills and accuracy. During labs the

students perform simple physiological measurements on each other, carry out short animal experiments either on living preparations or in computer simulated laboratories and discuss clinical case reports.

Each semester is completed by exams. However, students are encouraged to study continuously during the semester and their progress is checked by weekly tests. The results of these tests are incorporated into the exam grades.

In summary, the traditional physiology course relies on a repetition based knowledge transfer. Versatility in forms of communication and approaches to the subject maintains the curiosity and motivation of students. Thereby they attain firm understanding of human physiology.

S1-C5

Physiology teaching is a fully integrated Medical PBL curriculum in Maastricht

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The Medical curriculum in of the Faculty of Health, Medicine and Life Sciences (FHML), University Maastricht, lasting 6 years, is divided in a 3-years bachelor and 3-years master program. The bachelor program consists of a fully integrated Problem-based Learning curriculum, the first two years characterized by working groups consisting of 10 students and one tutor, studying case histories from block books of selected subjects and occasional lectures (at maximum two per week). Practical medical skills can be learned in the so-called Skillslab. The third year is bridging the gap between the bachelor and master program: the students combine studying basic and clinical disciplines in four blocks with subjects such as "Heart, Circulation and Respiration" with real life experiences with patients seen by them in the "student polyclinics". The master program consists of five clerkships (10 to 20 weeks in duration) in hospitals and/or practices of family doctors, two elective clinical clerkships of 10 weeks and one "Scientific Research Participation" and one "Health Care Participation" each of 18 weeks.

As Physiology is considered a key discipline in the Medical curriculum, physiologists participate in the curriculum at almost all levels. The department supplies members to the Organization committees of most of the individual blocks (taking care of constructing case histories seducing the students to study Physiology and Pathophysiology, providing multiple choice questions for the integrated block examination, etc), tutors to the tutorial groups and lecturers in the first two years. The participation in the third year of the bachelor program consists of providing interactive lectures, mentoring student groups, etc. During the master program, one day each week or each two weeks all master students return to the FHML in Maastricht and are following in groups of 14 to 16 students a teaching program organized by the basic disciplines varying from psychology to biophysics. Members of the Dept of Physiology are playing an important role at these so-called "home coming days". They provide interactive lectures on subjects, relevant for the student's stay in the clinic, such as "Disturbances in acid-base balance in patients" and guide discussion groups of students who are discussing the (Patho) physiological aspects of clinical cases. The

subjects to be discussed depend on the type of clerkship and the type of disease of the patients, seen by the students during the preceding weeks of their clerkship. Master students are entitled to participate in research programmes of the Dept of Physiology during the “Scientific Research Participating” block.

S2-A

Acta Review Symposium, Electrical Propagation in Smooth Muscle organs

S2-A1

Slow wave propagation in the stomach: Advances in experimental and modelling techniques

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Slow wave activity in the stomach is initiated and propagated by a network of interstitial cells of Cajal (ICC), located within the stomach musculature. Slow wave activity is responsible for coordinating the mechanical contractions of the stomach, necessary for the breakdown of ingested food. Degradation of the ICC network and the corresponding disruption of organised slow wave activity has been associated with functional motility disorders such as gastroparesis (delayed emptying of the stomach).

Cardiac electrophysiology leads the clinical use of extracellular measurements in the form of cutaneous ECG, catheter based measurements and more recently, non-contact endocardial mapping systems. In contrast, extracellular recordings from human gastrointestinal smooth muscle have had limited clinical impact and remain largely limited to research studies.

Until relatively recently, most experimental studies have used a limited number of electrodes (typically 4-8 electrodes). However, their limited coverage has meant that analysis techniques were primarily restricted to frequency dynamics and they were largely unable to accurately capture spatial dynamics. The introduction of high-resolution extracellular recording techniques (employing up to 250 electrodes at a 5 mm inter-electrode spacing) has accorded an improved understanding of the dynamic propagation patterns of slow wave activity. These techniques have subsequently translated to human studies resulting in the observation of a variety of complex spatial dysrhythmia patterns in patients with gastroparesis.

Advances in mathematical modelling techniques provide an alternative approach of interpreting and augmenting experimental data. The development of biophysically-based models capable of accurately simulating the electrical activity

of ICC and smooth muscle cells have enabled investigations into the structure-function relationships of ICC networks and to investigate the response to different electrical stimulation protocols.

These recent advances in both experimental and modelling techniques now provide the potential to bridge the current gap between laboratory research and clinical application.

S2-A2

Normal and abnormal electrical propagation in the small intestine

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As in other muscular organs, small intestinal motility is determined to a large degree by the electrical activities that occur in the smooth muscle layers of the small intestine. In recent decades, the interstitial cells of Cajal, located in the myenteric plexus, have been shown to be responsible for the generation and propagation of the electrical impulse: the slow wave. It was also known that the slow waves as such do not cause contraction, but that the action potentials (‘spikes’) that are generated by the slow waves are responsible for the contractions.

Recording from large number of extracellular electrodes simultaneously is one method to determine origin and pattern of propagation of these electrical signals. This review reports the characteristics of slow wave propagation through the intestinal tube, the occurrence of propagation blocks along its length, which explains the well-known decrease in frequency, and the specific propagation pattern of the spikes that follow the slow waves.

But the value of high-resolution mapping is highest in discovering and analyzing mechanisms of arrhythmias in the gut. Most recently, circus movements (also called ‘reentries’) have been described in the small intestine in several species. Moreover, several types of reentries have now been described, some similar to what may occur in the heart, such as functional reentries, but others more unique to the small intestine, such as circumferential reentry. These findings, also discussed in this review, seem to suggest the possibilities of hitherto unknown pathologies that may be present in the small intestine.

S2-A3

Electrical propagation in the uterine muscle during pregnancy and labor

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The uterine muscle plays its most evident role during pregnancy, when quiescence is required for adequate nourishment and development of the fetus, and during labor, when forceful contractions are needed to expel the fetus and

the other products of conception. The uterine muscle is composed of smooth muscle cells. Contraction is initiated by the spontaneous generation of electrical activity at the cell level in the form of action potentials. The mechanisms underlying uterine quiescence during pregnancy and electrical activation during labor remain largely unknown; as a consequence, the clinical management of preterm contractions during pregnancy and inefficient uterine contractility during labor remains suboptimal. In an effort to improve clinical management of uterine contractions, research has focused on understanding the propagation properties of the electrical activity of the uterine muscle. Different perspectives have been undertaken, from animal and in vitro experiments up to clinical studies and dedicated methods for noninvasive parameter estimation. A comparison of the results is not straight forward due to the wide range of different approaches reported in the literature. However, previous studies unanimously reveal a unique complexity in the pattern of uterine electrical activity propagation which necessarily needs to be taken into consideration for future studies to be conclusive.

The aim of this review is to structure current variegated knowledge on the properties of the uterus in terms of pacemaker position, pattern, direction, and speed of the electrical activity during pregnancy and labor.

S2-A4

Electrical propagation in the renal pelvis, ureter and bladder

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Under normal conditions, following the passage of urine from the collecting duct, the urine get stored briefly in the renal pelvis before being transported to the bladder via the ureter. In the bladder, the urine gets stored for a longer time (hours) before being voided through the urethra.

The transport of urine from the renal pelvis to the bladder occurs spontaneously due to contractions of the muscles in the wall of the pelvis and ureter. Spontaneous contractions also occur in the detrusor muscle and are responsible for maintaining the bladder shape during the filling phase. These muscle contractions occur as result of electrical impulses, which are generated and propagated through different parts of the urinary tract. The renal pelvis and the ureter differ from the bladder in relation to the origin, characteristics and propagation of these electrical impulses. In the ureter, the electrical impulses originate mainly at the proximal region of the renal pelvis and are transmitted antegradely down the length of the ureter. The electrical impulses in the bladder, on the other hand, originate at any location in the bladder wall and can be transmitted in different directions with the axial direction being the prominent one. In this presentation, an overview of the current research on the origin and propagation characteristics of these electrical impulses in the normal and pathological conditions will be provided.

S2-A5

Electrical propagation in the various sites of the pulmonary veins of mammalian

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OBJECTIVES. Pulmonary veins (PVs) are characterized by unique properties – interposition of the two types of electrical excitable tissues: smooth and cardiac muscle layers. Some mammals demonstrate tight interaction of the broad areas of extremely thin cardiac layer and smooth muscle in PVs “tree”. However, electrical contact and functional interaction between these tissues remains questionable. The aim of the present study is to investigate conduction of excitation in various sites of pulmonary veins tree and to determine possible influence of “smooth muscle factors” on the conduction.

METHODS. Multicellular preparations of left atrium (LA) or PVs of left lung lobe were dissected from rabbits (male, 2.6-3.2 kg, n=8), rats (male, 200-250 g, n=20) and guinea pigs (GP, male, 250-300 g, n=5). Preparations were perfused in standard conditions with Tirode solution. Conduction of excitation was estimated with use of optical mapping technique (potential sensitive dye - di-4-ANEPPS, CCD camera - WuTech Instruments, software – RedShirtImaging). Conduction parameters were separately defined in LA, LA and PV junction region (PVJ), in PVs on the level of bifurcations (PVB), intrapulmonary region of PV (IP). Also, action potentials (APs) were recorded in all sites of preparations with applying of standard microelectrode technique.

RESULTS. Rat and GP demonstrates excitability and conduction of excitation in PVJ, PVB, IP (in rat) regions of PVs. Conduction velocity, anisotropy of conduction were approximately similar in all PV sites and similar to those of the LA. Rabbit characterized by only small PVJ zone, which conduct the excitation. Removing of endothelium did not affect conduction or APs in any sites of rat PVs. Also, NO-donor application was unable to evoke significant conduction alteration. APs alteration in rat PVs sites, after NO-donor application, was indistinguishable from LA.

CONCLUSIONS. Thin layer of myocardium in rat and GP PVs tree, which is adjacent to smooth muscle, reveal conduction similar to LA. Probably, smooth muscle does not interact with or, at least, unable to significantly affect the bioelectrical activity of cardiac tissue in PVs.

S2-A6

Effects of methane inhalation on the nitrergic myenteric neurons and intestinal myoelectrical activity during mesenteric ischemia-reperfusion in rats

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We have recently demonstrated anti-inflammatory effects for exogenous methane after intestinal ischemia-reperfusion (IR). Gases in the gut lumen may influence gastrointestinal motility, but the effect methane (CH₄) inhalation on the IR-related increase of nitrergic myenteric neuronal density and on the intestinal myoelectric activity have not been investigated yet. Therefore, our aim was to follow the consequences of CH₄ inhalation on the quantitative parameters of myenteric neurons and on the intestinal myoelectrical activity changes in a rat model of experimental IR.

For the study control, IR, and methane-treated IR groups of male Sprague-Dawley rats (300-350g) were used (n=8-8). Ischemia was induced by the occlusion of superior mesenteric artery for 50 min, inhalation of normoxic artificial air with or without 2.2% CH₄ was started in the last 5 min of ischemia and maintained in the first 10 min of reperfusion. The myoelectric activity was monitored during IR and at the end of 120-min reperfusion, tissue samples were collected from the duodenum, ileum and colon for quantitative immunohistochemistry (HuC/HuD, nNOS and eNOS).

During ischemia the myoelectric activity increased, then decreased gradually until the end of the reperfusion period. The total number of myenteric neurons did not change, but the density of nitrergic neurons increased significantly exclusively in the duodenum at the end of reperfusion. CH₄ inhalation resulted in an increase in myoelectric activity in the early stages of reperfusion and prevented the IR-induced increase in nitrergic neuronal numbers.

Based on these results we hypothesize that due to the increased density of nitrergic myenteric neurons in IR the descending inhibition of intestinal peristalsis was enhanced. Normoxic CH₄ inhalation has significant biological effects by preventing the IR-related increase in nitrergic neuronal density and through changing of intestinal myoelectrical activity influences the intestinal motility.

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S3-A

Non-canonical Functions of the Endogenous Opioid Peptides

S3-A1

Pathophysiological consequences of endogenous opioids in injury and disease

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Endogenous opioid peptides are involved in a variety of normal physiologic functions. Alternatively, evidence suggests that, under pathophysiologic conditions, neuronal and glial-derived opioids cause inflammation and excitotoxicity leading to neuronal dysfunction, injury, and potentially death. Importantly, both normative and aberrant actions of opioids are highly contextual, differing among cell types, and even within subsets of the same cell type. Dynorphins, endogenous opioid neuropeptides derived from the prodynorphin gene, are exemplary in this regard. Dynorphins are well established as regulating a variety of normative physiologic functions via opioid receptors. Besides neurotransmission, at physiological levels, dynorphins can reduce microglial activation, enhance the survival of neurons, and promote oligodendroglial survival during maturation. By contrast, under pathophysiological conditions in which dynorphin levels are substantially elevated, or when the balance of prodynorphin biosynthetic peptide derivatives is disrupted, dynorphins can be excitotoxic. Although the mechanisms are incompletely understood, dynorphin excitotoxicity appears to be partially mediated through glutamate receptors, and possibly via direct biophysical interactions with cell membranes. Increased prodynorphin gene expression and/or altered prodynorphin processing occurs pathophysiologically (e.g., with stress, drug abuse, neuropathic pain, neuroAIDS, and neurotrauma). In addition, spontaneous mutations in dynorphin enhance synaptodendritic damage and have been demonstrated to underlie the etiology of spinocerebellar ataxia 23. Because the excitotoxic actions of dynorphins occur at supraphysiological concentrations or prolonged tissue exposure, there may be little evolutionary pressure to ameliorate the maladaptive, "non-opioid" consequences of dynorphins. Emerging evidence suggests that a variety of CNS pathologies are accompanied by altered dynorphin biogenesis. Such alterations are likely maladaptive and contribute to neuroinflammation, neuronal injury, and the pathogenesis of disease.

S3-A2

Glutamate mimicking mutant Dynorphin A causes spinocerebellar ataxia type 23

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Spinocerebellar ataxia type 23 (SCA23) is caused by mutations in PDYN, which encodes the opioid neuropeptide precursor protein, prodynorphin (PDYN). PDYN is processed into the opioid peptides, alpha-neoendorphin, and dynorphin (Dyn) A and B peptides, which play a role in pain signaling and addiction via opioid-receptor mediated actions. However, pathologically elevated Dyn A can also elicit non-opioid neurodegenerative functions via N-methyl-D-aspartate (NMDA) receptor signaling, leading to neuronal cell loss. Until now, it was not known if Dyn A can induce glutamate excitotoxicity in the cerebellum and whether this would cause motor dysfunction. Here, we show that elevated Dyn A levels lead to altered expression of glutamate and NMDA signaling components causing Purkinje cell loss and motor dysfunction in a mouse model of spinocerebellar ataxia type 23. Heterozygous SCA23-mutant PDYN-R212W mice reproduced the clinical features of SCA23, with gait deficits at three months of age, progressive loss of motor coordination and balance, and Purkinje cell loss by the age of 12 months. This coincided with pathologically elevated mutant Dyn A levels in the cerebellum and altered expression of ionotropic and metabotropic glutamate receptors and glutamate transporters. These alterations indicate an attempt to reduce glutamate-induced toxicity by the Purkinje cells, elimination of climbing fiber- Purkinje cell synapses and a decrease in long term depression. These changes in glutamate signaling are most likely due to glutamate mimicking, mutant Dyn A. In conclusion, the PDYN-R212W mouse is a novel model for spinocerebellar ataxia and our work suggests that the elevated mutant Dyn A levels are responsible for the initiation and progression of the disease. Furthermore, this model can aid in elucidating the molecular mechanism of, and developing a treatment for SCA23.

S3-A3

Opioid neuropeptides make pores in plasma membrane: possible mechanism of signal transduction

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Dynorphin opioid peptides exert their actions primarily by interacting with kappa-opioid receptors, but may also produce non-opioid-receptor-mediated effects causing

neurodegeneration, chronic pain and neurological dysfunctions. We have found that dynorphins (Dyn A, Dyn B and Big Dyn) interact with cellular membranes. Using quantitative methods with single-molecule sensitivity, fluorescence correlation spectroscopy and patch-clamp electrophysiology, we demonstrate that Big Dyn and Dyn A accumulate in the plasma membrane and induce a continuum of transient increases in its ionic conductance, which is consistent with formation of pores that are too large to possess ion selectivity (~27 Å in mode diameter). The pore forming potency of dynorphins correlates with their arginine content. The observed pore formation is not cell-type-specific and cannot be blocked by the general opioid receptor antagonist naloxone. However, it is greatly attenuated by increased external Ca²⁺ or by a high positive potential inside the cell. The potency of dynorphins to induce membrane pores correlates with their toxic effects on microbial cultures, mammalian cells, and with neuropathological effects in whole animals. Like other peptides disrupting the plasma membrane, dynorphins have powerful antimicrobial effects. Direct interaction of dynorphins with the plasma membrane may provide exchange of metabolites, RNAs and peptides between neurons and the extracellular milieu through large dynorphin-induced pores, thus potentially representing an ancestral form of intercellular or protosynaptic signaling. This novel mechanism may explain the unique actions of dynorphins at synaptic and extrasynaptic sites, as well as their neuropathological properties and ability to act as antimicrobial agents.

S3-A4

The left-right neurohormonal regulation in the brain: Lateralized endogenous opioid system

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In the 1920-1940s Anna Di Giorgio, an Italian scientist discovered that a unilateral injury to the brain induces the formation of postural asymmetry of the hind limbs. The asymmetry retained long after spinal cord transection suggesting the side-specific neuroplastic / molecular changes in the spinal cord. Our analysis of the neurochemical mechanisms underlying the postural asymmetry fixation identified the endogenous opioid peptides as factors that selectively modulate the left- and right-hind limb motor reflexes. Thus, Met-enkephalin induced flexion of the left leg, while Leu-enkephalin facilitated flexor reflex of the right hind limb. These effects were apparently mediated through the lateralized opioid receptors. Further studies demonstrated that the lesion to the left or right hemisphere differentially affects the opioid peptide levels; thus the opioid peptide-expressing neuronal networks may selectively mediate effects of left and right brain injury on the lateralized brain / spinal cord functions. The mechanism of the differential neurohormonal regulation of the left and right brain hemispheres / spinal cord segments is proposed and will be discussed. Recent findings on lateralization of the opioid peptides in the human anterior cingulate cortex that may underlie lateralization of positive and negative emotions will be also presented.

S3-A5

Investigation of possible interactions of pain modification actions of endogenous enkephalinergic and noradrenergic systems in Zymosan-induced chronic inflammatory pain model

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Chronic inflammatory pain, a nociceptive process associated with tissue damage due to inflammation mediated by increased sensitivity of nociceptive-specific neurons, is a feature of a large number of painful conditions for which no satisfactory treatment has been available yet. The aim of this study was to assess the possible analgesic affects of opiorphin, an endogenous enkephalinase inhibitor, and noradrenalin, known to be involved in the intrinsic modulation of nociceptive transmission, on inflammatory pain in rats. Timing the delay for the first hind paw lift to a focused thermal radiant heat, in vivo nociceptive behavioral "plantar test" was used for assessing pain sensitivity of adult male Sprague Dawley rats. After obtaining control nociceptive latency values, chronic inflammatory pain was induced by intraplantar injection of zymosan (6 mg in 200 uL) and heat stimulated nociceptive tests were repeated. Pain threshold values were determined and analyzed by a pairwise comparison between vehicle and each opiorphin treated group using a Dunnett's t-test on the ranked data. Zymosan caused a persistent significant increase in pain sensitivity as measured 15-180 minutes after its intraplantar injection ($P < 0.05$). Systemic administration of opiorphin (0.1-0.3 mg/kg) provided a dose dependent ($P > 0.05$ for 0.1 mg/kg opiorphin while $P < 0.05$ for 0.3 mg/kg i.p opiorfin at 30 and 40th min) inhibition of inflammatory pain. Combination of opiorphin with noradrenalin (1 mg/kg) provided a limited synergic action, and this effect was reversible by alpha-1 adrenergic blockage (prazosin 1 mg/kg). Results from this study provides some degree of overlap between endogenous enkephalinergic and noradrenergic analgesic modification which implicates potential for development of therapeutical strategies involving combined use of enkephalinase inhibitors and noradrenergic agonists for the management of persistent pain associated with inflammatory painful conditions. " tabindex="" maxlength="2200" onkeyup="charcheck()" style="height: 326px;width: 820px;" >0.05 for 0.1 mg/kg opiorphin while $P < 0.05$ for 0.3 mg/kg i.p opiorfin at 30 and 40th min) inhibition of inflammatory pain. Combination of opiorphin with noradrenalin (1 mg/kg) provided a limited synergic action, and this effect was reversible by alpha-1 adrenergic blockage (prazosin 1 mg/kg). Results from this study provides some degree of overlap between endogenous enkephalinergic and noradrenergic analgesic modification which implicates potential for development of therapeutical strategies involving combined use of enkephalinase inhibitors and noradrenergic agonists for the management of persistent pain associated with inflammatory painful conditions. " tabindex="" maxlength="2200" onkeyup="charcheck()" style="height: 326px;width: 820px;" >Chronic inflammatory pain, a nociceptive process associated with tissue damage due to inflammation mediated by increased sensitivity of nociceptive-specific neurons, is a feature of a large number of painful conditions for which no satisfactory treatment has been available yet. The aim of this study was to assess the possible analgesic affects of opiorphin, an endogenous enkephalinase inhibitor, and noradrenalin, known to be involved in the intrinsic modulation of nociceptive transmission, on inflammatory pain in rats. Timing the delay for the first hind paw lift to a focused thermal radiant heat, in vivo nociceptive behavioral "plantar test" was used for assessing pain sensitivity of adult male Sprague Dawley rats. After obtaining control nociceptive latency values, chronic inflammatory pain was induced by intraplantar injection of zymosan (6 mg in 200 uL) and heat stimulated nociceptive tests were repeated. Pain threshold values were determined and analyzed by a pairwise comparison between vehicle and each opiorphin treated group using a

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S3-A6

Hemokinin-1 is a potent inflammatory and pro-nociceptive peptide in acute and chronic mouse arthritis models

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The Tac4 gene-derived hemokinin-1 (HK-1) is expressed in the nervous system and inflammatory cells. Despite several similarities to substance P (SP), it is suggested to have different binding site at the NK1 tachykinin receptor, distinct activation mechanism and signal transduction pathways, but a specific own target was also proposed. We described its non-NK1-dependent inflammatory actions in the joints, therefore, in this study, the role of HK-1 was examined in arthritis models of distinct mechanisms using C57Bl/6 wildtype (WT) and Tac4 gene-deleted (Tac4^{-/-}) mice.

In acute mechanism models Complete Freund's adjuvant (CFA) or the protease-activated receptor 2 agonist mast cell tryptase (MCT) was injected into the knee joint. Edema and hyperalgesia were measured with digital micrometer and aesthesiometry after 2-24 or 2-6 hours, respectively. MCT-induced vasodilation was measured for 30 minutes by laser Speckle after topical administration. In the K/BxN serum-transfer chronic polyarthritis model, arthritogenic or control serum was injected i.p. on days 0 and 3. Paw volume was measured by plethysmometry, touch sensitivity by aesthesiometry, noxious heat threshold on hot plate, cold tolerance by paw withdrawal latency from 0°C water, joint function in the grid test and arthritis severity by weight loss during 2 weeks.

CFA and MCT evoked 10-15% knee joint edema and 30-35% mechanical hyperalgesia, which were significantly reduced in Tac4^{-/-} mice compared to the WTs. MCT-induced acute synovial vasodilation was also remarkably decreased after 15 minutes in the Tac4^{-/-} group. In the chronic immune-arthritis model 90% joint swelling and 40% mechanical hyperalgesia detected in the WTs were significantly smaller, and the thermonociceptive thresholds were higher in Tac4^{-/-} mice.

Cold allodynia, joint function and weight loss were not different.

Hemokinin-1 increases inflammatory swelling and hyperalgesia in both acute and chronic arthritis models. Identification of its receptors and molecular mechanisms of action might open new perspectives for arthritis therapy.

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S2-B

Nutrition and Cardiovascular Health: New Perspectives in Prevention and Therapy

S2-B1

Homocysteine and homocysteine-thiolactone induce cardiac and vascular damage: interplay with oxygen consumption, oxidative stress, and gasotransmitters

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This study deals with the effects of homocysteine (Hcy) isoforms on cardiodynamics, cardiac oxygen consumption, oxidative stress, and vascular reactivity. Firstly, cardiodynamics and oxidative stress were estimated following administration of any homocysteine isoform, i.e. DL-Hcy, DL-Hcy TLHC or L-Hcy TLHC (all 10 microM) in isolated hearts of rats (Wistar, male, groups n =6, age 8 weeks, b.m. 180–200 g, coronary perfusion pressure 70 cmH₂O). Oxidative stress markers were measured in coronary effluent spectrophotometrically through TBARS, nitrites (NO₂-), superoxide anion (O₂-), and hydrogen peroxide (H₂O₂) concentration. In the effects of DL-Hcy TLHC the role of gasotransmitters (NO, H₂S or CO) were estimated, through administration of L-NAME (30 microM), DL-propargylglycine (10 microM) or ZnPPR-protoporphyrin IX (10 microM). Any Hcy isoform induced decrease of cardiac contractility as well as decrease of coronary flow. NO and H₂S were involved in the effects of DL-Hcy TLHC but participation of CO was not registered. Regarding the effects of Hcy isoforms on oxidative stress markers, only L-Hcy TLHC significantly affected O₂- release. Secondly, transduction mechanisms were estimated following the application of 100 microM Hcy on isolated rat femoral artery ring (RFA). Hcy induced contractile response of intact RFA, which was significantly increased after endothelial denudation, while notably decreased after incubation of 10

microM urapidil, 0.1 microM nifedipine or 10 microM indomethacin. The initial RFA contraction evoked by 1 microM phenylephrine was further increased by single addition of Hcy, which was not the case when 100 microM ouabain was preincubated. Thirdly, oxygen consumption of rat cardiac homogenate was measured before and after administration of any homocysteine isoform. DL-Hcy, DL-Hcy TLHC or L-Hcy TLHC (all 10 microM) caused decrease of O₂ consumption rate. Also in the effects of DL-Hcy TLHC the influences of L-NAME, DL-PAG or ZnPPR IX (30, 10 or 10 microM, respectively) were tested. L-NAME or ZnPPR IX caused a higher decrease of O₂ consumption rate; however DL-PAG attenuated the effect of DL-Hcy TLHC.

S2-B2

Epigenetic modulation of cardioprotection with plant compounds

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Early reperfusion of occluded coronary arteries leads to additional myocardial injury for which effective therapy is a desirable achievement. Novel noninvasive strategies to reduce myocardial infarction size, to preserve cardiac function and to improve clinical outcomes in infarcted patients are still required. Over the past two decades, researchers have studied the ability of plant compounds to prevent or reverse the cardiac remodeling in translational models of myocardial infarction and heart failure. We have demonstrated that it is possible to increase the expression of cardioprotective factors (i.e.: VEGF, HGF, PIM-1) by inducing the acetylation of histone H4 with intramyocardial delivery of natural molecules (i.e: hyaluronan mixed ester of butyric and retinoic acid). Recently, we have also observed that lower dose of plant compounds with antioxidant property (i.e.: (1-3)beta-D-glucan) have the ability to promote angiogenesis in the presence of increased histone H4 acetylation, yet the cardiomyocyte survival is induced in an epigenetically independent manner. The same compounds have inhibitory effects on the cardiac cell viability and function at higher dose. The results from animal studies have been promising and suggest the pleiotropic effect of plant molecules to mediate cardioprotection in a dose-dependent manner. Although some mechanisms have been identified for the cardioprotective effect of selected plant compounds, there is a need for further research to identify the specific molecular mechanism of epigenetic cardioprotection of ischemic heart by bioactive compounds contained in foods of plant origin.

S2-B3

Dietary factors for favorable modulation of platelet function

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Thrombocyte activation and aggregation are crucial steps in the pathogenesis of atherothrombosis leading to severe complications, such as coronary artery disease, stroke and peripheral artery disease. The treatment of these clinical conditions is evidence-based, well established, antithrombotic agents are one of the basic drugs in therapy. A wide variety of pharmacological agents are available to inhibit thrombocyte aggregation, i.e. aspirin, ticlopidine, clopidogrel, prasugrel, etc., while others are under development. Secondary prevention of these diseases is also well described as published in the various guidelines. However, primary prevention of atherothrombosis is ambiguous. During drug prevention, the bleeding (and additional) risk should not exceed the benefit of drug use, and the decision between “to treat” and “not to treat” is not always easy. Thus when dealing with patients with a risk of atherothrombosis, it is important to consider non-pharmacological approaches that are effective and have a low side effect profile. Dietary plant polyphenols may have antithrombotic and therefore cardioprotective and vascular protective effects by interfering with platelet activation. They have been shown to inhibit platelet function in vitro and in vivo, in animal and in human studies. They also have been shown to improve endothel function.

The mechanism of actions seems to be complex. The major sources of polyphenols are cocoa, chocolate, wine, grape and berries. A significant antithrombotic effect is attributable to n-3 polyunsaturated fatty acid consumption as well. They exert their beneficial effects most probably via decreasing thromboxane A₂ formation. Despite the demonstrated beneficial effects, at the moment the mentioned (and other) nutrients are not considered an accepted antiplatelet tool for clinical purpose in healthy or diseased individuals, however, it seems that they efficiently can be used in primary prevention in low- and moderate-risk patients or as an adjunct in secondary prevention in cardiovascular disease.

S2-B4

The use of dietary flaxseed to promote cardiovascular health

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Hypertension is an important silent killer strongly associated with the incidence of myocardial infarctions and stroke.

Because of this, it is the leading global risk for burden of death in the world. The incidence of hypertension is rapidly increasing as well. Current medications used to control hypertension are costly, can induce unwanted side-effects and they are not always effective in controlling blood pressure (BP) in all hypertensive patients. Having a food that will control BP represents an alternative strategy that is more popular amongst patients than drugs. Flaxseed is enriched in the cardioprotective omega-3 fatty acid (alpha linolenic acid (ALA)), lignans and fiber. Peripheral arterial disease (PAD) is associated with hyperlipidemia and hypertension. The FlaxPAD Trial was initiated to determine if dietary supplementation with milled flaxseed in PAD patients could provide beneficial actions on a variety of cardiovascular disease parameters. The results of the trial will be reported here on the effects of dietary supplementation with milled flaxseed on BP regulation and circulating cholesterol levels in patients with PAD. The FlaxPAD Trial involved a clinical population with PAD who were randomized into a ground flaxseed or whole wheat placebo group. Individuals were required to consume 30g of the appropriate intervention which was incorporated into different foods daily. This FlaxPAD Trial was double-blinded and involved 110 patients. SBP was reduced by 10 mm Hg and DBP by 7 mm Hg in the flaxseed group relative to control following 6 months of intervention. Both total and LDL cholesterol levels were reduced by flaxseed by about 10-15%. The mechanisms for these effects will be discussed. We conclude that consuming ground flaxseed daily may offer a significant dietary strategy to lower both circulating cholesterol and BP.

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S2-B5

Interaction of clopidogrel and statins in secondary prevention after ischemic stroke

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AIMS. Variability in clopidogrel responsiveness is an emerging clinical problem in secondary prevention after ischemic stroke which has been postulated to be linked to competitive metabolism of cytochrome P450 (CYP) 3A4-oxidated statins such as simvastatin and clopidogrel. This study aimed to assess the hypothesis that simvastatin, in contrast to CYP 2C9-metabolized fluvastatin, reduced clopidogrel-mediated inhibition of platelet aggregation.

METHODS. A randomized, double-blind, double-dummy, crossover-study was performed in 13 patients with ischemic stroke (8 females, 5 males), ages 64.1±8.0 years (mean±SD). Following a 14-day run-in period during which all patients

received 75mg clopidogrel/die, patients additionally received either 20 mg simvastatin/die or 80 mg fluvastatin/die for 14 days. After a 14 day wash-out period, regimens were crossed over and switched regimens were continued for another 14 days. ADP-induced platelet aggregation, plasma concentrations of clopidogrel active metabolite (CAM) as well as routine laboratory parameters including prothrombin time (PT) Quick percent value were measured at baseline and after each treatment phase.

RESULTS. Clopidogrel reduced ADP-induced platelet aggregation in all patients as anticipated. Both, ADP-induced platelet aggregation and CAM plasma levels were unchanged when simvastatin or fluvastatin was added to treatment with clopidogrel. Simvastatin attenuated PT Quick percent value (reduction from $109 \pm 10.5\%$ to $103 \pm 11\%$, $p < 0.05$) when co-administered with clopidogrel but was unchanged following administration of fluvastatin and clopidogrel.

CONCLUSIONS. This study indicates that administration of CYP 3A4-metabolized simvastatin does not jeopardize clopidogrel-mediated platelet inhibition. In co-treatment of simvastatin and clopidogrel we observed attenuation of PT Quick percent value which could be explained by simvastatin-mediated reduction of prothrombin fragment 1+2 activity.

S2-B6

Effects of L-arginine, vitamin C and folic acid on coronary hemodynamics, oxidative stress markers and NO system in isolated rat heart

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The pathogenesis of all mechanisms endothelium-dependent coronary vasodilation in coronary blood vessels is still clearly not known. We examined whether supplementation with L-arginine, folic acid and vitamin C, with or without non-specific inhibition of nitric oxide synthase by L-NAME, improve endothelium-dependent coronary vasodilation and its effects on oxidative stress markers in isolated rat hearts. The hearts of male Wistar albino rats ($n=12$, age 8 weeks, body mass 180-200g) were retrogradely perfused according to the Langendorff technique at gradually increased coronary perfusion pressure (CPP=40-120 cmH₂O). Coronary flow and markers of oxidative stress: nitrite outflow, index of lipid peroxidation (TBARS) and superoxide anion production in coronary effluent were calculated. Study results suggest that L-arginine did not significantly improve coronary flow, and also did not significantly increase nitrite outflow, index of lipid peroxidation and superoxide anion production in isolated rat hearts. These effects were partially blocked by L-NAME. Intracoronary administration of vitamin C shows non-significantly improvement of coronary flow, nitrite outflow and decrease of lipid peroxidation and superoxide anion production. These effects were partially blocked by L-NAME.

The results also show that folic acid increased coronary flow, increased nitrite outflow, and index of lipid peroxidation and decreased superoxide anion production. These effects were blocked by L-NAME. Our study results demonstrated complicated mediation of many factors in coronary vasodilation. This confirms participation of L-arginine, vitamin C and folic acid in NO mechanisms. This study is also suggesting less known direct vasodilatory and antioxidative properties of folic acid.

S3-B

Signalling at Membrane Contact Sites

S3-B1

Coupling acidic organelles and the endoplasmic reticulum through Ca²⁺. A role for membrane contact sites?

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Cellular Ca²⁺ signals often derive from intracellular Ca²⁺ stores. By far the best characterized Ca²⁺ store is the endoplasmic reticulum. But acidic organelles such as lysosomes can also serve as mobilizable stores of Ca²⁺. These so called acidic Ca²⁺ stores are often tapped by the second messenger NAADP. Here I discuss the idea that Ca²⁺ released from acidic Ca²⁺ stores can trigger further Ca²⁺ release from the endoplasmic reticulum and that this process may involve a novel class of membrane contact sites between the two compartments.

S3-B2

Calcium and ROS signaling at the ER-mitochondrial interface

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Endoplasmic reticulum (ER) and mitochondria are functionally distinct with regard to membrane protein biogenesis and oxidative energy production, respectively, but cooperate in several essential cell functions, including lipid biosynthesis, cell signaling and organelle dynamics.

The interorganellar cooperation requires local communication that can occur at the strategically positioned and dynamic associations between ER and mitochondria. Recently, these

junctions have received attention because of their pivotal role in mediating calcium signal propagation to the mitochondria, which is important for both ATP production and mitochondrial cell death. Many of the ER-mitochondrial calcium transporters and signaling proteins are sensitive to redox changes and might be directly exposed to the reactive oxygen species (ROS) produced in the mitochondria and ER. Although ROS has been emerging as a novel signaling entity, the ER-mitochondrial redox signaling is yet to be elucidated.

We describe here a new fluorescence-based strategy to measure local calcium-ROS interactions at the ER-mitochondrial interface and provide some evidence for its relevance for the functions of mitochondria in both cell survival and death.

S3-B3

Broadband connections within the cell: How the mitochondria talk to the endomembrane?

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Mitochondria, like other organelles, need to communicate with neighboring subcellular compartments to exchange metabolites and to coordinate cellular function. This communication takes place, at least in part, at contact sites where the membranes of two organelles come to close apposition. These contact sites are organized and maintained by proteinaceous tethers. Contact sites between the mitochondria and the Endoplasmic Reticulum (ER) play major roles in lipid biosynthesis as they allow exchange of lipids between both organelles. This exchange provides lipids for the biosynthesis of both mitochondrial membranes and allows the distribution of phosphatidylethanolamine synthesized in mitochondria to the rest of the cell membranes. In yeast, one protein complex that establishes and maintain ER-mitochondria contact sites is the ER-Mitochondria Encounter Structure (ERMES), a multisubunit complex made of both ER and outer-mitochondrial membrane integral proteins. Although ERMES is involved in lipid exchange between the ER and mitochondria, its function appears to extend beyond that to mitochondrial DNA replication and protein import. How ERMES-mediated ER-mitochondria contact sites favor lipid exchange and how ERMES manages to coordinate all of its proposed functions is unknown. ERMES is regulated by the Miro GTPase Gem1, but the role of this regulation is unclear. Besides Gem1, all components of ERMES have been lost in metazoan, suggesting that the tethering role has been taken over by (an)other protein complex(es).

S3-B4

Investigating membrane contact sites between the endoplasmic reticulum and phagosomes

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Phagocytosis, the process through which immune cells engulf pathogens or foreign particles, is essential for an effective immune response. Endoplasmic reticulum (ER) membranes are recruited to phagosomes, but the role the ER plays in phagocytosis is not entirely understood. Recently, we showed that ER membranes are recruited to the vicinity of phagosomes for signaling purposes by STIM1, a key regulator of the ubiquitous store-operated Ca^{2+} entry pathway. Using a combination of fluorescence and electron microscopy in neutrophils from STIM1-deficient mice and in phagocytic fibroblasts lacking STIM1, we showed that STIM1 is recruited to phagosomes and helps generate membrane contact sites (MCS) between the ER and phagosomes. Our findings indicate that these MCS are required for high-efficiency phagocytosis by generating localized intracellular Ca^{2+} hotspots through the opening of phagosomal Ca^{2+} channels. Our current research efforts are focused on further dissecting the mechanisms that drive ER-phagosome MCS formation as well as their function, through both candidate as well as unbiased proteomic approaches. Our preliminary data identify novel molecules that comprise ER-phagosome contact sites and/or regulate their function as platforms that allow precise spatial localization of signaling events within the cell.

S3-B5

Differential impact of 5-phosphatase-mediated and phospholipase C-induced plasma membrane PtdIns(4,5)P₂ depletion on G protein-coupled receptor endocytosis

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Phosphatidylinositol 4,5-bisphosphate (PtdInsP₂), a minor lipid constituent of the plasma membrane, plays an important role in several cell functions including signal transduction and membrane trafficking. We have previously shown that depletion of PtdInsP₂ by the acute plasma membrane recruitment of a 5-phosphatase (5-ptase) domain inhibits the endocytosis of various G protein-coupled receptors (GPCRs). In this study we compared the impact of this method on GPCR endocytosis with that of phospholipase C (PLC)-mediated PtdInsP₂ depletion.

In our 5-ptase-based system we used the inducible heterodimerization of FKBP and FRB to translocate the PtdInsP₂-degrading enzyme domain (FKBP-5-ptase) to the plasma membrane (PM-FRB); whereas wild type (wt) and internalization-incompetent mutant forms of the Gq protein-coupled type 1 angiotensin receptor (AT1R) were used for PLC β -mediated PtdInsP₂ depletion. We also created and tested AT1R-FRB fusion proteins that were capable of both 5-ptase recruitment and PLC β activation. The rate of PtdInsP₂ depletion was measured with the help of the PLC δ 1 PH domain which binds PtdInsP₂ specifically. We applied bioluminescence resonance energy transfer (BRET) between the luciferase-labeled β 2 adrenergic receptor (β 2AR) and the fluorescently tagged early endosome marker Rab5 as an indicator for receptor endocytosis in HEK-293T cells.

Confirming our previous results, β 2AR internalization was inhibited after PtdInsP₂ depletion by our 5-ptase-based system. A similar inhibition occurred after the activation of wt AT1R.

However, PtdInsP2 depletion by internalization-incompetent AT1R forms caused very little inhibition of β 2AR internalization, despite the higher rate of lipid depletion compared to the wt receptor.

Our data suggest that stimulation of wt AT1R inhibits β 2AR endocytosis, possibly through competition for the endocytic machinery. Using internalization-incompetent AT1R mutants we found that the effect of plasma membrane PtdInsP2-depletion on β 2AR endocytosis depends on the method of lipid degradation suggesting the importance of local phosphoinositide pools in the regulation of receptor endocytosis.

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S3-B6

Glycine modulates membrane potential, cell volume, and phagocytosis in murine microglia

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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). Phagocytosis of polystyrene microspheres was visualized by scanning electron microscopy (SEM). Gene expression was analyzed by reverse-transcriptase (RT)-PCR. Cell membrane potentials (V_{mem}) and Na^+ currents were measured using the perforated patch clamp technique. CV was measured by flow cytometry. In BV-2 cells and pMG, RT-PCR analysis revealed expression of SNATs (Slc38a1, Slc38a2), but not of GlyRs (Gla1–4). In BV-2 cells, glycine (1 and 5 mM) led to a rapid Na^+ -dependent depolarization of V_{mem} . Furthermore, glycine (0.3, 1 and 5 mM) increased CV up to about 10%. Visualizing of phagocytosis of polystyrene microspheres revealed that glycine (1 mM) increased the number of BV-2 cells containing at least one microsphere by about 13%. Glycine-dependent increase in phagocytosis was suppressed by the SNAT inhibitor α -(methylamino)isobutyric acid, by replacing extracellular Na^+ 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. From our findings we conclude that the neutral amino acid glycine increases phagocytosis in iso- and hypertonic conditions by activation of Na^+ -coupled neutral amino acid transporters (SNATs).

S2-C

New Therapeutic Targets in Acute Pancreatitis

S2-C1

Characterization of pancreatic acinar Ca^{2+} influx pathway leads to potential new therapy for pancreatitis

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In the exocrine pancreatic acinar cells, the physiological secretagogues evoke release of Ca^{2+} from internal stores, which in turn triggers opening of store-operated Ca^{2+} entry channels in the plasma membrane. The resulting repetitive short-lasting elevations (spikes) of the cytosolic Ca^{2+} concentration then activate fluid and enzyme secretion (1). Pathological stimulation of pancreatic acinar cells with bile acids or combinations of alcohol and fat induce massive intracellular Ca^{2+} release, followed by excessive entry of Ca^{2+} through the store-operated channels, resulting in a sustained elevation of the cytosolic Ca^{2+} concentration. This causes intracellular trypsin activation and necrotic cell death (1). There are probably several components of store-operated Ca^{2+} entry in these cells (1), but recent work indicates that the most important pathway is through classical Ca^{2+} release-activated Ca^{2+} (CRAC) channels, which are extremely Ca^{2+} -selective (2). This conclusion, based on the detailed characterization of the Ca^{2+} influx pathway, gave rise to the hypothesis that specific blockade of CRAC channels could be used as a therapy against severe acute pancreatitis. The relatively selective CRAC channel blocker GSK7975A dramatically inhibited store-operated Ca^{2+} entry and therefore prevented the sustained elevation of the cytosolic Ca^{2+} concentration that normally follows emptying of intracellular stores. Most importantly, the CRAC channel blocker markedly inhibited the intracellular protease activation evoked by palmitoleic acid ethyl ester – a non-oxidative product of palmitoleic acid and ethanol, which is an important mediator of alcohol-related pancreatitis – as well as the resulting necrosis (2). These data (2) provide proof in principle that specific CRAC channel blockade can be the basis of a drug-based therapy of acute pancreatitis (3).

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S2-C2

How to target the inflammatory response in pancreatitis

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Premature intracellular protease activation is known to be the primary event in acute pancreatitis. However, severe acute pancreatitis is characterised by an early inflammatory immune response syndrome (SIRS) and a subsequent compensatory anti-inflammatory response syndrome (CARS) contributing to severity as much as protease activation does. CARS suppresses the immune system and facilitates nosocomial infections including infected pancreatic necrosis, one of the most feared complications of the disease. A number of attempts have been made to suppress the early systemic inflammatory response but even if these mechanisms have been found to be beneficial in animal models they failed in daily clinical practice. Since T-cells are known to balance immune response we have addressed the role of the Teff and Tregs in severe acute pancreatitis. In mice depleted for the co-inhibitory surface molecule CTLA-4 T-cell deregulation resulted in complete destruction of the pancreas within weeks displaying morphological features of chronic pancreatitis. When studying severe acute pancreatitis in either CTLA-4 depleted animals or animals depleted for Tregs we found that T-reg cells play a dominant role in controlling the early immune response (SIRS) in severe acute pancreatitis whereas a subsequent Th-1 activation is crucial for preventing nosocomial infections and to overcome CARS. Blockade of CTLA-4 after the onset of severe acute pancreatitis, at the peak of CTLA-4 expression, resulted in the activation of T-effector cells and helped to overcome CARS. It was beneficial with regard to survival and bacterial translocation. Thus, inhibition of the co-inhibitory surface molecule CTLA-4 represents a promising therapeutic strategy aimed at reversing the immune deficit during the most critical phase of pancreatitis.

S2-C3

Insulin protects pancreatic acinar cells from pancreatitis-inducing agents

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Acute pancreatitis is a serious and sometimes fatal inflammatory disease where the pancreas digests itself. The non-oxidative ethanol metabolites palmitoleic acid (POA) and POA-ethylester (POAEE), are reported to induce pancreatitis due to impaired mitochondrial metabolism, cytosolic Ca²⁺ ([Ca²⁺]_i) overload and necrosis of pancreatic acinar cells. Metabolism and [Ca²⁺]_i are linked critically by the ATP-driven plasma membrane Ca²⁺-ATPase (PMCA) important for maintaining low resting [Ca²⁺]_i. Our previous study showed that insulin protected pancreatic acinar cells against

hydrogen peroxide-induced cellular injury which mimics many of the features of pancreatitis. The aim of the current study was to test the protective effects of insulin on cellular injury induced by the pancreatitis-inducing agents, ethanol, POA and POAEE.

Rat pancreatic acinar cells were isolated by collagenase digestion and [Ca²⁺]_i was measured by fura-2 imaging. An *in situ* [Ca²⁺]_i clearance assay was used to assess PMCA activity. Magnesium green (MgGreen) and a luciferase-based ATP kit was used to assess cellular ATP depletion. Propidium iodide fluorescence was used to assess acinar cell injury (necrosis).

Ethanol (100 mM) and POAEE (100 μM) induced a small but irreversible Ca²⁺ overload response but had no significant effect on PMCA activity. POA induced a concentration dependent ATP depletion leading to inhibition of PMCA activity and the consequent cytotoxic Ca²⁺ overload and necrotic cell death. Insulin pretreatment (100 nM for 30 minutes) prevented the POA-induced ATP depletion, inhibition of the PMCA, Ca²⁺ overload and necrotic cell death. Moreover, the insulin-mediated protection of the cytotoxic overload was partially prevented by the PI3K inhibitor, LY294002, suggesting that protection was due at least in part to the activation of the PI3K/Akt pathway.

These data provide the first evidence that insulin directly protects pancreatic acinar cell injury induced by *bona fide* pancreatitis-inducing agents, such as POA. This may have important therapeutic implications for the treatment of pancreatitis.

S2-C4

Inhibition of CFTR function is critical in the development of pancreatitis

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Acute pancreatitis is one the most common cause of hospitalization for non-malignant gastrointestinal diseases. The mortality of the disease is unacceptably high, moreover no specific pharmaceutical therapy is currently available. A major reason stated to account for the inability to develop effective treatments is that there are multiple pathobiologic pathways activated during pancreatitis making it difficult to choose molecular targets for therapeutic strategies. However recent advances highlighted the crucial role of pancreatic ductal epithelial cells in the pathogenesis of acute pancreatitis. This cell type secretes the HCO₃⁻ rich, alkaline pancreatic juice, which maintains the intraluminal pH and washes the digestive enzymes out from the ductal system playing an important role in the pancreatic physiology. The damage of the pancreatic HCO₃⁻ secretion can lead to pancreatic damage and to the development of acute pancreatitis, as described in the genetic disease cystic fibrosis. Recently we showed that the most common pancreatitis inducing factors (bile acids, ethanol and ethanol metabolites) and active trypsin impair the pancreatic ductal HCO₃⁻ secretion via the functional inhibition of CFTR Cl⁻ channel. Moreover exposure of pancreatic ductal cells to ethanol decrease the cell surface expression of CFTR as well via decreased protein maturation and increased plasma

membrane turn over. We also showed that the autoactivation of trypsinogen is a pH dependent process, with increased activity in acidic environment, which means that HCO₃⁻ secretion prevents the untimely trypsinogen autoactivation. During the development of acute pancreatitis the intraductal pH drops due to the impaired ductal HCO₃⁻ secretion resulting in accelerated trypsinogen autoactivation, which will further inhibit HCO₃⁻ secretion. These observations highlight the central role of CFTR Cl⁻ channel in the pancreatitis pathogenesis; therefore, correcting CFTR function should offer therapeutic benefit.

S2-C5

ER-PM junctions in pancreatic acinar and pancreatic cancer cells: from structure to function

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Junctions between the endoplasmic reticulum (ER) and the plasma membrane (PM) are important platforms for Ca²⁺ and cAMP signalling. In pancreatic acinar cells ER-PM junctions preferentially concentrate near the basolateral regions of the plasma membrane. Upon the reduction of Ca²⁺ concentration ([Ca²⁺]) in the ER lumen stromal interaction molecule 1 (STIM1), which serves as a [Ca²⁺] sensor in the ER, translocates into ER-PM junctions, interacts with Orai 1 (channel-forming protein) and activates Ca²⁺ entry. In pancreatic acinar cells ER-PM junctions are stationary. The number of junctions is not modified by the store depletion. In pancreatic ductal adenocarcinoma cells (PANC-1 cells in our experiments) ER-PM junctions are dynamic; they preferentially decorate the leading edge of migrating cells and undergo continuous cycles of formation and dissolution. The functional implications of the positioning and dynamics of ER-PM junctions will be discussed.

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S2-C6

The effect of taurocholic acid on ryanodine receptor and SR calcium pump activity

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The earliest critical event of pancreatitis is a long lasting high amplitude rise of intracellular Ca²⁺ concentration in the acinar cell, which can be triggered by secretagogue overstimulation or high concentration of bile acids. Although, Ca²⁺-release through ryanodine receptors (RyR) is involved in the process, the significance and the exact mechanism of bile acid's action on RyR has not been fully elucidated yet. Therefore, we aimed to test whether taurocholic acid (TCA) exerts a direct effect on RyR and SERCA pump. We show that TCA enhanced RyR's 3H-ryanodine binding in the pathologically relevant 25-500 μM range. We also found that 500 μM TCA triggered robust Ca²⁺-release from RyR-enriched vesicles, which was prevented by the application of 12 μM dantrolene. Single channel current analysis of RyRs reconstituted into lipid bilayers demonstrated that 200 μM TCA induced a 5- fold increase in channel open probability (K_d=180 μM). Furthermore, we show that TCA suppressed Ca²⁺-uptake and ATP-ase activity of SERCA- enriched vesicles.

Overall, our data strongly suggest that TCA activates RyR and inhibits SERCA with an allosteric mechanism, which might contribute significantly to TCA-induced pathologic Ca²⁺-leak from the endoplasmic reticulum in pancreatic acinar cells.

S3-C

Epithelial Function and Repair

S3-C1

Epithelial fluid and HCO₃⁻ secretion

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HCO₃⁻ secretion is a key function of secretory epithelia and involves HCO₃⁻ entry at the basolateral membrane and exit across the luminal membrane. In most epithelia the bulk of HCO₃⁻ entry is mediated by the Na⁺-HCO₃⁻ co-transporter NBCe1-B and HCO₃⁻ exit is mediated by the combined and regulated action of CFTR and members of the SLC26 transporters. The function, regulation and interdependence of CFTR/SLA26 complexes are critical for the secretory process. In this presentation, the properties of and function of the HCO₃⁻ transporters by IRBIT and by intracellular Cl⁻ will be discussed in the context of synergism in epithelial fluid and HCO₃⁻ secretion and intracellular Cl⁻ as a key regulator of the transport process.

S3-C2

HCO₃⁻ secretory function of pulmonary epithelial cells

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Bicarbonate serves many functions in our body. It is the predominant biological buffer maintaining acid-base balance in the blood as well as inside all of our cells. Bicarbonate is also secreted from the blood to the mucosal surfaces of most epithelial tissues where it plays a crucial role in a myriad of processes ranging from sperm capacitation to mucosal protection and even innate defence. In the airways, HCO₃⁻ secretion likely contributes to important lung defence mechanisms, such as secretion and solubilisation of mucins, bacterial killing and regulation of ciliary beat frequency. Aberrant HCO₃⁻ secretion would therefore be predicted to predispose the lungs to mucus blockage, bacterial infection, and disease. An important challenge in epithelial biology has been to understand the molecular coordination of transepithelial bicarbonate secretion and how this simple anion impacts on such varied physiological processes. Through studies on the inherited disease cystic fibrosis (CF), it has become clear that the plasma membrane protein encoded by the CF gene, the cystic fibrosis transmembrane conductance regulator (CFTR), is essential for bicarbonate secretion, but it is only recently that we have begun to fully appreciate the complex role this anion channel plays. This talk will describe some of our recent work in airway epithelial cells that helps define the role of CFTR and other putative bicarbonate transporters in this fundamental process, as well as how pathological situations, such as hypercapnia, exposure to cigarette smoke and bacterial infection, impacts on bicarbonate secretion.

S3-C3

Physiological and pathophysiological roles of pancreatic ducts

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The pancreas is a glandular organ that has both endocrine and exocrine functions. The exocrine pancreas, including acinar and ductal cells, in humans secretes about 2.5 l of bicarbonate-rich fluid daily. Interestingly, a large proportion of this fluid is actually produced by the ductal cells which make up less than 5% of the pancreatic tissue. Therefore, it is no wonder that besides providing a structural framework, duct cells play an essential role in maintaining the homeostasis of the pancreas. The exact mechanism of ductal HCO₃⁻ secretion is unknown, but cystic fibrosis transmembrane conductance regulator (CFTR) is a key protein in orchestrating this process. In fact, CFTR not only acts as an anion channel, but also interacts with other (e.g. solute carrier 26) transporters involved in bicarbonate secretion. The importance of CFTR is highlighted by the fact that its functional defect can result in the development of cystic fibrosis (CF), which is the most

common lethal autosomal recessive disorder in Caucasians. Therefore, it is not surprising that bicarbonate secretion is impaired in CF. It has been shown that the alteration of ductal bicarbonate secretion is also present in other diseases such as acute and chronic pancreatitis which may influence their course.

This presentation will focus on some interesting developments in the fields of physiology and pathophysiology of pancreatic ductal secretion. Experimental evidence suggests that the stimulation of ductal secretion may provide new prophylactic and therapeutic strategies in the treatment of pancreatic disease and inflammation.

S3-C4

Epithelial transport processes of ameloblasts leading to dental enamel formation

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Dental enamel, the hardest tissue in mammals, is produced by ameloblasts, which are electrolyte-transporting epithelial cells. Although the end product is very different, they show many similarities to transporting epithelia of the pancreas, salivary glands and kidney. Ameloblasts transport calcium and phosphate ions, the principal building blocks of hydroxyapatite crystals, into the enamel space. Enamel is produced in a multi-step epithelial secretory process. First, “secretory” ameloblasts form the entire thickness of the enamel layer, but with low mineral content. Then they differentiate into “maturation” ameloblasts, which remove organic matrix from the enamel and in turn further build up hydroxyapatite crystals. To form dental enamel large amounts of mineral ions should be transported across the ameloblast layer into the enamel space. The protons generated by hydroxyapatite formation need to be buffered, otherwise enamel will not attain full mineralization. Buffering requires a tight pH regulation and secretion of bicarbonate by ameloblasts. The whole process has been the focus of many studies but, perhaps surprisingly, the data available are almost exclusively based on ultrastructural, immunohistochemical and gene expression techniques, and the use of knock-out mice. Up till now, no functional data existed for mineral ion transport by ameloblasts. However, most recent studies including ours provided evidence for the nature of molecular mechanism of mineral transport, as well as for the bicarbonate secretory processes in enamel formation. The secretory regulation is not completely known as yet, but its significance is crucial. Impairing regulation retards or prevents completion of enamel mineralization and results in the development of hypomineralized enamel that easily erodes after dental eruption. Factors that impair this function are fluoride and disruption of pH regulators. Revealing these factors may eventually lead to the treatment of enamel hypomineralization related to genetic or environmentally induced malformation.

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S3-C5

Aquaporin-3 (AQP3) in cell proliferation, a potential target for therapeutic drugs

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Objective: Numerous studies demonstrated an abnormal AQP3 expression in tumor cells of different origins, suggesting a role for this enhanced protein expression in the tumor process. Recently we verified that the gold (III) complex Auphen, described as a very selective and potent inhibitor of AQP3's glycerol permeability, significantly inhibits the cell proliferation of AQP3 expressing cells. Then to further understand the role AQP3 may play in the cell proliferation process we explore the effect that stable over expression of AQP3 produce over the proliferation and cell cycle of PC12 cells as cellular model.

Methods: Cell cycle by flow cytometry with propidium iodide (PI) and cell proliferation rate through cell counting and BrdU staining were used. Using Nocodazole, a typical drug used to induce cell death for apoptotic mechanisms, we evaluated the cell response to arrest its cell cycle and the resistance to apoptosis by Annexin V staining. Auphen effect over cell cycle progression and cyclins was also analyzed, and proteomic and transcriptomic techniques were performed with the goal of highlight key molecules implicated in cell proliferation process which expression may be altered by overexpression of AQP3.

Results: Cells with overexpression of AQP3 showed higher cell proliferation rate and larger percentage of cells in phases S and G2/M. After 24h in the presence of Nocodazole, overexpressing cells exhibited less modification of the cell cycle pattern and lower Annexin V specific staining consistent with a higher resistance to apoptosis in these cells. Additionally, in AQP3-expressing cells treated with Auphen, strong arrest of the cell cycle in the S-G2/M phases, supported by analysis of cyclins (A, B1, D1, E) levels, was observed. The RT-qPCR analysis comparing AQP3 overexpressing cells to wild type revealed interesting changes in the expression of molecules related with cell proliferation, tumor and cell cycle progression, such as, Zeb2, Jun, JunB, NF- κ B, Cxcl9, Cxcl10, TNF, and TNF receptors.

Conclusions: The significant role of AQP3 in the cell proliferation process seems to be connected to increments in the cell cycle turnover. Our results support the view that larger expression of AQP3 confers to the cell a more tumoral like phenotype and contributes to explain the presence of this protein in many different tumors. A potential therapeutic effect of Auphen in tumors where cell proliferation can be associated with AQP3 seems promising, but more studies are necessary to clarify this issue.

S3-C6

Basal ciliary activity depends on ATP release in respiratory epithelium of mouse trachea

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In mucociliary epithelia from respiratory tract, ciliary beat frequency (CBF) is the main factor that determines the effectiveness of mucociliary clearance (MCC). ATP is known to increase CBF in ciliated cells, effect that is mediated by purinergic receptor activation. The increase of CBF induced by ATP depends on intracellular Ca^{2+} . In respiratory epithelial cells, ATP can be released constitutively or following mechanical stimulation (MS) through a mechanism possibly mediated by pannexin 1 (Pannx1) or connexin 43 (Cx43) hemichannels (HCs). The aim of this study was to determine if extracellular ATP (eATP) release contributes to regulate basal ciliary activity. We used ciliated cells from primary cultures of mouse trachea. CBF was recorded using videomicroscopy and Sisson Ammons Video Analysis software, eATP was measured by luminometric assay using luciferin/luciferase and $[\text{Ca}^{2+}]_i$ were measured using FURA 2AM. Apyrase (50 U/mL), an ectonucleotidase that hydrolyzes ATP, significantly lowered eATP levels compared to vehicle (3.8 ± 1.4 versus 8.1 ± 0.8 pmol/cm² after 1 min. treatment, * $p < 0.05$). Apyrase also reduced CBF in a 45.5 ± 2.3 % (n=4), effect that correlates with a $[\text{Ca}^{2+}]_i$ reduction. Simultaneous treatment with carbenoxolone (CBX) (100 μ M), a HCs inhibitor, and oxidized ATP (oATP) (100 μ M), a P2X7-R antagonist, produced a reduction of CBF compare to basal activity of 57.5 ± 3.0 % after 5 min of incubation, returning to baseline after 20 min. This reduction was prominent compared with oATP alone (6.8 ± 1.7 %, * $p < 0.05$) and CBX alone (24.9 ± 6.8 %, n=12). Furthermore, concomitant treatment with CBX, oATP and apyrase reduced the basal CBF in 85.2 ± 4.8 % (n=10), in concordance with a reduction on eATP levels (19.9 ± 9.5 pmol/cm² for vehicle, n=11, versus 2.6 ± 0.1 pmol/cm² for treatment, n=4, * $p < 0.05$).

These results suggest that ATP release from epithelial ciliated cells is necessary to maintain basal ciliary activity associated to $[\text{Ca}^{2+}]_i$ homeostasis. The underlying molecular mechanism might involve HCs and P2X7-R, through an autocrine mechanism that regulates basal ciliary activity in respiratory epithelium. Millenium Scientific Iniciative P10-035-F.

S2-D

Lipid Homeostasis: What We Learnt from Sex Hormone Estrogens

S2-D1

Estrogen more than a sex hormone

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17 β -estradiol (E2), the most effective female estrogen, controls many aspects of human physiology, including development, reproduction and homeostasis in general. The spectacular diversity of this hormone effects relies on different estrogen receptor subtypes (e.g., ER α and ER β), which are differentially expressed in many if not all human tissues as well as on a repertoire of molecular mechanisms that are activated in E2 target cells both in female and in male individuals. Indeed, the ERs are ligand-activated transcription factors that differentially modulate gene expression in different tissues by recruiting a vast array of transcriptional co-factors (i.e., co-activators and co-repressors). However, research over the last two decades have clearly revealed that ERs are extrinsic plasmalemmal proteins with the ability to signal through many different intracellular transduction languages (i.e., kinases, ubiquitin-ligases, calcium) to the regulation of many physiological functions. Structure-function analysis have linked this so-called extra-nuclear signalling with specific ERs biochemical determinants and revealed an intimate cross-talk with the ERs-based ability to regulate gene transcription. More recently, the availability of mouse model systems strongly demonstrated the importance of the ERs extra-nuclear signalling for the body homeostasis in vivo. Therefore, the pleiotropic nature of this sex steroid hormone resides on the different and somewhat opposite mechanisms of the E2-based signalling, which also cross-talks with many other hormonal networks. In this perspective, it is not surprising that deregulation of ERs-based signalling can be a cause of many hormone-based diseases and, on the other hand, E2 can exert also protective effects in many different body districts (e.g., central nervous system). Here an updated overview of the above-mentioned E2-based mechanisms will be given with the perspective of regulation of physiological and protective functions of estrogen in male and female organs.

S2-D2

Estrogen suppresses lipid synthesis: Role of membrane estrogen receptors

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Lipid synthesis is an important part of overall lipid homeostasis. Estrogen has been reported to repress low density cholesterol synthesis and augment clearance of this lipid, while enhancing high density cholesterol synthesis. This is attributed in part to the regulation of key genes, presumably from nuclear estrogen receptor (ER) α action. To investigate a possible role of extra-nuclear ER action in these regards, we compared ovariectomized wild type (WT), membrane ER α -only (no nuclear ER α) (MOER), and total ER α deleted (ERKO) mice responses to injection for 3 days of an ER α agonist, PPT. Comparisons included DNA microarray in the liver that showed suppression of many lipid synthesis genes, comparably in WT and MOER mice, but absent in ERKO mice. This correlated to suppression of liver cholesterol, triglyceride, and the fatty acid palmitate content in both mice. Cultured hepatocytes from WT and MOER mice responded to insulin with increased lipid synthesis, suppressed by estradiol or PPT, only and comparably in WT and MOER mice. Further investigation implicated membrane ER α signaling through AMP kinase, phosphorylating SREBP1 to sequester this key transcription factor in the cytoplasm, thereby preventing lipid synthesis gene expression stimulated from insulin action (Science Signaling, 2013). More recently, we also asked whether estrogen suppressed triglyceride synthesis and prevented differentiation of pre-adipocytes (3T3-Li cells), and found an important role for membrane ER α action in these regards. Estrogen fails to suppress fat cell development in-vivo in both MOER mice and mice that lack the membrane receptor but retain nuclear ER α (NOER mice, Developmental Cell, 2014).

In summary, extra-nuclear (membrane-localized) ER α plays a prominent role in suppressing lipid synthesis in several organs, at times exclusive of nuclear ER α .

S2-D3

Complex interplay between estrogens and polyunsaturated fatty acids in hippocampal lipid homeostasis: Relevance for Alzheimer's disease

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After the adipose tissue, the brain has the highest lipid content in the body. Moreover, the brain is unique in that it contains the largest amount of long chain polyunsaturated fatty acids (LCPUFA), in particular docosahexanoic acid (DHA), which is extremely well preserved in the nervous tissue. These fatty acids play a crucial role in maintaining a number of nerve cell functions from neurogenesis and synaptic transmission to memory consolidation, and their depletion has been linked to the development of Alzheimer's disease (AD). Along with cholesterol, LCPUFA modulate the physicochemical

properties of plasma membranes and provide the lipid environment for signaling platforms known as lipid rafts. Data from different laboratories, including ours, have demonstrated that n-3 LCPUFA and cholesterol levels are altered in AD brains, a phenomenon that may greatly contribute to membrane destabilization and perturbation of neuronal homeostasis during the development of AD.

Recently, we have demonstrated that estradiol is an important modulator of brain lipid homeostasis. Thus, physiological doses of this hormone increase cholesterol and DHA levels in nerve tissues through different mechanisms, but especially by activating the DHA mobilization from the liver to the brain across the blood-brain barrier. Moreover, we also demonstrate that the brain is also capable to produce LCPUFA and cholesterol through the local synthesis from dietary precursors, and that this is probably a crucial mechanism contributing to the preservation of brain lipid matrix. Moreover, the brain capacity to synthesize LCPUFA and cholesterol is under hormonal and dietary influences. Interestingly, estrogens and DHA might act synergistically to stabilize brain lipid structure by modulating both hepatic and neuronal synthesis, even in conditions of low LCPUFA intake. Our lipidomic results in the hippocampus of wild-type and APP/PS1 transgenic mice (a familial model of AD), suggest that part of the neuroprotective effects elicited by estrogens occur through the homeostatic preservation of LCPUFA and cholesterol levels in the brain.

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S2-D4

Bisphenol-A as a potential environmental factor that alters the development

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The endocrine disrupting compound Bisphenol A (BPA) has been reported to act as an obesogen in rodents exposed perinatally. Indeed, a number of studies have determined that rodents exposed to environmentally relevant doses of BPA during prenatal and postnatal development are more susceptible to becoming obese. Our work has confirmed these effects and we have determined that early life BPA exposure alters the development of the hypothalamic pro-opiomelanocortin (POMC) system leading to altered adult expression of hypothalamic peptides associated with food intake and energy balance. For instance, Males exposed to the high dose of BPA showed impaired glucose tolerance on both the normal and high-fat diets. This was correlated with increased Neuropeptide-Y (NPY) and Agouti Related Peptide (AgRP) expression in the arcuate nucleus (ARC) and reduced pro-opiomelanocortin (POMC) fiber innervation into the paraventricular nucleus (PVN) of the hypothalamus. Females exposed to the high BPA dose were heavier, ate more and had increased adiposity and leptin concentrations with reduced POMC mRNA expression in the ARC when consuming a high fat diet. The alterations in the developmental course of the melanocortin system appear to be related to a shift in the

timing of the early postnatal leptin surge that is required for the full development of the melanocortin system projections stemming from the hypothalamic arcuate nucleus (ARC) to other hypothalamic nuclei. While some of these effects were also observed in mice exposed to estrogen during development, some were only observed in mice exposed to BPA. These data support the idea that BPA at environmentally relevant doses can alter the development of the hypothalamic circuits regulating food intake and energy balance. Furthermore, they suggest that BPA may pre-program susceptibility to develop obesity in both a steroid dependant and independent manner.

S2-D5

Cholesterol homeostasis in the brain: A sex and age viewpoint

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Although a great knowledge about the patho-physiological roles of cholesterol metabolism perturbation in several organs has been reached, scarce information is available on the regulation of cholesterol homeostasis in the brain where this lipid is involved in the maintenance of a variety of neuronal processes. Currently, no study is available in literature dealing how and if sex and age may modulate the major proteins involved in the regulatory network of cholesterol levels in different brain regions. In this study we investigated the behavior of 3-hydroxy 3-methylglutaryl Coenzyme A reductase (HMGR) and Low Density Lipoprotein receptor (LDLr) in adult (3-month-old) and aged (12-month-old) male and female rats. The analyses were performed in four different brain regions: cortex, brain stem, hippocampus and cerebellum which represent brain areas characterized by different neuronal cell types, metabolism, cytoarchitecture and white matter composition.

The results show that in hippocampus HMGR is lower (30%) in adult female rats than in age-matched males. Differences in LDLr expression are also observable in old females with respect to age-matched males: the protein levels increase (40%) in hippocampus and decrease (20%) in cortex, displaying different mechanisms of regulation.

The obtained data demonstrate that age- and sex-related differences in cholesterol homeostasis maintenance exist among brain regions, such as the hippocampus and the prefrontal cortex, important for learning, memory and affection. Some of these differences could be at the root of marked gender disparities observed in clinical disease incidence, manifestation, and prognosis.

S2-D6

The determination of sodium salicylate effect to body weights and fatty acid values in the frontal lobe of brain on the immobilized rats

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The aim of this study is effects of sodium salicylate to body weights and fatty acid values in frontal lobe of brain in rats which were exposed immobilization. According to literature, sodium salicylate has protective effect against oxidative stress in rat tissues. Ethics committee approval was taken from Faculty of Medical Ethics Committee in Firat University (Turkey) and experimental applications were fulfilled in Experimental Research Unit of Firat University. 12 numbers Wistar-Albino rats that were 100-120 days and weigh 200-220g were used for this applications. Study was separated to three groups: control group (C, physiological solution, n=4), immobilization group (I, physiological solution+immobilization-15min./day, n=4) and immobilization+sodium salicylate group (I+SS, 200mg/kg+immobilization-15min./day, n=4). Fatty acids that were obtained from brain tissues were analyzed by gas chromatograph device (GC). According to experimental results, 15:1 (pentadecanoic acid), 16:0 (palmitic acid), 16:1 n-7 (palmitoleic acid), 18:0 (stearic acid), 18:1 n-9 (oleic acid), 18:2 n-6 (linoleic acid), 20:1 n-9 (eicosenoic acid), 20:4 n-6 (arachidonic acid), 22:2 (docosadienoic acid), 22:6 n-3 (docosahexaenoic acid) and 24:0 (lignoceric acid) fatty acids were calculated. 18:0 and 24:0 fatty acid values of immobilization group was statistically lower than control group (p <0.05), but Σ MUFA value of immobilization groups was higher than control groups (p <0.05). Σ SFA value of I+SS group was lower statistically than control group, but Σ USFA value of I+SS group was higher than control (p <0.05). The average body weight changes of animals in the I group (6.4g rising) according to control group (11.86g) showed statistically decreasing (p <0.05). On the other hand, the average body weight changes of animals in the I+SS (9.12g rising) was lower compared with control group (p <0.05). As a result, Both immobilization and sodium salicylate affected the weight of animals and the fatty acid composition in the brain frontal lobes of rats. We suggest that immobilization may affect the fatty acids metabolism directly or indirectly in the brain.

S3-D

Novel Mechanisms Contributing to Aging

S3-D1

Functional, morphological and molecular changes in arteries as a function of age

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Background: In the present study we hypothesized that due the continuous presence of hemodynamic forces the contractile capacity of arteries increases as a function of age. Potassium chloride (KCl) was used to assess the contractile capacity of arteries, because it elicits contraction of smooth muscle without receptor mediation (which could also be affected by aging).

Methods: Carotid arteries from 8 days (0.25m), 2 months (2m), 6 months (6m), 12 months (12m), 24 months (24m) and 30 months (30m) old WKY rats were isolated and their isometric wall tensions were measured. Arterial contractions were elicited by KCl (60mM). The arterial level of oxidative stress was assessed by HPLC. The expressions of some of the key signaling molecules of contractile activity of smooth muscle were also assessed, such as PKC-pCPI-17, MLCP and RhoA-ROCK2.

Results: There were three phases of KCl-induced arterial contractions as a function of age: contractions increased from 8d to 2 months (phase I.) than it did not change until 12m (phase II.), then it increased again to the age of 30 months (phase III.). Wall thickness increased with age, most significantly in the media and adventitia. Expressions of PKC-pCPI-17, MLCP and RhoA-ROCK2 proteins showed also an age dependency. PKC-CPI17 increased substantially from newborn to senescence age, whereas RhoA-Rock2 first increased than decreased as function of age.

Conclusions: Because KCl increases smooth muscle [Ca²⁺]_i via voltage operated Ca²⁺-channels and release of Ca²⁺ from the sarcoplasmic reticulum thereby stimulating the PKC-pCPI-17 and the RhoA-ROCK2 pathways, the increased magnitude of arterial contraction in older age is likely due to the upregulation of these pathways, findings which may have physiological importance regarding the age dependent regulation of peripheral vascular resistance.

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S3-D2

Mechanisms of extension of cognitive health span with IGF-1

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Insulin-like growth factor-1 (IGF-1) is an important anabolic hormone that decreases with age. In the past two decades, extensive research has determined that the reduction in IGF-1 is an important component of the age-related decline in cognitive function in multiple species including humans. Deficiency in circulating IGF-1 results in impairment in

processing speed and deficiencies in both spatial and working memory. Replacement of IGF-1 or factors that increase IGF-1 to old animals and humans reverses many of these cognitive deficits. Despite the overwhelming evidence for IGF-1 as an important neurotrophic agent, the specific mechanisms through which IGF-1 acts have remained elusive. Recent evidence indicates that IGF-1 is both produced by, and has important actions on, the cerebrovasculature as well as neurons and glia. Nevertheless, the specific regulation and actions of circulating, vascular- and brain-derived IGF-1 are poorly understood. The diverse effects of IGF-1 discovered thus far reveal a complex endocrine and paracrine system essential for integrating many of the functions necessary for brain health. In this presentation the latest studies of the complex actions of IGF-1 on the cerebrovasculature, neurons and glia will be discussed. Identification of the mechanisms of IGF-1 actions will undoubtedly provide critical insight into regulation of brain function in general and the causes of cognitive decline with age.

S3-D3

Novel model of age-related cognitive impairment and molecular mechanism of synaptic failure

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Vascular cognitive impairment, Alzheimer's and other neurodegenerative diseases affect more than 40 million elderly patients world-wide. As prevalence of cognitive impairment and dementia cases is accelerating in our aging population, more efficient therapies are urgently needed. According to recent results in dementia research, a key event in the pathomechanism of dementia is the disruption of synaptic connections among neurons. Synapses are the structural elements for information processing, neuronal communication in the brain, and are essential for learning and memory as well as other cognitive processes. The core mechanism for transmitter release from synaptic vesicles requires the SNARE (SNAP Receptor) complex. Three proteins form the synaptic SNARE complex in the brain: SNAP-25, syntaxin1 and synaptobrevin.

Using the knock-out mouse strains as a novel model of dementia and fluorescence imaging functional assays, we have found that the levels of SNARE proteins significantly correlate with synaptic release. Synaptobrevin2 (syb2) and Insulin-like Growth Factor (IGF)-1 have been implicated in a transcriptional network analysis as strongly associated with brain aging and dementia. IGF-1 is an important trophic hormone and its expression decreases with age. Deficiency of this hormone therefore may influence cognitive decline in the elderly. We hypothesized direct synaptic effects of IGF-1 and tested its role in memory improvement. We performed electrophysiological and fluorescence imaging experiments on primary neuronal cultures of the synaptobrevin2 knock-out mice and present novel data on synaptic mechanisms of learning and memory after IGF-1 treatment.

Taken together, our data indicate that the level of syb2 is a limiting factor in synaptic transmission and its expression in the brain is hindered during physiological aging. Based on these new results on the effects of IGF-1 on synaptic communication, we propose the IGF-1/PI3K/Akt pathway as a possible therapeutic target and a novel approach to improve cognitive function for the elderly that could overcome synaptic dysfunction.

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S3-D4

Determination of biological age in humans: Results from the EU FP7 MARK-AGE project

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The rate of ageing in humans is not uniform, due to genetic heterogeneity and the influence of environmental factors. Age-related changes in body function or composition that could serve as a measure of "biological" age and predict the onset of age-related diseases and/or residual lifetime are termed "biomarkers of ageing". Many candidate biomarkers have been proposed but in all cases their variability in cross-sectional studies is considerable, and therefore no single measurement has so far proven to yield a useful biomarker of ageing on its own. The MARK-AGE Consortium, comprising 26 partners from 14 European countries, has therefore conducted a population study (3,300 subjects) aiming at the identification of a set of biomarkers of ageing that could serve as a measure of biological age. Two larger groups of subjects have been recruited, i.e. (i) randomly recruited age-stratified individuals from the general population covering the age range 35-74 years and (ii) subjects born from a long-living parent belonging to a family with long living sibling(s) already recruited in the framework of the GEHA project. For genetic reasons such individuals (termed GEHA offspring) are expected to age at a slower rate. They have been recruited together with their spouses as controls, thus allowing initial validation of the biomarkers identified. (iii) A small number of patients with progeroid syndromes have also included in the study. A wide range of candidate biomarkers were tested, including (a) classical ones for which data from several smaller studies have been published; (b) new ones, based on recent preliminary data, as well as (c) novel ones, based on recent research on mechanistic aspects of ageing. Bioinformatic analyses have been performed to extract a robust set of biomarkers of human ageing from the large amounts of data generated. Data on the top 10 biomarkers will be shown. Based on our results we have developed a strategy to determine biological age of men and women, respectively, which also will be shown.

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S3-D5

Age-related changes in response to ischemia and adaptation in male rat hearts: Potential molecular mechanisms behind

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Reduced tolerance to ischemia/reperfusion (IR) injury was shown in aged human and animal hearts, however, precise cellular mechanisms underlying this unfavorable phenotype are incompletely understood. Moreover, aging may interfere with the mechanisms of innate myocardial protection (preconditioning, PC) and cause defects in protective cell signaling cascades that may shift the balance from cell survival to cell death. We studied effects of aging on myocardial function and response to ischemia, as well as selected molecular pathways in the hearts from juvenile (1,5 months), younger adult (3 months), mature adult (6 months) and senescent (24 months) male Wistar rats. In Langendorff-perfused hearts exposed to 30-min I/120-min R without or with prior PC induced by one cycle of 5-min I/5-min R, we measured the size of myocardial infarction (IS), susceptibility to ventricular arrhythmias and contractile function (LVDP). In parallel groups, LV tissue was sampled at baseline (BL) and after IR, for the detection of protein levels (WB) of protein kinase B (Akt, an effector of PI3-kinase), phosphorylated (activated) Akt (pAkt), its target endothelial NO synthase (eNOS) and protein kinase C ϵ (PKC ϵ) as “pro-survival” pathways linked with attenuation of apoptosis. Maturation impaired heart response to lethal IR injury (increased IS) and promoted arrhythmogenesis, and these changes were more markedly evident in the younger groups of animals, while functional recovery was not affected by aging. PC suppressed the occurrence of malignant arrhythmias, reduced the IS and improved LVDP recovery in the younger animals, while its efficacy was attenuated in the mature ones. Loss of cardioprotective effects of PC was associated with reduced BL and post-IR levels of total Akt, pAkt, eNOS and PKC ϵ in the elder groups. Age-dependent decline in PC-induced upregulation of these proteins in the hearts of mature animals compared with younger adults was also observed. In conclusion, aging-related alterations in myocardial response to ischemia may be caused by loss of innate cardioprotection due to dysfunction of proteins involved in protective cell signaling.

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S3-D6

Aging dependent GDNF induction by hypoxia in Carotid Body: Implications for antiparkinsonian cell therapy

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Intraatrial carotid body (CB) grafts induce trophic protection and restoration of the dopaminergic nigrostriatal pathway in rodent and primate models of Parkinson's disease (PD) by the release of glial cell line-derived neurotrophic factor (GDNF). Moreover, CB cells are physiologically resistant to hypoxia, a normal environmental condition in the brain that is accentuated inside intracerebral grafts. In this study, we analyse whether the age of the CB tissue could modify the regulation of GDNF expression under chronic hypoxia. Chronic environmental hypoxia (PO₂ ~75 mmHg) induced an up-regulation of CB GDNF expression in young mice (2-3 months old) but the same treatment resulted in decreased CB GDNF expression in aged mice (>14 months old). This differential regulation of GDNF expression with aging was also observed in intraatrial CB grafts and affects its efficacy in antiparkinsonian cell therapy. In addition, we have demonstrated that young CB implants are able to induce trophic protection of dopaminergic nigrostriatal neurons of aged host parkinsonian mice. These findings are in accord with previous clinical trials, where the efficacy of CB autotransplantation in PD patients was inversely related to patient age. To further demonstrate this issue, we are performing human CB xenografts in chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated PD mice model. Finally, we also are studying age-related epigenetic modifications of the GDNF promoter to unravel the molecular mechanism involved in the differential hypoxic response. In conclusion, these findings will provide new insight on critical factors that could affect clinical efficacy of antiparkinsonian CB cell therapy.

S2-E

Regulation of Mitochondrial Function in Heart Failure: From Health to Dysfunction

S2-E1

Alternations in mitochondrial structure and function in rat myocardium in chronic heart failure

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During the disease progression of chronic heart failure, a profound remodelling process takes place in the cardiac myocyte. Alterations occur in cell size, glycogen storage, enzyme content and mitochondrial shape and function, all affecting cardiac energetic function. Cardiac mitochondrial function is not only determined by its volume density, but also mitochondrial quality. However, little is understood about the influence of alterations in mitochondrial respiration during the transition from cardiac hypertrophy (CH) to failure (HF). Recently, we developed a photometry-based technique to simultaneously measure contractile function and autofluorescence of NADH (reduced nicotinamide adenine dinucleotide) and FAD (oxidized flavin adenine dinucleotide) in thin cardiac trabeculae from rats with and without CH or HF. The contraction-energetic coupling (i.e. increased contractile output linked to an increased mitochondrial function) was disturbed in HF. High-resolution respirometry revealed that mitochondrial complex I-stimulated respiration and maximal oxidative capacity were severely affected in HF and could not be explained by changes in mitochondrial volume density or protein content. Other factors, such as ultra-structural alterations and mitochondrial super-complex destabilisation are likely contributing to a bio-energetic failure in HF.

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S2-E2

Diffusion obstacles shape the environment surrounding mitochondria in heart

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Intracellular environment in the heart cells is highly packed which could lead to the formation of diffusion obstacles. In particular, diffusion of ATP and ADP is rather slow when estimated on the basis of respiration kinetics measurements on permeabilized cardiomyocytes. Intriguingly, using kinetic experiments to estimate diffusion restrictions, it has been shown that they change significantly in disease. We have recently demonstrated that this estimation of slow diffusion is caused by intracellular structures and is not induced by possible side-effects, such as clamping of the cells or unstirred water layers. For that, we used the autofluorescence of cardiomyocytes and followed redox state of mitochondria by recording nicotinamide adenine dinucleotide (NADH) and flavoprotein (FP) signal changes in a permeabilized cell. By positioning the permeabilized cells in flow chamber, following NADH and FP changes induced by variation of ADP and ATP concentration in solution, and analyzing the data with

mathematical models, we were able to distinguish contribution of unstirred water layers and significant intracellular diffusion restrictions. Significant intracellular diffusion restrictions have been revealed when following diffusion of fluorescent molecules using raster image correlation spectroscopy. However, characteristics and localization of these restrictions is yet to be elucidated. While a mitochondrial outer membrane has been frequently suggested as a main diffusion restriction estimated by the kinetic measurements, recent data points to existence of diffusion restrictions in cytoplasm grouping mitochondria and ATPases.

The aim of this work is to distinguish the contribution of mitochondrial outer membrane and obstacles in cytoplasm to overall intracellular diffusion restrictions. For that, we recorded high resolution images of mouse cardiomyocyte autofluorescence changes due to ADP titration. These recordings allow us to analyze the heterogeneity of autofluorescence response. Experimental data is analyzed by mathematical modeling that enables us to estimate intracellular ADP diffusion coefficient and resistance of mitochondrial outer membrane.

S2-E3

Myocardial mitochondrial respiration in human heart failure

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Heart failure (HF) is a clinical disease entity defined by the presence of symptoms related to structural or functional abnormality of the heart, which impairs its pumping capability. The cellular mechanism associated with HF is not fully elucidated. Decreased phosphocreatine (PCr) to ATP ratios in human dilated cardiomyopathy contributed to the notion that the failing heart is “an engine without fuel” in recognition of the inability of the mitochondria to meet the energetic requirements of the contractile elements in the myocytes. Bioenergetic studies of human cardiomyocytes is limited by the availability of tissue samples (in particular left ventricular), but some data are available. In patients with and without left ventricular systolic dysfunction (LVSD) biopsies from the left ventricle was studied using high resolution respirometry. A pronounced reduction in oxidative phosphorylation capacity (OXPHOS) was seen in the patients with LVSD EF, when the respiration was sustained by both non-fatty acid as well as fatty-acid substrates. Creatine addition displayed marked effect on respiration, and revealed differences between groups in apparent K_m from ADP titrations. The limited fatty acid sustained respiration was supported by decreases in proteins involved in uptake, transport and combustion of fatty acids, as well as a decrease in proteins in the respiratory chain. Promising data on a feline model of hypertrophic cardiomyopathy are now emerging, and this model may actually serve as a substitute for human disease.

S2-E4

Ca²⁺ microdomains and interaction between mitochondria and ER/SR: Open questions

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Mitochondria are highly pleiotropic organelles, involved in the control of many intracellular processes, ranging from ATP synthesis to programmed cell death. They play a key role in the maintenance of Ca²⁺ homeostasis, since they can accumulate Ca²⁺ within their matrix through the so-called mitochondrial Ca²⁺ uniporter (MCU). Being the affinity of MCU well above the Ca²⁺ concentration reached in the bulk of the cytoplasm following its release from the Endoplasmic Reticulum (ER), the ability of mitochondria to take up Ca²⁺ was highly debated. This apparent paradox was explained by hypothesizing the presence of microdomains of high Ca²⁺ concentration at the interface between the two organelles. Experimental evidence to support this theory lacked until the recent discovery of the first mitochondria-ER molecular tether (Mitofusin 2, whose ablation impairs mitochondrial Ca²⁺ uptake) and our demonstration that [Ca²⁺] hot-spots exists at the ER-mitochondria contact sites, as independently confirmed by Hajnoczky and colleagues.

Since then the field of mitochondria-ER/SR interaction gained more attention, and nowadays its importance for the control not only of Ca²⁺ homeostasis but also of the overall cell physiology is widely recognized.

Despite this, and despite the evidence that impaired mitochondria-ER interactions are present in several disease models, a lot of open questions still remain: which are the molecules that mediate the physical interaction between these organelles? Which are the signalling pathways that control, or in turn are controlled by, mitochondria-ER/SR communication? How does impairment of inter-organellar communication contribute to the onset and progression of pathological conditions, i.e. does it play a causative role? Answering these questions will improve our understanding of physiological and pathological processes, and likely open the way to the discovery of druggable targets and novel therapeutical strategies.

S2-E5

The effects of Simvastatin on skeletal muscle treated with LPS in rats

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Aim: Sepsis model in animal is comprised experimentally to intraperitoneally injection of Lypopolysaccharide (LPS). LPS causes release of proinflammatory cytokines and alters mitochondrial activities together with the changes in muscle

structure. In our study, we aimed to investigate the effects of Simvastatin on muscle tissue treated with LPS in rats.

Material-Method: Male Wistar albino rats were divided into four groups: Control, LPS, Simvastatin, Simvastatin+LPS. Mitochondrial electron transport chain enzymes; citrate synthase, Complex I, Complex I+III, Cytochrome c oxidase (COX) were measured in spectrophotometer. Muscle tissue was evaluated for myopathic changes, Succinate dehydrogenase (SDH) and Cytochrome Oxidase (COX) under light microscopy.

Results: We found statistically significant increased levels of serum TNF- α and IL-10 in LPS and Simvastatin+LPS groups compared to Controls (p <0.01). In all experimental groups, Complex I and Citrate synthase activities were increased compared to controls. In control and LPS groups, COX activities were significantly higher than in the Simvastatin groups. In Simvastatin and Simvastatin+LPS groups, Complex I-III activity was higher than control and LPS (p <0.05). More than half of the animals treated with LPS showed myopathic changes.

Conclusions: Our findings demonstrated that simvastatin improves the alterations on enzyme activities and muscle structure after treating with LPS.

S2-E6

Exercise performed before and during sub-chronic Doxorubicin treatment mitigates cardiac mitochondrial alterations (morphology, OXPHOS, biogenesis, oxidative stress, permeability transition and apoptotic signaling)

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The role of exercise performed before and during treatment against heart mitochondrial dysfunction and cell death associated with sub-chronic Doxorubicin (DOX) is unknown. Two chronic exercise models (endurance treadmill training – TM and voluntary free-wheel activity – FW) were used. Mitochondrial ultra-structure, OXPHOS protein subunits, biogenesis, permeability transition pore (mPTP), apoptotic signaling and oxidative stress were analyzed. Thirty-six male Sprague-Dawley rats were divided into six groups (n=6 per group): saline sedentary (SAL+SED), SAL+TM (12-wks treadmill), SAL+FW (12-wks voluntary free-wheel), DOX+SED [7-wks sub-chronic DOX treatment (2 mg·kg⁻¹·wk⁻¹)], DOX+TM and DOX+FW. Heart mitochondrial ultra-structural alterations, OXPHOS proteins content and in gel activity, as well as proteins involved in mitochondrial biogenesis (PGC1 α and TFAM) and oxidative stress (carbonyl groups, SIRT3 and p66shc) were evaluated. Apoptotic signaling and mPTP opening regulation were followed by measuring Bax, Bcl2, and cofilin expression and calcium-induced variations of osmotic swelling. DOX

treatment resulted in ultramitochondrial alterations, decreased OXPHOS, decreased complex I activity and content, mitochondrial biogenesis (TFAM) and increased oxidative stress (carbonyls, SIRT3 and p66shc(Ser36)/p66shc ratio). Moreover, DOX treatment also augmented of calcium-induced mPTP susceptibility and apoptotic signaling (Bax/Bcl2 ratio). TM and FW mitigated DOX-induced alterations in mitochondrial ultra-structure, OXPHOS, increased oxidative stress and the decrease in complex I activity and content. DOX-induced decreased TFAM and SIRT3 content were only prevented by TM and both exercise modalities increased the content of cofilin in comparison with DOX+SED group. Both TM and FW mitigated DOX-increased mPTP susceptibility and apoptotic signaling. In summary, both chronic models of physical exercise performed before and during the course of sub-chronic DOX treatment translated into improved mitochondrial fitness and limited cardiac mitochondrial-driven apoptotic signaling, which may result in part from the prevention of oxidative stress and damage.

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S3-E

Current Trends in Respiratory Physiology: From Lung Function Towards System Biology Approaches

S3-E1

The links between lung function and cardiovascular diseases

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Lung function measurements provide an insight into the mechanical function of the respiratory system. Forced vital capacity (FVC) and forced expiratory volume in one second (FEV1) are spirometric measures that reflect lung volume and airway calibre and not only used to diagnose and monitor pulmonary diseases, but can also be applied for the prediction of cardiovascular disease mortality. Loss of tissue elasticity with aging is associated with loss in FVC and lung age is suggested as a potential marker of vascular remodelling. Both lung function and vascular stiffness have heritable determinants and are also influenced by environmental factors. Furthermore, there is a potential genetic influence on parenchymal and airway changes in chronic obstructive pulmonary disease (COPD) (Tarnoki DL et al, *Acta Physiol Hung*, 2014) together with a genetic predisposition for accelerated arterial stiffening (Horvath T et al *Hypertens Res* 2014). Recent findings show that FVC and FEV however are

phenotypically, but not genetically, associated with augmentation index, a measure of wave reflection (Tarnoki DL et al, *J Breath Res*, 2013) emphasizing the importance of environmental and life-style factors. Furthermore, in hypertension increased cardiovascular risk and reduced lung function coexists, but the pathophysiological mechanisms behind this feature are not clear. Further investigation of this relationship may lead to a better understanding of the close interplay in the cardiopulmonary system.

S3-E2

Oxidative stress pathways as new therapeutic opportunities: from infection to lung ageing

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Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, such as peroxide and oxygen ions. It has been widely accepted that the moderate concentrations of ROS have important roles in cell signalling, immune cell function and homeostasis, particularly host defence. However, an excess or chronic exposure of ROS or imbalance between ROS and anti-oxidants results in significant damage of molecules, cells and tissue. Thus oxidative stress is often blamed for the development of cancer and chronic inflammatory diseases such as chronic obstructive pulmonary disease (COPD) and severe asthma. Oxidative stress cascade is not simple, and recent system biological approach revealed that oxidative stress is amplified by mitochondrial dysfunction and secondary reaction of pollutants, smoke and infection rather than direct response. Elevated oxidative stress then affects inflammation, virus/fungus infection as well as lung ageing. Chronic exposure of ROS also reduced expression or function of anti-ageing molecules, such as histone deacetylase 2 (HDAC2), sirtuin 1, DNA repair protein Ku86 in lung, and this causes reduced endogenous antioxidants via defect of the large forkhead family of proteins (FOXO) and nuclear factor erythroid 2-related factor 2 (Nrf2). Defect of Nrf2 is also reported to causes an increase in influenza or respiratory syncytial virus infection, or virus infection-induced systemic effects, such as exacerbation. Even more importantly, Nrf2 activators, sulforaphane and metformin restored function of anti-ageing molecules and inhibited respiratory infection. Thus oxidative stress causes respiratory inflammation as well as accelerating lung ageing and respiratory infection in an even more complex manner. This vicious downward spiral of oxidative stress is a key factor of pathogenesis of COPD, and novel approach to oxidative stress will provide great opportunities to develop new treatment for chronic inflammatory disease, ageing or infection rather than simple synthetic anti-oxidants.

S3-E3

Mast cell biology in the human lung

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Allergic asthma is associated by involuntary constriction of the bronchial smooth muscle. This is often caused by mast cell activation. Allergen activation of mast cells causes most symptoms of allergic asthma through release of histamine, and synthesis of PGD2 and Cysteine leukotrienes, and later secretion of cytokines and chemokines. Expression of FcεRI on lung mast cells varies greatly by microlocation. It is known to depend on plasma IgE concentration.

We culture mast cells from human peripheral CD133+ blood stem cells with a 7 week protocol, in which we add IL4 and human specific IgE (total IgE 80 kU/L) for the last two weeks. Mast cells cultured this way can be activated with allergen under near-physiological conditions. Mast cells cultured from both non-allergic controls and allergic asthmatics respond similarly in this model, which suggests that allergen activation is largely a function of IgE rather than an intrinsic mast cell property. The sensitivity of PBMC depends to some extent on IgE concentration, and the fraction of cells responding depends on the complexity of IgE.

The relative contribution of mast cells and IgE to allergic responses is not yet elucidated, and may vary from person to person (and patient to patient). With this model, we can keep the IgE component constant and explore properties of the mast cell per sé.

S3-E4

Systems medicine and big data to phenotype and treat chronic airway diseases

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The most prevalent respiratory diseases exhibit far from simple pathophysiology. The complex biology of asthma and COPD is currently fueling the discovery of various disease phenotypes. Nevertheless, current guidelines for diagnosis and treatment are almost exclusively based on clinical and functional characteristics. Even though single biomarkers (e.g. sputum eosinophils) are gradually introduced in the management of these diseases (based on evidence provided by ‘algorithm studies’), it can be postulated that composite biomarker fingerprints will provide more comprehensive patho-biological information of these diseases and their underlying phenotypes¹. High-throughput “omics” technologies based on unbiased systems biology approaches, including transcriptomics, proteomics, lipidomics, and metabolomics are increasingly used for biomarker discovery in asthma². This has led to unravelling and validation of e.g. transcriptomic phenotypes of asthma based on epithelial, sputum or bronchial biopsy analysis. The leading principle here is to strictly obey the recent guidelines on omics analysis in clinical medicine, thereby purposely limiting false discovery with proper (external validation)³.

There are two challenges when bringing this to clinical practice. First, the measurements should be feasible at point of care with real-time availability of results. Metabolomics of exhaled air (breathomics) qualifies for this purpose and is currently applied in longitudinal, monitoring studies. Second, the ‘omics’ fingerprints have to be integrated with clinical data, requiring a quantitative multi-scale systems approach. The latter is key of the U-BIOPRED project that, after recently completing its longitudinal follow-up of (severe) asthma patients, is delivering its first unbiased, bio-clinical phenotypic fingerprints (www.ubiopred.eu). It is envisaged that this will lead to effective integration of molecular fingerprints in patient phenotyping and clinical management.

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S3-E5

Bronchoconstriction and alveolar derecruitment following extracorporeal circulation: Good by(e)pass?

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Background: While cardiopulmonary bypass (CPB) exerts its adverse pulmonary effects via inducing partial ischemia-reperfusion in the lung and systemic inflammatory response leading to surfactant damage, the changes in the lung mechanics and ventilation have not been characterized. Therefore, we aimed at elucidating the effects of CPB on the airway and lung tissue mechanics, ventilation heterogeneities, and dead space parameters.

Methods: Anaesthetized, mechanically ventilated patients (n=46) undergoing elective heart surgery were studied in open-chest condition before, and 5 min after weaning from CPB. Forced oscillations were applied to measure airway resistance (Raw), inertance (Iaw), lung tissue damping (G) and elastance (H). Mainstream capnography was performed to assess third phase slope of the expired CO2 (SIII) and respiratory dead space indices. Fowler’s dead space (VDF) reflecting the volume of the

conductive airways, Bohr's dead space (VDB) including also the unperfused alveolar volume, and Enghoff's dead space (VDE) comprising additionally the volume of the perfused but not ventilated alveoli were determined. The VDE VDB difference reflecting the intrapulmonary shunt was also calculated.

Results: CPB induced significant increases in Raw (143 ± 15 [SE]%), G ($130 \pm 6\%$), H ($7 \pm 0.5\%$) and SIII ($38 \pm 17\%$, $p < 0.02$ for all). Conversely, Iaw ($-178 \pm 25\%$) and the ventilation dead space variables decreased after CPB ($-12 \pm 0.1\%$ and $-10 \pm 0.1\%$ for VDB and VDF, respectively; $p < 0.01$). These changes caused significant elevation in the intrapulmonary shunt ($38 \pm 0.6\%$, $p < 0.001$).

Conclusions: After CPB, the narrowing of the airways and their volume loss is revealed by the rises in Raw and the drops of Iaw and VDF. The increases in SIII, G and H suggest atelectasis development with concomitant augmentation of ventilation heterogeneities. Elevated intrapulmonary shunt is substantiated from the increased VDE-VDB. These findings demonstrate the deleterious pulmonary effects of CPB, with providing evidence for the rapid development of disperse mechanical and ventilation defects affecting the central and peripheral lung compartments.

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S3-E6

Metabolic risk factors in insulin resistant vs. insulin sensitive asthma patients

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The current study assesses the metabolic risk status of asthma patients visiting the outpatient unit of Dept. of Pulmonology between August 15 2012 and October 15 2013. The metabolic state was characterized by the HOMA index (insulin resistance: HOMA index ≥ 4.4). Lung function was quantified by whole body plethysmography. Quality of life (QoL) was characterized using the St. George Respiratory Questionnaire. Overall 164 asthmatic patients were included in our sample (90 male, 74 female, average age 46.38 ± 14.87 years), of whom 36 proved to be insulin resistant. There was no significant difference between the insulin sensitive and insulin resistant group regarding the age, gender, smoking habits, total cholesterol, LDL-C, Lp(a) and ApoB levels. In contrast, several laboratory parameters were higher in insulin resistance: HgA1c ($p=0.0002$), triglyceride ($p < 0.001$), CRP ($p=0.048$) and uric acid ($p=0.027$). In turn, HDL-C ($p=0.003$) and ApoA1 ($p=0.022$) levels were lower for the insulin resistant group. Body mass index ($p < 0.001$) and waist circumference ($p < 0.001$) were higher among insulin resistant patients. Lung function differed as well: the residual volume compared to the reference value was lower in the insulin sensitive group ($p=0.02$). Of the four domains assessing the QoL, the activity ($p=0.043$) and total score ($p=0.039$) showed significant differences. It is concluded that asthmatic patients with insulin resistance have several untoward metabolic

derangement and poorer quality of life compared to those asthmatic patients who are insulin sensitive. This advocates the need for multidisciplinary action in the disease management of asthma.

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S4-A

Sodium Signalling in Astroglia

S4-A1

The mitochondrial $3\text{Na}^+/\text{Ca}^{2+}$ exchanger NCLX is a hub for cellular and mitochondrial Ca^{2+} signaling in astrocytes. or Na^+

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Powered by the mitochondrial membrane potential Ca^{2+} permeates into the mitochondria via the mitochondrial Ca^{2+} uniporter and pumped out by the activity of a $3\text{Na}^+/\text{Ca}^{2+}$ exchanger. This mitochondrial Ca^{2+} shuttling is playing a key role in coupling Ca^{2+} signal to ATP production or regulation of Ca^{2+} signals in the ER and plasma membrane micro domains while its breakdown leads to mitochondrial Ca^{2+} overload, the hallmark of brain and cardiac diseases. Despite the importance of mitochondrial Ca^{2+} shuttling the molecular identity of the uniporter and exchanger remain elusive. In the first part of my talk I will describe the identification of the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger termed NCLX and the tools we use to control and monitor its activity. In the second part of my talk I will focus on the physiological role of NCLX in regulating Ca^{2+} and Na^+ signal thereby mediating executive functions ranging from neurotransmission release to proliferation of glial cells. Finally I will describe a novel communication pathway between NCLX with the plasma membrane store operated Ca^{2+} channel.

S4-A2

Exocytotic glutamate release from astrocytes: Intracellular Ca^{2+} and Na^+ dynamics

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Astrocytes can exocytotically release the gliotransmitter glutamate. Increased cytosolic Ca^{2+} concentration is

necessary and sufficient in this process. The source of Ca^{2+} for the Ca^{2+} -dependent mechanically-induced exocytotic release of glutamate from astrocytes predominately comes from endoplasmic reticulum (ER) stores; both inositol trisphosphate (IP_3)- and ryanodine-sensitive receptors are involved. An additional source of Ca^{2+} in this process comes from the extracellular space through the plasmalemmal canonical transient receptor potential 1 protein (TRPC1), which forms channels that are activated by depletion of internal Ca^{2+} stores, and $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) operating in reverse mode. Mitochondria can modulate cytosolic Ca^{2+} levels by affecting two aspects of the cytosolic Ca^{2+} kinetics in astrocytes. They play a role in immediate sequestration of Ca^{2+} during the cytosolic Ca^{2+} increase in stimulated astrocytes using calcium uniporter. As cytosolic Ca^{2+} declines due to activity of pumps, such as the smooth ER Ca^{2+} -ATPase, free Ca^{2+} is slowly released by mitochondria into cytosol via the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCLX) and transient openings of the mitochondrial permeability transition pore. Taken together, ER, extracellular space and mitochondria, can vary concentration of cytosolic Ca^{2+} which in turn can regulate Ca^{2+} -dependent vesicular glutamate release from astrocytes. In parallel to this Ca^{2+} excitability, there are fluctuations in astrocytic cytosolic Na^+ levels. While the plasmalemmal Na^+/K^+ -ATPase and Ca^{2+} -ATPase are the major Na^+ and Ca^{2+} extruders in resting astrocytes, they appear less important during times of Ca^{2+} and Na^+ cytosolic loads caused by mechanical stimulation. Unexpectedly, NCX in reverse mode appears as a major contributor to overall Ca^{2+} and Na^+ homeostasis in astrocytes both at rest and when these glial cells were mechanically stimulated. This stimulation of astrocytes results in increases in cytosolic Ca^{2+} and Na^+ levels that are in part due to entry of extracellular cations through TRPC1 containing channels. TRPC channels seem amenable to changes in selective filtering as antibody binding to the TRPC1 pore different

S4-A3

Astrocytic Na^+ influences extracellular GABA/glutamate balance in the neocortex

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In the CNS, glutamatergic neurotransmission proceeds with high temporal and spatial resolutions. Synaptically released glutamate diffuses through the synaptic cleft and activates postsynaptic receptors, eliciting a postsynaptic current. Glutamate effects on receptors are terminated by diffusion and by the actions of glutamate transporters (EAATs). Glial EAATs strongly contribute to glutamate removal, and synaptically-activated, glutamate transporter-mediated currents (STCs) can be recorded. In this study, STCs elicited by local electrical stimulation in cortical layer 4 were recorded from layer 2/3 astrocytes. When low $[\text{Na}^+]_i$ (5 mM) intra-pipette solution was used, STCs demonstrated paired-pulse facilitation (PPF) at short (<250 ms) inter-stimulus intervals (ISIs) and paired-pulse depression (PPD) at longer ISIs. Elevation of intra-pipette $[\text{Na}^+]_i$ to 20 mM, i.e. close to its physiological value, reduced PPF of STCs at short ISIs, while

PPD at longer ISIs was not affected. STC decay kinetics were slowed in the presence of high $[\text{Na}^+]_i$. Exogenous GABA increased astrocytic $[\text{Na}^+]_i$, reduced the mean STC amplitude, decreased PPF at short ISIs, and slowed STC kinetics. All GABA-induced changes were blocked by NO-711 and SNAP-5114, GABA transporter (GATs) antagonists. GAT blockade decreased PPF at short ISIs under control conditions, provided low $[\text{Na}^+]_i$ intra-pipette solution was used. Dialysis of single astrocyte with low $[\text{Na}^+]_i$ solution increased the amplitude and reduced paired-pulse ratio of evoked field potentials recorded in the vicinity of the astrocyte. Thus, endogenous GABA can influence EAAT functioning via GATs and astrocytic $[\text{Na}^+]_i$ modulates the short-term plasticity of STCs and in turn the efficacy of glutamate removal.

S4-A4

Sodium signalling in astroglia

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Astrocytes exhibit their excitability based on variations in cytosolic Ca^{2+} levels, which leads to variety of signalling events. Only recently, however, intracellular fluctuations of more abundant cation Na^+ are brought in the limelight of glial signalling. Indeed, astrocytes possess several plasmalemmal molecular entities that allow rapid transport of Na^+ across the plasma membrane: (i) ionotropic receptors, (ii) canonical transient receptor potential cation channels, (ii) neurotransmitter transporters, and (iv) sodium-calcium exchanger. Concerted action of these molecules in controlling cytosolic Na^+ may complement Ca^{2+} signalling to provide basis for complex bidirectional astrocyte-neurone communication at the tripartite synapse.

S4-A5

A putative counter-apoptotic role of the non-gastric H^+/K^+ -ATPase ATP1A1 (ATP12A)

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The non-gastric H^+/K^+ -ATPase ATP12A (ATP1A1) is expressed in a broad variety of tissues, but the knowledge about its function is sparse. We found by RTPCR and/or western blotting, intracellular pH measurements, electron microprobe analysis, cell volume (CV) measurements and flow cytometry that ATP12A is expressed in human myelomonocytic HL60 cells, rat insulinoma Ins-1E cells, human pancreatic islets, the prostate cancer cell lines LNCaP, PC3 and DU-145 as well as in normal and cancerous human prostate tissue. Treatment of HL60 cells with low (1mM) concentrations of butyrate leads to monocyte-directed differentiation whereas higher (5-10mM) concentrations

induce apoptosis as assessed by flow cytometric determination of CD86 expression, CV, cell granularity, caspase activity, phosphatidylserine exposure on the outer plasma membrane leaflet, cell cycle analysis and cell proliferation. Transcriptional up-regulation of ATP12A is evident during apoptosis in HL60 and Ins-1E cells and both cell types exhibit apparent apoptotic volume decrease (AVD). An effect of the H⁺/K⁺-ATPase inhibitor SCH28080 is not evident in untreated HL60 cells, but becomes visible in cells are stimulated with 1mM butyrate. Moreover, AVD from 24-72h is not affected by SCH28080 in the untreated non-apoptotic cells. Inducing the cells with 1 mM butyrate reveals the same behavior in PS⁻ cells, however PS⁺ cells resist to shrink and have finally a significantly higher CV than PS⁻ cells after 72h and the CV of PS⁺ cells is now sensitive to SCH28080. i.e. PS⁺ cells treated with SCH28080 have the same cell volume as butyrate treated non-apoptotic cells. This indicates that AVD may depend on differentiation and/or exposure of phosphatidylcholine to the outer leaflet of the cell membrane. Moreover ATP12A expression is altered in tissue from benign hyperplasia of human prostate and in prostate cancer. In summary it is shown that ATP12A is functionally active, plays a role during early apoptosis in HL60 cells and Ins-1E cells, is differently expressed in normal and pathological prostate tissue.

S5-A

From Macro- to Microvessels: Function, Structure and Molecular Mechanisms

S5-A1

Role of metabolites of arachidonic acid in regulation of vascular function

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Eicosanoids, metabolites of arachidonic acid (AA), are crucial in maintenance of vascular tone and reactivity to various physiological stimuli, contributing to blood pressure regulation, appropriate tissue perfusion, and protection of, or susceptibility to atherosclerosis and thrombosis. While the importance of prostaglandin (PGs)/TX balance is well documented, there is an increasing body of evidence that hydroxylation or epoxygenation of AA via CYP450 enzymes significantly contribute to physiological maintenance of vascular tone and vascular reactivity of resistance vessels. 20-HETE mediates arterial myogenic constriction and arteriolar response to increased pO₂ in vitro and the autoregulation of cerebral blood flow in vivo.

On the other hand, increased levels of 20-HETE mediates cerebral microvessels' vasoconstriction and increases tissue damage after stroke in rats and in human, while increasing levels of EETs or decreasing levels of 20-HETE have beneficial effects. EETs are anti-inflammatory, sustain endothelium-dependent vasodilation in human essential hypertension, and decreased EETs are associated with salt-sensitive hypertension.

The increased production of reactive oxygen species (ROS) underlies disbalance of vasodilator vs. vasoconstrictor eicosanoids. ROS are generated by a variety of enzymes in vascular tissues, but also in circulating and adhering leukocytes. There is a circuitous relationship between ROS and cyclooxygenases (COX1,2) upregulating each other. 20-HETE significantly increases vascular production of ROS. ROS mediate non-enzymatic peroxidation of AA, which gives rise to isoprostanes, particularly to potent vasoconstrictors, 8-epi-PGF₂α and 8-epi-PGE₂. In oxidative stress, blood levels of isoprostanes are much higher than those of COXs' products.

In conclusion, there is a communication network among various eicosanoids. In physiological conditions eicosanoids are important in maintenance of vascular tone and reactivity, but in condition with increased ROS/isoprostanes formation, the imbalance of the PGI₂/TXA₂ system and/or in 20HETE/EETs system can occur, which may become deleterious to vascular function, such as in stroke and hypertension

S5-A2

Angiotensin II and leukocyte trafficking: New insights for an old vascular mediator

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Angiotensin-II (Ang-II) is implicated in atherogenesis. We demonstrated that 4h exposure to Ang-II in vivo caused arteriolar leukocyte adhesion in the mesenteric microcirculation of the rat through the interaction with its AT1 receptor (1). While mononuclear cells were the main leukocytes attached to the arteriolar endothelium, neutrophils were predominantly cells interacting with the venular endothelium. Since, the same cell adhesion molecules (CAMs) were expressed on both the arteriolar and venular endothelia in response to Ang-II (1), other mechanisms seemed to be responsible for the differential cellular distribution. Indeed, arteriolar mononuclear leukocyte recruitment by Ang-II was found to be largely mediated by tumor necrosis factor-α (TNFα) (2) and, fractalkine (CX3CL1) expression was detected in both cremasteric arterioles and post-capillary venules 24h after Ang-II intrascrotal injection (3). Arteriolar leukocyte adhesion was the unique parameter significantly reduced (83%) in animals lacking CX3CL1 receptor (CX3CR1). When human arterial and venous umbilical endothelial cells (HUAEC and HUVEC) were stimulated with 1 μM Ang-II increased CX3CL1 expression was detected, yet neutralization of CX3CL1 activity only significantly inhibited Ang-II-induced mononuclear cell-HUAEC interactions. The use of siRNA revealed the involvement of TNFα in Ang-

II-induced CX3CL1 up-regulation and mononuclear cell arrest. Nox5 knock down with siRNA or pharmacological inhibition of ERK1/2, p38 MAPK and NFκB also abolished these responses. These results suggest that targeting CX3CL1-CX3CR1 axis may constitute a new therapeutic strategy in the treatment of Ang-II-associated cardiovascular disorders.

- 1.- Alvarez et al., Blood 104:402-8 (2004).
- 2.- Mateo et al., Blood 110:1895-902 (2007).
- 3.- Rius et al. Arterioscler Thromb Vasc Biol 33:96-104 (2013).

S5-A3

Microvascular mechanisms of age-related cognitive decline

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Hypertension in the elderly substantially contributes to cerebrovascular damage and promotes the development of vascular cognitive impairment. Despite the importance of the myogenic mechanism in cerebrovascular protection, it is not well understood how aging affects the functional adaptation of cerebral arteries to high blood pressure. Hypertension was induced in young (3 months) and aged (24 months) C57/BL6 mice by chronic infusion of angiotensin II (AngII). In young hypertensive mice, the range of cerebral blood flow autoregulation was extended to higher pressure values, and the pressure-induced tone of middle cerebral artery (MCA) was increased. In aged hypertensive mice, autoregulation was markedly disrupted, and MCAs did not show adaptive increases in myogenic tone. In young mice, the mechanism of adaptation to hypertension involved upregulation of the 20-HETE (20-hydroxy-5,8,11,14-eicosatetraenoic acid)/transient receptor potential cation channel, subfamily C (TRPC6) pathway and this mechanism was impaired in aged hypertensive mice. Downstream consequences of cerebrovascular autoregulatory dysfunction in aged AngII-induced hypertensive mice included exacerbated disruption of the blood-brain barrier and neuroinflammation (microglia activation and upregulation of proinflammatory cytokines and chemokines), which were associated with impaired hippocampal dependent cognitive function. Collectively, aging impairs autoregulatory protection in the brain of mice with AngII-induced hypertension, potentially exacerbating cerebrovascular injury and neuroinflammation.

S5-A4

Role of antioxidant genes and microRNAs in revascularisation after hind limb ischemia

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Nuclear factor, erythroid 2-like 2 transcription factor (Nrf2, encoded by NFE2L2) and heme oxygenase-1 (HO-1, encoded by HMOX-1 gene), one of its major targets, are crucial mediators of cellular defence response against oxidative stress. Accordingly, their involvement in numerous diseases has been widely investigated. However, it appears that the functions of both Nrf2 and HO-1 extend much beyond their canonical protective activity. We and others have elucidated that the HO-1 is playing a role in blood vessels formation and such an effect of HO-1 in regeneration after skin injury, hind limb ischemia and myocardial infarction has been demonstrated. Our recent studies showed also that angiogenic response of endothelial cells and bone-marrow derived proangiogenic cells is impaired in the absence Nfe2l2 and Hmox-1. Accordingly, lack of HO-1, as evidenced by the studies of Hmox-1 deficient animals attenuates regeneration after hind limb ischemia and even haplodeficiency of Hmox-1 decreased revascularisation in diabetic animals. Genetic overexpression of Hmox-1 by hypoxia-regulated vectors provides faster restoration of vascularisation and, additionally, combined overexpression of HO-1 and VEGF-A is able to fully restore hind limb circulation in Hmox-1 knockout mice. Surprisingly, in contrast to HO-1, the lack of Nrf2 promotes blood vessel regeneration after hind limb ischemia, indicating for the context-dependent role of Nrf2 in inflammatory and non-inflammatory angiogenesis.

Recent studies indicate for the significant role of non-coding microRNAs in blood vessel formation. Among angiomiRs the miR-378 appears to be crucial for revascularisation. The cross-talk interactions of this miRNA with HO-1 have been recently described by us. Interestingly, the lack of miR-378 appears to significantly affect the vasculature regeneration after hind limb ischemia.

Understanding of the role of antioxidant genes and their interactions with non-coding RNAs can provide the rationale for novel approaches in revascularisation therapies.

S5-A5

A new functional role of Ca²⁺ sensitization mechanisms in the regulation of vascular smooth muscle contraction

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Introduction: Cardiovascular diseases related to sustained arterial contraction are an important cause of mortality and morbidity in humans. Although the increase in [Ca²⁺]_i can cause contraction of smooth muscle, arterial contraction can be regulated even if the concentration of this ion remains constant. This mechanism, called "Ca²⁺ sensitization", participates in the maintenance of arterial contraction through the inhibition of the activity of myosin light chain phosphatase (MLCP). Although RhoA/Rho kinase (ROCK) and PKC can both inhibit MLCP, these two pathways not only work in parallel, but they can also interact. We have studied the role of both, PKC and RhoA/ROCK, sensitization pathways in PDBu-treated arteries.

Materials and Methods: Isometric force was measured in rat femoral arterial rings. Immunofluorescence experiments were performed on isolated myocytes. PKC and RhoA were analyzed by Western blot and G-LISA in arterial rings precontracted with PDBu, a PKC activator.

Results: PDBu induced the activation and translocation of PKC α to the plasma membrane. PDBu also evoked an increase in cytosolic RhoA, suggesting its inhibition. Simultaneous application of ROCK and PKC α inhibitors induced a synergistic, non-additive, vasorelaxant effect on sustained contraction in PDBu-treated arteries.

Discussion: Here we describe a new form of crosstalk between PKC α and RhoA pathways. As PKC α negatively regulates RhoA activity, PKC α inhibitors could facilitate the vasorelaxant effect of ROCK inhibitors. Simultaneous treatment with ROCK and PKC α inhibitors could provide a new strategy to optimize the therapeutic treatment of pathological processes that mediate arterial vasospasm.

S5-A6

Myeloperoxidase promotes the vasoconstrictive effects of hydrogen-peroxide

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Myeloperoxidase (MPO) is a central participant in inflammatory processes; however recent studies emphasize the involvement of MPO in the development of cardiovascular diseases. MPO catalyzes the reaction of hydrogen-peroxide (H₂O₂) with chloride (Cl⁻) to form hypochlorous acid (HOCl).

We detected the changes of internal diameter (id.) of cannulated and pressurized (80 mmHg) skeletal muscle arterioles (SKA, id.: 160±6 μm) and coronary arterioles (CA, id.: 178±14 μm), moreover we examined the changes of the contractile force of the basilar artery (BA). The changes in [Ca²⁺]_i by Fura-2 fluorescence ratio (F340/380) were also determined in SKA.

H₂O₂ has a biphasic effect in the SKA and the BA (constriction at lower, and dilation at higher concentrations) however; it evokes only vasodilation in the CA. In the presence of MPO (5 nM) the H₂O₂-induced constriction was increased in SKA (47±11% constriction vs. 50±21% dilation at 1 mM H₂O₂, P=0.004), and in the BA (114±14% vs. 88±6% of KCl evoked maximal constriction measured at 100 μM H₂O₂). The H₂O₂-evoked dilation turned into constriction in CA (6±3% constriction vs. 13±4% dilation at 100 μM H₂O₂, P=0.006) after incubation with MPO. The mechanism of the MPO evoked increased constriction was investigated in SKA. The HOCl scavenger L-methionine inhibited the MPO and H₂O₂ induced constriction (91±2% dilation vs. 47±11% constriction at 1 mM H₂O₂, P <0.0001). In the absence of endothelium the constriction was still observed in lower concentrations of H₂O₂ (31±8% vs. 26±4% constriction at 100 μM H₂O₂) but it was abolished in higher concentrations of H₂O₂ (28±21% dilation vs. 47±11% constriction at 1 mM H₂O₂). Furthermore, the constriction was converted to

dilation (70±21% dilation vs. 47±11% constriction at 1 mM H₂O₂, P=0.001) by thromboxane A₂ (TXA₂) receptor inhibition. MPO and H₂O₂ induced constriction was not accompanied by significant changes in F340/380.

Our results suggest that MPO increases the H₂O₂-evoked constriction by the generation of HOCl which causes the synthesis of TXA₂ in the endothelial and smooth muscle cells leading to an increase in the Ca²⁺ sensitivity of the vascular smooth muscle.

S4-B

Lipid GPCRs in Physiology and Disease

S4-B1

Novel GPCRs for lysophosphatidylserine; their structure and function

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It is now accepted that lysophospholipids (LPLs) have a wide variety of functions as lipid mediators that are exerted through G protein-coupled receptors specific to each lysophospholipid. While the roles of some LPLs, such as lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), have been thoroughly examined, little is known about the roles of several other LPLs, such as lysophosphatidylserine (LysoPS), lysophosphatidylthreonine (LPT), lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI) and lysophosphatidylglycerol (LPG). Recently, three GPCRs (GPR34/LPS1, P2Y10/LPS2 and GPR174/LPS3) were found for LysoPS. In this talk, I will focus on these newly identified GPCRs and summarize the actions of LysoPS as lipid mediators.

S4-B2

Cannabinoid type 1 receptor in noradrenergic/adrenergic cells and its role in metabolism and stress

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Neuropeptide Y (NPY) and stress has been associated with obesity. However, it is still unknown why some people lose weight under stress. Our study suggests that specific deletion of cannabinoid type 1 receptor (CB1) from dopamine

β -hydroxylase (dbh) expressing neurons in mice disturbs the homeostatic equilibrium between β -adrenergic and NPY transmission, making mutant mice resistant to diet induced obesity through increased β -adrenergic transmission in white and brown adipose tissue (BAT). However, these mutant mice when exposed to chronic stress gain weight through increased NPY transmission in visceral fat independent of ongoing higher thermogenesis in BAT.

Our study suggests CB1 signaling in dbh neurons may be crucial for sensitivity to stress-induced weight gain or loss. Furthermore, stress- and NPY-induced obesity cannot be corrected by increasing thermogenesis alone and requires inhibition of NPY transmission in visceral fat.

S4-B3

New functions for short chain fatty acid and prostanoid receptors

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G-protein-coupled receptors (GPCRs) have traditionally been regarded as receptors for hormones, neurotransmitters, and other mediators which are produced solely for the purpose of carrying a signal and to serve cell-cell communication. This view has changed during the last decade, as a growing number of GPCRs are being identified, for which the ligands are energy substrates or metabolic intermediates. Among these ligands are saturated and unsaturated free fatty acids (FFAs), which exert cellular effects through GPCRs named FFA1-FFA4. These receptors are widely expressed in the human body and regulate the metabolic, endocrine or immune system to maintain homeostasis under changing dietary conditions. Data will be presented on the role of FFA2 and FFA3 in the regulation of metabolic functions. In addition, recent research on the identification of novel lipid ligands of prostanoid receptors will be discussed.

S4-B4

Control of gastrointestinal epithelial integrity by lysophosphatidic acid GPCR

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Lysophosphatidic acid (LPA) is a pleiotropic growth factor-like lipid mediator that acts via at least six GPCR. Although LPA is produced in blood and other biological fluids from lysophosphatidylcholine and lysophosphatidylserine by the lysophospholipase D autotaxin, it is also present in foods. The LPA2 GPCR is abundantly expressed in gastrointestinal epithelial cells and for this reason we have studied its role in the maintenance of gastrointestinal epithelial function. In this

talk we will review the following aspects of LPA2 signaling in the GI epithelium:

- 1) Inhibition of CFTR-mediated secretory diarrhea by the LPA2-NHERF2 macromolecular complex.
- 2) Protection of the small intestinal stem cells from radiation- and chemotherapeutic-induced apoptosis by the LPA2-Siva-1 and LPA2-NHERF2-TRIP6 macromolecular signalosome.
- 3) Enhancement of DNA repair mediated by LPA2.
- 4) Protection against NSAID-induced gastric erosions by LPA2-selective agonists.

Our results point to a comprehensive role of the LPA2 GPCR in the maintenance of the functional integrity of the gastrointestinal epithelium.

S4-B5

Signaling pathways of thromboxane receptor-mediated vasoconstriction: Major role of phospholipase C epsilon

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We aimed to elucidate the intracellular signaling pathways of the sustained vasoconstriction and hypertension induced by thromboxane A2 (TXA2) via stimulation of TP prostanoid receptors. Isometric tension recording was performed in thoracic aortic segments (TAs) isolated from wild-type mice (WT) as well as from mice deficient in TP receptors (TP-KO), the alpha subunits of q/11 or 12/13 G-proteins (Gq/11-KO and G12/13-KO) or PLC ϵ (PLC ϵ -KO). Phosphoinositol-hydrolysis was monitored by measurement of 3H-inositol-phosphate (3H-InsP) formation in TAs. Changes of intracellular calcium levels [Ca²⁺]_i in TAs and primary cultures of vascular smooth muscle cells (VSMC) were assessed by ratiometric measurement of Fura-2-AM fluorescence. Arterial blood pressure was determined in ketamine/xylazine anesthetized animals. 12/13-KO mice showed decreased contraction and [Ca²⁺]_i elevation in response to the TP receptor agonist U-46619, which failed to induce any effect in TP-KO vessels. Interestingly, the U-46619 induced Ca²⁺ signal in Gq/11-KO VSMC persisted in Ca²⁺-free medium and was unaffected by the Rho-kinase (ROCK) inhibitor Y-27632, but almost completely abolished by the RhoA inhibitor TAT-C3. Since RhoA can activate reportedly PLC ϵ we further investigated this pathway. TAs from PLC ϵ -KO mice showed significantly decreased vasoconstriction, 3H-InsP accumulation and [Ca²⁺]_i elevation upon administration of U-46619 whereas the effects of the alpha1-adrenoreceptor agonist phenylephrine remained unchanged. In accordance, the hypertensive effect of U-46619 but not that of norepinephrine decreased in PLC ϵ -KO as compared to WT mice. α TAs from both Gq/11-KO and G Our results indicate that q/11 and 12/13 G-proteins are

simultaneously involved in the mediation of TXA₂-induced vasoconstriction. While the Gq/11-dependent effect is likely to be mediated by PLC β , the Gq/11-independent pathway also involves phosphoinositol-hydrolysis and subsequent intracellular Ca²⁺ release in a RhoA-dependent, but ROCK-independent manner.

Our results suggest that G12/13- and RhoA-mediated activation of PLC ϵ plays a major role in TXA₂-induced vasoconstriction and hypertension.

S4-B6

Mutations in the conserved 'DRY' motif of the CB1 cannabinoid receptor result in functionally selective receptor conformations

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The role of the highly-conserved 'DRY' motif in the signaling of the CB1 cannabinoid receptor (CB1R) was investigated by introducing single, double and triple alanine mutations into this site of the receptor. We found that the CB1R-R3.50A (CB1R-DAY) mutant displays a partial decrease in its ability to activate heterotrimeric G α proteins (~85% of wild-type CB1R (CB1R-WT)). Moreover, this mutant showed impaired β -arrestin binding in response to agonist stimulus, although its basal β -arrestin binding was enhanced. More strikingly, the double mutant CB1R-D3.49A/R3.50A (CB1R-AAY) was biased toward β -arrestins, as it gained a robustly increased β -arrestin2 binding ability compared to the wild-type receptor, while its G protein activation was substantially decreased. In contrast, the double mutant CB1R-R3.50A/Y3.51A (CB1R-DAA) proved to be G protein-biased, as it was practically unable to recruit β -arrestin2 in response to agonist stimulus, while still activating G proteins, although at a lowered level (~75% of CB1R-WT). Agonist-induced internalization as well as ERK1/2 activation of the CB1R mutants showed good correlation with their β -arrestin binding ability, but not with G protein activation. Our results suggest that G protein activation and β -arrestin binding of the CB1R are mediated by distinct receptor conformations and that the conserved 'DRY' motif plays different roles in the stabilization of these conformations, thus mediating both G protein- and β -arrestin2-mediated functions of CB1R.

S5-B

Physiology and Regulation of K2P Channels

S5-B1

Excitability tuning by two-P-domain channels: From inhibitory potassium-selective channels to excitatory cationic channels

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Potassium channels set the resting membrane potential, favouring hyperpolarization and inhibition of cell excitability. Gene mutations leading to a loss of selectivity for potassium in different potassium channels are related to severe disorders in mouse and human.

We have shown that such a change in ion selectivity is not only observed in pathological conditions but also in normal physiology. The background K2P channel TWIK1 is the very first example of a channel that reversibly shifts from a potassium-selective state to a sodium-permeable state upon physiological stimuli. This result suggests that a single channel can fulfill opposite roles traditionally attributed to different classes of ion channels: either hyperpolarizing and inhibitory such as potassium and chloride channels, or depolarizing and excitatory such as sodium, calcium and cationic channels. Dynamic ion selectivity also occurs in other mammalian K2P channels, as well as likely in those of *Caenorhabditis elegans*. This broad distribution and evolutionary conservation among K2P channels indicates that dynamic selectivity constitutes a new regulatory mechanism of cellular excitability, whose significance is only now becoming appreciated.

S5-B2

The intracellular traffic of the two-pore-domain potassium channel TASK-1

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Background. It is still unclear to what extent membrane proteins determine the itinerary and the destination of the transport vesicles in which they reside. In many cases the intracellular traffic of membrane proteins is controlled by specific interacting proteins. We have shown previously that the K2P-channel TASK-1 interacts with 14-3-3 proteins and with p11 (S100a10). Recently, we found that TASK-1 also interacts with the endosomal SNARE protein syntaxin-8 and studied the functional relevance of this interaction.

Principal findings. TASK-1 and syntaxin-8 were expressed in *Xenopus* oocytes and mammalian cell lines. Co expression of syntaxin-8 caused a four-fold reduction in TASK-1 current, a marked reduction in the expression of TASK-1 at the cell surface and an increase in the rate of endocytosis of the channel. Co-expression of the SNARE protein syntaxin-7 had no effect on TASK-1 currents. Systematic mutagenesis experiments showed that the C-terminus of TASK-1 interacts with a 40 amino-acid region proximal to the SNARE domain of syntaxin-8. The stimulatory effect of the SNARE protein on the endocytosis of the channel was abolished when both an endocytosis signal in TASK-1 and an endocytosis signal in syntaxin-8 were mutated. A syntaxin-8 mutant which cannot

assemble with other SNARE proteins had virtually the same effects as wild-type syntaxin-8. TIRF microscopy showed formation and endocytosis of vesicles containing fluorescence-tagged clathrin, TASK-1 and/or syntaxin-8.

Conclusions. Our results suggest that the unassembled form of syntaxin-8 and TASK-1 are internalised via clathrin-mediated endocytosis in a co-operative manner. The adaptor protein AP-2 possesses separate binding sites for tyrosine and di-leucine-based endocytosis signals. In vitro binding assays suggested that AP 2 exhibits tighter binding to a membrane harboring both di-leucine- and tyrosine-based sorting signals [6]. Now we have shown for the first time that this is indeed the case in intact cells. This implies that syntaxin-8 regulates the endocytosis of TASK-1, and vice versa. In addition, our study shows that SNARE proteins can have functions unrelated to membrane fusion.

S5-B3

Pharmacological and genetic recovery of current through truncated and mutated K2P channels

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Two pore domain potassium (K2P) channels contribute to background potassium conductances in many cell types, being active over the entire voltage-range. However their activity can be up or down regulated by a variety of physiological and pharmacological mediators (Enyedi & Czirják, 2010). Using molecular modelling, site directed mutagenesis and electrophysiological approaches, my laboratory is investigating how both gain of function mutations and physiological and pharmacological mediators can give insight into the functional properties of K2P channels and what regions of the channel are important for both gating and the transduction of a regulatory signal to the gate. In this presentation, I will focus on our recent experiments on two K2P channels, TREK1 (TWIK-related K channel 1, K2P2.1, KCNK2) and TASK3 (TWIK-related acid-sensitive K channel 3, KCNK9, K2P9.1).

TREK1 can exist in two forms following alternative translation initiation (Thomas et al 2008). Each of these forms is expressed as protein in both neurons and expression systems. The shorter, N-terminus truncated form of TREK1 gives rise to a current with a much reduced open probability, a reduced potassium selectivity and a measurable permeability to sodium (Thomas et al., 2008). We have found that the current through this short form of TREK1 is greatly enhanced by non-steroidal anti-inflammatory drugs such as flufenamic acid (FFA). In addition, FFA alters the ion selectivity of this channel so that it becomes a K selective channel, like the long form of TREK1. The effects of these drugs are also seen following gain of function mutations of the short form of the channel (Veale et al., 2014b).

TASK3 channels are the only K2P channels known to be genetically imprinted, in that they are expressed only from the maternal allele. Indeed a mutation in TASK3 (G236R) is responsible for a maternally transmitted developmental disorder, Birk Barel mental retardation dysmorphism

syndrome (Barel et al., 2008). We have considered the functional properties of G236R mutated TASK3 channels in some detail. Expression of TASK3_G236R channels gives rise to small but detectable functional current. In contrast to WT TASK3 channels, the current is inwardly rectifying and this current is differentially sensitive to a number of regulators such as extracellular acidification and activation of G protein coupled receptors. Importantly, the reduced current through mutated TASK3_G236R channels can be overcome, at least in part, by both application of FFA and by a gain of function mutation of TASK3 channels (Veale et al., 2014b).

Our work on truncated TREK1 channels and mutated TASK3 channels shows that it is possible to restore current through these channels through manipulations that alter channel gating. Pharmacological manipulation of these channels, in particular, may have potential therapeutic applications.

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S5-B4

TRESK background K⁺ channel is regulated by calcineurin and other interacting proteins

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Members of the K2P channel family are controlled by a great diversity of regulatory mechanisms. Excitable cells typically express different combinations of K2P channels in order to adjust their resting membrane potential and counter excitatory stimuli. TRESK (K2P18) is predominantly expressed in the sensory neurons of dorsal root and trigeminal ganglia, together with TREK-2 (K2P10) and minor amounts of other K2P channels. At present, the specific physiological roles of these K2P components in pseudounipolar neurons are incompletely understood, although the inactivating mutation of TRESK was reported to be linked to a rare form of familial migraine, suggesting the functional relevance of the channel.

In the past decade we have investigated the regulatory mechanisms of TRESK in vitro and in heterologous expression systems. We have established that in contrast to the other K2P channels, TRESK is regulated by the elevation of the cytoplasmic calcium concentration. This activation depends on the calcium/calmodulin-dependent protein phosphatase calcineurin. Alanine-scanning mutagenesis indicated three serines in TRESK as the putative targets of dephosphorylation. Calcineurin directly associates to TRESK via a Nuclear Factor of Activated T cells (NFAT)-like binding site located in the cytoplasmic loop of the channel. Such a mechanism of calcineurin targeting is unique within the ion channel superfamily. In the present lecture, we are going to reveal a previously unknown level of the intimate interaction of the channel with the phosphatase.

Basal channel activity is maintained, and calcineurin-dependent activation is reversed after the cessation of the

calcium signal, by inhibitory kinases. One regulatory serine is phosphorylated by protein kinase A (PKA) and this phosphorylation allows the anchoring of 14-3-3 adaptor protein to the channel. 14-3-3 may have inhibitory effect by preserving the phosphorylation of the channel under resting conditions. We have also detected the interaction of a third protein with TRESK by a recent affinity chromatography approach, however, it remains to be established whether this interaction is relevant *in vivo*.

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S5-B5

Amusing functions of TWIK-1 in the brain

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TWIK-1 is the first identified K2P channel which is highly expressed in the brain. However, its physiological role has been elusive because it has been demonstrated as a non-functional channel. Recently, we found that TWIK-1 form a heterodimer with TREK-1 to be functional in astrocytes. These heterodimeric channels via disulfide bridge formation mediate the potassium passive conductance and G β γ -induced glutamate release in astrocytes. In my talk, I will discuss the role of TWIK-1 in astrocytes and present some of our recent data on its function in CNS neurons.

S5-B6

Stable gene silencing of TASK-3 channels in melanoma cells induce intrinsic apoptosis

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We have previously demonstrated a primarily mitochondrial localisation of the TASK-3 potassium channels in cultured melanoma cells. We hypothesised that mitochondrial TASK-3 channels may exert antiapoptotic effects via contributing to mitochondrial function, most likely by maintaining mitochondrial membrane potential. To confirm this hypothesis and to study possible other functions of TASK-3 channels, we employed RNA interference. Our present experiments were conducted on WM35 cells in which TASK-3 biosynthesis was stably knocked-down. WM35 cells that were stably transfected with a scrambled RNA sequence served as control. To monitor mitochondrial function, Jc-1 fluorescent dye was applied at a concentration of 5 μ g/ml. Mitochondrial depolarisation was evoked by carbonyl cyanide m-

chlorophenylhydrazone (50 μ mol/l CCCP). TASK-3 knock-down cells had depolarised mitochondrial membrane potential. In addition, their mitochondrial membrane could be more easily depolarised, suggesting that melanoma cells having reduced TASK-3 expression are less capable of increasing their mitochondrial activity in response to metabolic challenges. An MTT assay, that measures mitochondrial reducing capacity, also indicated reduced mitochondrial function in the knock-down cell cultures. In addition, TASK-3 gene-silenced cells showed slower proliferation rate (confirmed by Cyquant assay) and increased Annexin V binding. The latter observation indicates that knock-down melanoma cells are more prone to apoptotic cell death. Knock-down cells also had decreased cell volume which may be the result of apoptotic volume decrease. During the apoptotic events the translocation of AIF from mitochondria to cytosol and cell nuclei occurs.

Our data indicate that reduced TASK-3 expression of the melanoma cells results in mitochondrial depolarisation, reduced mitochondrial function, decreased rate of proliferation, and a markedly increased rate of intrinsic apoptosis.

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S4-C

Physiology and Pathophysiology of Bicarbonate secretion in the Airways – Key to Therapy of Cystic Fibrosis

S4-C1

Anion secretion by calcium-activated anoctamin chloride channels: A direct or indirect mechanism?

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Cystic fibrosis (CF) is characterized by reduced Cl⁻ secretion due to impaired function of the cystic fibrosis transmembrane conductance regulator (CFTR) and probably enhanced Na⁺ absorption through amiloride-sensitive epithelial sodium channels (ENaC). Electrolyte secretion by the airways is necessary to produce the airway surface liquid that allows for mucociliary clearance of the lungs. More recently the requirement of CFTR for airway bicarbonate secretion has been increasingly recognized, leading a decrease of the pH in the airway surface liquid of CF patients and CF-animal models. Anion secretion is driven by opening of Cl⁻ selective ion channels in the apical membrane of airway epithelial cells,

through either receptor mediated increase in intracellular cAMP or cytosolic Ca^{2+} . Traditionally cAMP-dependent and Ca^{2+} -dependent secretory pathways are regarded as independent. However, this concept has been challenged recently. With identification of the Ca^{2+} activated Cl^- channel TMEM16A (anoctamin 1) and with increased knowledge of the cAMP-regulated cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel, it became possible to look more closely into the relationship of both ion channels. Mice lacking expression of anoctamin 1 in the airways demonstrate a CF-like lung phenotype, suggesting that anoctamin 1 and possibly additional anoctamin paralogues also expressed in airway epithelial cells are essential in mouse airways for Cl^- and possibly bicarbonate secretion. Thus anion secretion by anoctamins may occur by forming separate apical exit pathways, or indirectly by supporting the secretory function of CFTR.

S4-C2

HCO₃⁻, fluid, mucus and the structure of small airways

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The surfaces of the lumens of small airways are continually exposed to exogenous debris and pathogens as well as accumulated endogenous products. Since these surfaces, excepting the alveoli, represent the largest surface of the lung, extremely efficient mechanisms of clearance must operate to defend the tissue from infection, inflammation, and obstruction while maintaining lumens patent for airflow. Toward that end, the surfaces maintain a liquid layer that normally flows easily along the luminal surface without obstructing the lumen.

Since secretory glands are few or absent from the smaller airways, the structures must perform both fluid absorption and fluid secretion to maintain a critical balance between dehydration and inundation of the airway. Microanatomy shows that these airways are anastomosing tubes whose luminal surface is corrugated into longitudinal folds and pleats that run in parallel from the respiratory bronchioles to the larynx. Accepting that the anatomy allows expansion and contraction of the tubes without a change in the epithelial surface, our data indicate that the folds and pleats, respectively, are also the sites of concurrent fluid absorption and fluid secretion. Moreover, in general, mucin-secreting goblet cells tend to be aggregated in the basal zones of the pleats.

Results also show that: a.) small airways continuously secrete HCO₃⁻, b.) both cAMP and Ca^{++} mediated agonists can modulate HCO₃⁻ secretion, and c.) like fluid secretion, such HCO₃⁻ secretion likely occurs in the basal regions of pleats as well. Given the recent reports that HCO₃⁻ is critical for bactericidal properties of innate anti-microbial peptides and that HCO₃⁻ is essential for transforming mucins compacted in granules into hugely expanded networks of transportable mucus gels, the structures of pleats and folds appears to be

crucial for local integration of: a.) epithelial HCO₃⁻ secretion, b.) water and solute coupling for isotonic fluid secretion, and c.) strategic maintenance of surface fluid volumes, into a local mechanism for clearing pathogenic material from small airway surfaces.

S4-C3

The importance of bicarbonate and proteases for mucin secretion and mucus formation

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In contrast to previous ideas, mucin is packed in the mucin granulae of goblet cells in a highly organized way by calcium and low pH mediated interactions of the N-terminal part of the mucin (1). Further structural studies have revealed molecular details that explain how the mucin packed on concatenated rings are organized in relation to each other. This will allow an organized release that is triggered by removal of calcium ions. The natural way of accomplishing this is by sufficiently high concentrations of bicarbonate. Cystic Fibrosis (CF) is caused by a non-functional chloride and bicarbonate ion channel (CFTR) and mice lacking functional CFTR have to humans similar ileal problems with mucus stagnation. Interestingly, the ileal CF mucus adhered to the epithelium, was denser, and less penetrable than that of wild-type mice (2). The adherent ileal CF mucus was normalized by secretion into a high concentration sodium bicarbonate. However, deeper studies of this process revealed that the unfolding of the main mucus component, the MUC2 mucin, was not sufficient to detach the mucin as a specific enzyme was also necessary to cleave in the MUC2 mucin. The lower levels of secreted bicarbonate in CF hindered this cleavage as the mucin was not sufficiently unfolded to allow the cleavage to take place. This knowledge allows novel studies of novel ways of testing mucus release and therapeutic options for a mucus stagnation.

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S4-C4

Effects of bicarbonate and pH on bacterial growth and MIC of Erythromycin

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In cystic fibrosis (CF), defective transepithelial anion transport is associated with retention of thick mucus, lower pH and reduced bacterial killing in the airways. Aerosolizing bicarbonate into CF pig airways increased bacterial killing in vivo. Thus, we investigated the effects of external pH and bicarbonate on the growth of different bacteria, as well as on the efficacy of erythromycin against *S. aureus*. We compared the effects of adding NaHCO₃ or NaCl to media on the growth of *E. coli*, *S. aureus*, *S. agalactiae*, *E. faecalis* and *H. influenzae*. The growth rate of bacteria was followed by measuring the optical density. Erythromycin MIC was determined by agar dilution: a) in the presence and absence of NaHCO₃, b) in ambient air versus 5%/20% CO₂, and c) in a pH range of 7.1-9.0. We found that NaHCO₃ significantly inhibited bacterial growth in all strains, whereas NaCl did not influence growth. Bicarbonate was bacteriostatic rather than bactericidal. Alkalinizing external pH reduced the MIC values of erythromycin significantly. However, bicarbonate did not alter erythromycin MICs when both external pH and CO₂ concentrations were held constant. The data indicate that bacterial growth is inhibited by HCO₃⁻ per se rather than by HCO₃⁻-induced alkaline pH or higher osmolality. Increased efficacy of erythromycin appeared to be due more to alkaline pH and not solely to bicarbonate alone. Administration of aerosolized bicarbonate may transiently raise airway pH, inhibit bacterial growth and improve the efficacy of antibiotic therapy in CF and other inflammatory lung diseases.

S4-C5

HAT-7 cells, a new model to study the intracellular pH regulation and bicarbonate transport of ameloblasts

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Formation and growth of hydroxyapatite crystals during amelogenesis by ameloblasts generates a large number of protons that must be neutralized. Ameloblasts express a number of transporters and channels involved in bicarbonate transport in other epithelia. However, to date there is no functional evidence for bicarbonate transport in these cells. Therefore, we aimed to develop a novel two dimensional monolayer to model the bicarbonate transport by ameloblasts using HAT-7, a rat cell line of ameloblast origin. HAT-7 cells were seeded onto Transwell permeable filters. We used three different media to test tight junction formation. Transepithelial resistance (TEER) was measured by an epithelial volt-ohm meter. The expression of transporters and tight junction proteins was investigated by RT-PCR. Intracellular pH regulation and bicarbonate transport was measured by microfluorometry. HAT-7 cells formed polarized epithelia on permeable supports. TEER was significantly higher in Hepatostim medium and, to a lesser extent, in Differentiation medium (DMEM-F12 containing 2.1

mM calcium and 10 nM dexamethasone) compared to control DMEM-F12, indicating the formation of tight epithelia. HAT-7 cells in all three media expressed tight junction proteins and key transporters previously identified in ameloblasts. The intracellular pH increased rapidly in response to basolateral CO₂/HCO₃⁻ containing solution, as bicarbonate entered the cells. The bicarbonate uptake was sensitive to 0.1 mM acetazolamide (carbonic anhydrase inhibitor) and 0.5 mM H2DIDS (AE/NBC inhibitor) indicating intracellular HCO₃⁻ accumulation in HAT-7 cells by anion transporters and/or carbonic anhydrases. Furthermore, cells were polarized for membrane CO₂ permeability as the apical membrane has much higher permeability than the basolateral one. In conclusion, HAT-7 cells i) can form tight junctions, ii) express typical tight junction proteins and electrolyte transporters, iii) are functionally polarized and iv) can accumulate bicarbonate from the basolateral side. Therefore, the HAT -7 cell line is suitable to study bicarbonate transport by ameloblasts.

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S4-C6

The role of aquaporins in pancreatic ductal cells

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Background. Acute pancreatitis (AP) is a multicellular disease in which pancreatic ductal cells play an important role. Toxic agents inducing AP inhibit pancreatic ductal bicarbonate secretion, however, no information is available concerning their effects on the regulation of aquaporins (AQPs). Therefore, the aim of this study was to investigate the effects of bile acids, ethanol and its metabolites on the expression of AQPs.

Methods. CAPAN-1 cells were treated with ethanol (EtOH; 1-100 mM), chenodeoxycholate (CDC; 0.1-0.5 mM), glycochenodeoxycholate (GCDC; 0.1-0.5 mM) palmitoleic acid (POA; 10, 100 and 200 uM) and palmitoleic acid ethyl ester (POAEE; 10, 100 and 200 uM) for 6, 12, 24 and 48 hours and the expression of AQP isoforms (AQP1-12) was examined by real-time RT-PCR and immunocytochemistry.

Results. All 12 AQPs were expressed in the CAPAN-1 cell line to a certain degree. AQP1, 3, 5, 6 and 11 were expressed at the highest levels while AQP2 and 4 were hardly detectable. In almost all treated group, the expression of AQPs decreased both at mRNA and protein levels dose- and time-dependently. Notably, a 72-hour incubation in culture media restored the expression of AQPs in the 6- and 12-hour CDC- and GCDC-treated groups and in the 24-hour EtOH-treated group.

Conclusion. The role of AQP in the pathogenesis of AP needs further investigations.

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S5-C

From Cell Signalling to Bioenergetics and Cell Damage

S5-C1

Pathways to calcium mediated neuronal injury: Starvation in the midst of plenty

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Neuronal cell death is attributed to abnormal calcium signalling in a number of different pathological states in the CNS. We have shown that amyloid beta and glutamate toxicity both cause abnormal calcium signals that culminate in neuronal death. In both cases, calcium mediated toxicity is associated with oxidative stress. Our data suggest that the enzyme PARP plays a key role in these pathophysiological models as an intermediary between oxidative stress and mitochondrial dysfunction. I will show data to suggest that activation of PARP mediated by oxidative stress causes consumption of cytosolic NAD⁺, inhibition of mitochondrial substrate supply and impaired mitochondrial function suggesting a number of potential therapeutic targets that are neuroprotective in these model systems and that may be considered in the associated pathologies.

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S5-C2

The crucial role of mitochondrial damage and consequent breakdown of bioenergetics in acute pancreatitis

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Acute pancreatitis is an inflammatory disease with no specific treatment. One of the main reasons behind the lack of specific therapy is that the pathogenesis of acute pancreatitis is poorly understood. During the development of acute pancreatitis, the

disease-inducing factors can damage both cell types of the exocrine pancreas, namely the acinar and ductal cells. Because damage of either of the cell types can contribute to the inflammation, it is crucial to find common intracellular mechanisms that can be targeted by pharmacological therapies. Despite the many differences, recent studies revealed that the most common factors that induce pancreatitis cause (1) uncontrolled Ca²⁺ release leading to intracellular Ca²⁺ overload and toxicity and (2) mitochondrial damage with the consequent breakdown of bioenergetics, that is, ATP depletion in both cell types.

This presentation summarizes the variety of Ca²⁺ signals and the mitochondrial function and damage within both pancreatic acinar and ductal cells. We also suggest that colloidal ATP delivery systems for pancreatic energy supply may be able to protect acinar and ductal cells from cellular damage in the early phase of the disease. An effective energy delivery system combined with the prevention of further mitochondrial damage may, for the first time, open up the possibility of pharmacological therapy for acute pancreatitis, leading to reduced disease severity and mortality.

S5-C3

The mitochondrial calcium uniporter: Molecular identity and physiological role

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Mitochondria rapidly accumulate Ca²⁺ through a low-affinity uptake system (the mitochondrial Ca²⁺ uniporter, MCU) because they are exposed to high [Ca²⁺] microdomains generated by the opening of ER Ca²⁺ channels. These rapid [Ca²⁺] changes stimulate Ca²⁺-sensitive dehydrogenases of the mitochondrial matrix, and hence rapidly upregulate ATP production in stimulated cells. On the other hand, Ca²⁺ also sensitizes to cell death mediators, e.g. ceramide, and anti-apoptotic oncogenes reduce the Ca²⁺ transfer from the ER to mitochondria. Molecular identification of the mitochondrial Ca²⁺ transporter(s) was thus much awaited. In my presentation, I will present the most recent molecular information on MCU, identified by our group in 2011, and the newly identified regulators (MCUb, MICU1, MICU2). I will also show how the availability of molecular tools for MCU now allows to carry out experiments in intact cells and whole organisms that highlight and clarify the importance of mitochondrial calcium homeostasis in physiology and pathophysiology.

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S5-C4

Imaging incretin-regulated bioenergetics in intact pancreatic islets

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Type 2 diabetes currently affects ~8% of the population worldwide and involves impaired insulin secretion and action. Incretins such as glucagon-like peptide 1 (GLP-1) are released from the gut and potentiate insulin release in a glucose-dependent manner, in part by increasing the connectivity between individual beta cells in the islets. While this action is generally believed to hinge on cAMP and protein kinase A (PKA) signalling, up-regulated beta cell intermediary metabolism may also play a role in incretin-stimulated calcium dynamics and insulin secretion. By employing recombinant probes to image ATP dynamically in situ within intact mouse and human islets, we have shown that GLP-1 engages a metabolically-coupled subnetwork of beta cells to increase cytosolic ATP levels, an action independent of prevailing energy status. We also demonstrate that inactivation of the type 2 diabetes genes *ADCY5*, encoding adenylyl cyclase 5, interferes with normal glucose, but not incretin-stimulated ATP changes and calcium increases, providing a mechanism through which polymorphisms in this gene affect disease risk. Optogenetic approaches are currently being used to identify and analyse individual beta cells responsible for coordinating beta cell behaviour islet-wide.

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S5-C5

Cardiac calsequestrin and heart failure

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Cardiac calsequestrin (CSQ2) is located in the junctional sarcoplasmic reticulum (SR) of mammalian cardiac myocytes. To further characterize the role of CSQ2 for regulation of cardiac contractility we generated CSQ2-null (CSQ2^{-/-}) mice by targeted deletion of the first exon (heterozygous: CSQ2^{+/-}; wild type: CSQ2^{+/+}). In the targeted CSQ2 allele the coding region of exon 1 is replaced by a loxP site leading to a nonsense mutation. Chimeric mice were crossed into a C57BL/6 background to get CSQ2^{+/-} mice for further breeding. Successful targeting of the CSQ2 allele was confirmed by Western blotting. CSQ2^{-/-} mice are viable. At an early time point (6 months of age) normal ejection fraction was measured using echocardiography by means of a Vevo 2100 system and isofluran anesthesia. However, even at this

time point alterations in ventricular function as assessed by Langendorff perfusion were noticed: basal contractility in CSQ2^{-/-} mice was higher than in CSQ2^{+/-} and CSQ2^{+/+} mice. Furthermore, the contractility in CSQ2^{-/-} mice could not be stimulated by perfusion of the isolated heart with isoprenaline, a β -adrenoceptor agonist, at concentrations that greatly elevated force of contraction in CSQ2^{+/-} and CSQ2^{+/+} mice. Additionally, at six months gross morphology of the heart as well as absolute and relative heart weight were not different in CSQ2^{-/-} mice compared to CSQ2^{+/+} or CSQ2^{+/-}. However 20-22 months old mice we measured profound impairment of cardiac function as assessed by decrease ejection fraction in CSQ2^{-/-} mice ($29 \pm 6.1\%$, $n=4$) compared to six month old CSQ2^{-/-} mice and to 20-22 months old CSQ2^{+/-} mice ($68 \pm 6.4\%$, $n=5$, $p < 0.05$), whereas heart rates were not significantly different. In summary, we present the first evidence that not only overexpression but also but also deletion of CSQ can lead to cardiac failure.

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S4-D

Revealing the Prominent Role of Neuroglia in Neurodegeneration

S4-D1

Neuroglial morphological and metabolic alterations during the progression of Alzheimer's disease and ageing

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Neuroglial cells are fundamental for brain homeostasis and therefore represent the intrinsic brain defence system. Thus, all forms of neuropathological processes inevitably involve glial cells. Neurodegenerative diseases, including Alzheimer's disease (AD) disrupt brain connectivity affecting neuronal-neuronal, neuronal-glial and glial-glial interaction. Furthermore, neurodegenerative processes trigger universal and conserved glial reactions classically represented by astrogliosis and microglial activation. The recently acquired knowledge allows us to regard the neurodegenerative diseases as primarily gliodegenerative processes, in which glial cells determine the progression and outcome of neuropathological processes such as AD and ageing. We have recently probed this active pathological role, by showing: (i) an astroglial generalised atrophy with a concomitant astrogliosis just restricted to A β plaques presence, (ii) alterations in glutamate glial metabolism, (iii) changes in S-100 β trophic factor and (iv) an early resting microglial recruitment in the affected areas,

even before the presence of activated/macrophagic microglial cells. These glial alterations, which are complex and region dependent are fundamental for the disruption of neural networks connectivity as well as with the neurotransmitters imbalance that underlie the mnesic deficits associated with AD. However, we have recently demonstrated that psychostimulative processes, such as exposure to enriched environment and voluntary running, can not only revert this generalized astrocytic hippocampal and cortical atrophy but also potentiate their hypertrophy which could trigger new insights into the search for a potential therapeutic treatment of AD and pathological ageing.

S4-D2

Dysfunction of AMPA-type glutamate receptors in microglia may cause neurodegeneration

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Microglia express AMPA (alpha-amino-hydroxy-5-methylisoxazole-4-propionate)-type of glutamate (Glu) receptors (AMPA), which are highly Ca²⁺ impermeable due to the expression of GluA2. Glu-induced currents in the presence of cyclothiazide (CTZ), an inhibitor of AMPAR desensitization, showed time-dependent decrease after activation of microglia with lipopolysaccharide (LPS) in GluA2^{+/+} microglia, but not in GluA2^{-/-} microglia. Upon activation of microglia, expression level of surface GluA2 subunits significantly increased, while expression of GluA1, A3 and A4 subunits on membrane surface significantly decreased. These results suggest that nearly homomeric GluA2 subunits were the main reason for low conductance of AMPAR in activated microglia. Increased expression of GluA2 in microglia was also detected partially in brain slices from LPS-injected mice. Cultured microglia from GluA2^{-/-} mice showed higher Ca²⁺-permeability, consequently inducing significant increase in the release of proinflammatory cytokine, such as TNF- α . The conditioning medium from KA-treated GluA2^{-/-} microglia had more neurotoxic effect on wild type cultured neurons than that from KA-treated GluA2^{+/+} microglia. These results suggest that membrane translocation of GluA2-containing AMPARs in activated microglia has functional importance and thus, dysfunction or decreased expression of GluA2 may accelerate Glu neurotoxicity via excess release of proinflammatory cytokines from microglia. Since low expression of GluA2 was reported in some neurodegenerative diseases, it may be one of the reasons of the neuronal death.

S4-D3

Does innate immunity contribute to the pathogenesis of Alzheimer's disease?

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Generation of neurotoxic amyloid- β peptides and their deposition along with neurofibrillary tangle formation represent key pathological hallmarks in Alzheimer's disease (AD). Recent evidence suggests that inflammation may be a third important component which, once initiated in response to neurodegeneration or dysfunction actively contributes to disease progression and chronicity. Microglia is being activated by binding of aggregated proteins or aberrant nucleic acids to pattern recognition receptors which elicit an innate immune response. The latter is characterized by the release of inflammatory mediators including complement activators and inhibitors, chemokines, cytokines, radical oxygen species and enzyme systems. Exogenous as well as endogenous factors may promote and facilitate neuroinflammation in the AD brain. Thus, degeneration of aminergic brain stem nuclei including the locus ceruleus and the nucleus basalis of Meynert may drive inflammation in their projection areas given the antiinflammatory and neuroprotective action of their key transmitters norepinephrine and acetylcholine. Inflammation may not just occur secondary to degeneration, but actively drive amyloid beta aggregation and APP processing. Inhibition of the microglia driven innate immune response at key signalling steps may provide protection. Therefore, antiinflammatory treatment strategies should be considered. Data on microglial activation in AD along with suggestions to modify and alter the pro- into an antiinflammatory phenotype will be reviewed and discussed.

S4-D4

The response of NG2-glia (oligodendrocyte precursors) to aging in an animal model of Alzheimer's Disease

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Oligodendrocytes are the myelinating cells of the CNS. Myelin enables the rapid transmission of signals that underlies the massive computing power of the human cortex. However, there is a loss of myelin and white matter shrinkage in the ageing brain that is associated with cognitive decline. Notably, oligodendrocytes are continuously generated throughout life by endogenous oligodendrocyte precursors (OPs), or NG2-glia. NG2-glia (OPs) are unique amongst glia in that they form direct synapses with neurons, which are proposed to regulate the differentiation of NG2-glia (OPs) into oligodendrocytes. A diminished capacity for NG2-glia (OPs) to regenerate oligodendrocytes is proposed to be a critical factor underlying myelin loss in the ageing brain. Moreover, myelin loss is accelerated in Alzheimer's Disease (AD), suggesting the function of NG2-glia (OPs) is disrupted in this disease. We have examined this in the 3xTg mouse model of

AD and provide evidence that NG2-glia (OPs) display atrophic changes at an early stage of the disease. In contrast, at later stages of the disease NG2-glia (OPs) appear hypertrophic closely associated with amyloid beta (Abeta) plaques, and their proliferation may be decreased. Notably, there is evidence that neuronal/synaptic loss and myelin disruption occur at the earliest signs of Abeta plaques, which correlates with the observed changes in NG2-glia (OPs). The mechanisms regulating NG2-glia (OPs) and oligodendrogenesis in the adult and aging brain are unresolved, but Wnt signalling is essential for synaptic function and is deregulated in AD. Significantly, we have identified a profound role for Wnt signalling in driving oligodendrogenesis in vivo in the mouse brain and demonstrate that this is deregulated in ageing white matter. These studies suggest a sequence of interrelated events in which deregulation of Wnt signalling is associated with disruption of synaptic function and reduced differentiation of NG2-glia (OP) into oligodendrocytes with resulting myelin loss, which in turn leads to axon/neuron degeneration and a vicious cycle of events that accelerates cognitive decline in AD.

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S4-D5

Release of 4-hydroxynonenal and 4-hydroxyhexenal-modified proteins in exosomes

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Exosomes are vesicles released from mammalian cells. They contain a diversity of proteins and siRNAs. Presumably, they play an important role in intercellular communication. 4-hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE) are 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 microglial cells, one target of 4-HNE are swelling-activated Cl⁻ channels (see Abstract by Schmörlzer and colleagues). In the present study, we documented the intra- and extracellular distribution of HHE-modified proteins in the primary murine microglial cells. 4-HHE-modified proteins were detected using an antibody to 4-HHE-modified proteins. Membranes were stained with rhodamin-labeled concanavalin A. Immune-labeled as well as concanavalin A-labeled areas were visualized using confocal laser microscopy. HNE-modified proteins were visualized in discrete areas of the cell. Most immune-labeled patches were localized close to the plasmamembrane or close to the nucleus. In addition, we localized HHE-modified proteins and apolipoprotein E (ApoE) in exosomes. ApoE is involved in oxidative stress and neurodegenerative processes. Whether release of exosomes containing HNE and HHE-modified proteins is cellular strategy to expel waste proteins or may have additional biological functions remains to be analyzed.

S4-D6

Galanin is a modulator for phagocytosis in microglial cells

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The biological significance of inflammation is clearance of affected areas. The bioactive peptide galanin, is up-regulated during inflammatory processes in the brain. Notably, microglial cells, monocyte-derived immune cells in the central nervous system, reveal galanin-dependent release of cytokines. Galanin suppresses LPS-induced release of the pro-inflammatory cytokine TNF alpha, and induces up-regulation of class II major histocompatibility complex. In the present study, we characterized galanin-receptors using RT-PCR as well as the consequences of galanin on phagocytosis in murine microglia. In BV-2 cells, a murine microglial cell line, RT-PCR revealed mRNA transcripts for galanin receptor (GalR)2, GalR3, and galanin, but not for GalR1, alarin- or galanin-like peptide. For phagocytosis experiments, microglial cells were exposed to uncoated and IgG-coated polystyrene beads (microspheres, MB) for 15 minutes. In primary microglial cells, galanin in the pM and nM range impaired uptake of MBs compared to basal phagocytic activity. In contrast, in BV-2 cells, galanin facilitated engulfment of MBs. Since the widely used GalR3 specific antagonist SNAP 37889, induced cell blebbing, an indicator for cell death processes, the contribution of GalR2 and GalR3 to phagocytosis could not be distinguished. 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.

S5-D

Pulmonary Surfactant: From Molecule to Function

S5-D1

Exocytosis of the lamellar body, a calcium mobilizing secretory lysosome

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Lamellar bodies (LBs) are large vesicles ($\emptyset \approx 1 \mu\text{m}$) within the type II pneumocyte, an epithelial cell type of the pulmonary alveolus, the terminal structure of the airways where gas exchange takes place. LBs contain surfactant, a lipid-rich, lipoprotein-like substance reducing the surface tension of alveoli and preventing their collapse. LBs are lysosome-related organelles, and live cell fluorescence imaging experiments revealed that they are secreted into the alveolar lumen by regulated exocytosis, i.e. by low threshold elevations of the cytoplasmic Ca^{2+} concentration above $\approx 320 \text{ nmol/l}$. The exocytotic process consists of a pre-, hemi-, and post-fusion step, and during the post-fusion step, fusion pores expand slowly and discontinuously. Laser tweezer experiments revealed that LB fusion pores may represent a significant mechanical barrier for the release of surfactant into the extracellular space. This necessitates active extrusion mechanisms by actin coat formation and contraction around fused LBs. Although the molecular mechanisms involved herein are still not entirely resolved, some unique principles have been elucidated:

Ca^{2+} entry from fused LBs into the cytoplasm through P2X4 receptors located within the limiting LB membrane plays an important role for fusion pore dilation. We termed this mechanism fusion-activated cation entry (FACE), and demonstrated that it is activated by ATP from the extracellular (apical) compartment, when ATP is able to diffuse into the LB lumen through the exocytotic fusion pore. FACE induces an expansion of the fusion pore FACE, in part by Ca^{2+} binding to synaptotagmin 7. FACE also leads to osmotic cell swelling and fluid reabsorption, which may facilitate fusion pore expansion as well and support surfactant incorporation at the air-liquid interface.

S5-D2

Misfolding of surfactant protein C and how it is solved by Nature and by rational design

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Lung surfactant protein C (SP-C) is unique in many aspects; it is the only known secreted transmembrane protein, it lacks

homologous proteins and it is only synthesized by one cell type - the alveolar type 2 cell. Moreover, SP-C spontaneously converts from α -helix to amyloid-like β -sheet polymers, but the helical state is kinetically stabilized by an unusually high activation barrier for unfolding. Thus, in contrast to the general behavior of protein helices, the SP-C α -helix is not able to unfold and refold. These features suggest an elegant explanation to the aggregation encountered by many groups when synthesizing SP-C for formulation of an artificial surfactant for treatment of respiratory distress syndrome (RDS). We solved this problem by replacing all Val residues in SP-C (with high β -strand propensity) with Leu (with high α -helix propensity), which yields a thermodynamically stable helix peptide, which can be synthesized in large amounts without aggregation. This SP-C analogue is active in animal models of RDS and is currently undergoing clinical trials for treatment of RDS in premature infants. By an innovative approach based on spider silk proteins we have recently developed a method to make large amounts of the SP-C analogue recombinantly. The inability of the SP-C α -helix to refold in vitro raises questions about its formation in vivo.

We have discovered that a BRICHOS domain of proSP-C works as a molecular chaperone that prevents aggregation of the transmembrane SP-C part, and promotes its folding into a helix. Intriguingly, mutations that segregate with human lung fibrosis are localized to the BRICHOS domain, and our recent determination of the first crystal structure of a BRICHOS domain can explain how mutations inactivate its chaperone function. We have found that BRICHOS mutations lead to amyloid disease; BRICHOS is thus the first described example of an endogenous factor that guards extraordinarily amyloidogenic peptides. Such factors are attractive candidates to harness for treatment of amyloid diseases, and recent findings have motivated us to explore its ability to treat and prevent Alzheimer's disease.

S5-D3

The role of surfactant in host defence

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Surfactant coats the alveolar surface which is an enormous surface and interface towards the environment. Millions of bacterial, viral and fungal pathogens are inhaled by all of us every day. Our immune system must handle the challenge to avoid the invasion of pathogens into the lung parenchyma and the blood stream on the one side without constantly overreacting on the other side. Surfactant plays a crucial role in this context.

The mechanical properties of surfactant keep the airways open and facilitate mucociliary clearance. The phospholipids that are spread on the surface exert a mechanical barrier function. Surfactant in itself and antibacterial peptides bound to surfactant have been demonstrated to interfere with bacterial growth in vitro. In vivo experiments with newborn rabbits demonstrate that surfactant limits bacterial growth and translocation to the blood stream e.g. in group streptococcal and E. coli pneumonia. Surfactant protects the airways from ventilator

induced lung injury and lack or dysfunction of surfactant seems to play a role in the pathophysiology of ventilator associated pneumonia. In addition, surfactant has a potential for use as carrier for antibiotics or immunoglobulins in experimental pneumonia.

A focus in surfactant research has been directed to the role of surfactant proteins in host defense. The hydrophobic surfactant proteins SP-B and SP-C play a major role for the biophysical function of surfactant and both the natural proteins or synthetic analogues are contained in the surfactant preparations that are in clinical use. The hydrophilic “large” surfactant proteins SP-A and SP-D have a collectin structure. They are water soluble and thus removed during the extraction/production procedure of the commercially available surfactants. SP-A and SP-D exert a variety of important functions in the pulmonary immune defense. However, results on SP-D knock mice demonstrate that SP-D may be of importance both for “stability and sterility”. Animal derived surfactants have in use for more than 2 decades, but clinical trials with preparations containing both synthetic lipids and synthetic proteins are under way.

S5-D4

Surfactant inhibition and its reversal

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Pulmonary surfactant is a mixture of phospholipids and specific proteins that reduces surface tension of the alveolar lining layer, prevents transudation of oedema fluid into the alveolar spaces and plays a role in local immune mechanisms. Therapy with exogenous surfactant is an established part of routine clinical management of newborns with respiratory distress syndrome (RDS). In recent years the indications have been widening to the “non-RDS” respiratory disorders with the secondary inhibition of surfactant function. Inhibition, or inactivation, refers to those processes that decrease or abolish the normal surface activity of pulmonary surfactant. Surfactant may be inactivated by wide spectrum of substances that appear in the alveolar space under pathological conditions, e.g. plasma proteins, unsaturated membrane phospholipids, free fatty acids, cholesterol, meconium, reactive oxygen species, and enzymes. Currently, there is an evidence that also inhaled nanoparticles interfere with the biophysical function of the surfactant system.

At least two distinct inhibitory mechanisms arise from in vitro studies: competitive adsorption of plasma proteins and fluidizing surfactant film by lipids.

Preventing surfactant inactivation is an important approach that could increase the therapeutic effects of exogenous surfactants in various forms of lung disease. The harmful effect of some inhibitors can be counterbalanced by increasing surfactant concentration and/or enrichment of the surfactant preparations with SP-A, or by addition of polymers such as dextran, polyethylene glycol or hyaluronan. Also polymyxin B increases the resistance of natural modified surfactant to inactivation by albumin. On the other side, the effect of plasma proteins, e.g. fibrinogen is not always inhibitory. At

high phospholipid concentration used in clinical practice fibrinogen has a protective effect on properties of natural modified surfactant subjected to surface area cycling.

For the further development of surfactant therapy it is important to study and better understand behaviour of pulmonary surfactant both in vitro and in vivo.

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S5-D5

Lymphatic function is required prenatally for lung inflation at birth

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Mammals must inflate their lungs and breathe within minutes of birth to survive. A key regulator of neonatal lung inflation is pulmonary surfactant, a lipoprotein complex which increases lung compliance by reducing alveolar surface tension. Whether other developmental processes also alter lung mechanics in preparation for birth is unknown. We identify prenatal lymphatic function as an unexpected requirement for neonatal lung inflation and respiration. Mice lacking lymphatic vessels, due either to loss of the lymphangiogenic factor CCBE1 or VEGFR3 function, appear cyanotic and die shortly after birth due to failure of lung inflation. Failure of lung inflation is not due to reduced surfactant levels or altered development of the lung but is associated with an elevated wet/dry ratio consistent with edema. Embryonic studies reveal active lymphatic function in the late gestation lung, and significantly reduced total lung compliance in late gestation embryos that lack lymphatics. These findings reveal that lymphatic vascular function plays a previously unrecognized mechanical role in the developing lung that prepares it for inflation at birth. They explain respiratory failure in infants with congenital pulmonary lymphangiectasia, and suggest that inadequate late gestation lymphatic function may also contribute to respiratory failure in premature infants.

S5-D6

The N-terminal domain of spider silk proteins for synthetic surfactant production

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Lung surfactant keeps the alveoli from collapsing at end expiration and spreads rapidly over a mucosal surface.

Animal-derived surfactant preparations have successfully been employed for treating neonatal children with respiratory distress syndrome (RDS), but due to high cost and limited supply, lung surfactant is not available for other uses, e.g. surfactant could act as vehicle for drug delivery to the alveoli after topical administration. Artificial surfactant preparations based on designed, synthetic surfactant protein analogues work in animal models of RDS and are in clinical development, but peptide synthesis is costly and generates unwanted by-products.

Spider silk proteins are large and prone to aggregate, but their N-terminal domain prevents precocious aggregation during storage in the spiders' silk glands. By an innovative approach, using the spider silk proteins' N-terminal domain as a solubility tag, large amounts of surfactant protein analogues (SP-C33 and KL4) can for the first time be produced in heterologous hosts. The bacterial production of SP-C33 and KL4 fused to NT results in high yields of soluble fusion proteins and by a simple precipitation procedure, a 99% pure and functional target peptide can be obtained without the use of chromatographic steps. A surfactant made from our recombinant SP-C33 mixed with synthetic phospholipids has the appropriate spreading capabilities and is effective in animal models of respiratory distress syndrome.

S4-E

Cardiovascular Exercise Physiology

S4-E1

Use of virtual patients in teaching veterinary physiology at the Faculty of Veterinary Science, Szent István University, Budapest

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According to the teachers' experiences and feedback from the learners, the first and second year students of the veterinary and medical universities often wonder why they need to study basic sciences. They want to learn more clinical related subjects and do not want to "waste" their time on biochemistry or physiology as they applied to learn veterinary medicine.

This known approach raised the demand to include modern methods in the teaching of basic sciences based on the needs and different attitude of the new generation.

An EU project "Use of virtual problems/virtual patients in veterinary basic sciences" (vetVIP) was granted and started in 2012. Three universities are involved namely the University of Veterinary Medicine Hannover Germany (TiHo), the University of Life Sciences, Faculty of Veterinary Medicine, Lublin Poland

and the Szent István University, Budapest Hungary. The TiHo has a long history of using different e-learning methods in the teaching, but the Polish and the Hungarian faculties are novices in this field.

The aim was to use case-based learning to connect the basic sciences with clinical aspects and to use a "real" clinical case to explain the processes in physiology or biochemistry.

The national teams are working together in the project to prepare 30 virtual patient cases for the students. These cases cover important fields of veterinary biochemistry or physiology such as blood coagulation, metabolism of carbohydrates or lipids, hormonal dysfunctions, inflammatory processes or even bone formation.

All the cases have listed teaching aims on the first card, and they follow similar structure and length. The cases are designed to motivate self-study as they are based on a current, often seen clinical case and have a basic story with an animal patient and its owner to simulate an everyday veterinary situation. The cases are planned to have an authentic veterinary aspect, to be relevant for the curriculum of veterinary students and to integrate theoretical with clinical knowledge. All of them are based on citable literature, illustrated with pictures and videos. To check the improvement of the students different types of multiple choice questions are integrated. The right and even the wrong answers are explained in the Comments. Further, more detailed information can be found in the Expert comments.

The cases undergo different levels of evaluation: technical, didactic and meritoric checking. The authors together with clinicians evaluate the cases of the other two national teams in terms of content. The acceptance of the new methods was measured with online surveys, the learning success of the students is analysed based on pre- and post-tests showing the improvement after using the cases.

The cases are prepared in the CASUS learning system provided by one of the partners, INSTRUCT AG which is a small enterprise specialized in e-learning, located in Munich Germany and has its roots in the academic world.

S4-E2

The effect of detraining on the characteristics of the athlete's heart

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Relatively short-term detraining often occurs during an athlete's career due to injuries, holidays, army service etc. It is a very important question how the well defined characteristics of the athlete's heart move back to the non-trained values. Different authors (1-3) describe that different characteristics do not change equally after cessation of regular training.

In the present study data of non-athletes (113 males, 117 females), of actually trained athletes (948 males, 486 females) and of athletes after detraining (99 males 101 females) were analysed in the function of the days of detraining (1-20, 21-40, 41-60, 61-180, more than 180).

The earliest (20-40 days) finding during detraining was a slightly higher cardiac output and stroke volume, the next modification was a decrease of the relative left ventricular

(LV) muscle mass and an impairment of the diastolic function characterized by the E/A quotient, the resting heart rate (HR) seem to elevate after half a year cessation of regular training only.

We suppose that stop of regular training first induce an increase of the reduced sympathetic tone inducing an enhanced activity of the LV myocardium, which doesn't have parasympathetic innervation. LV musculature seems to lose athletic characteristics (hypertrophy, improved diastolic function) after two months of detraining, the late modification of the HR suggest a stability of parasympathetic regulation. Modifications are more marked in the males than in the females and athletic characteristics are more stable in the top-athletes than in the lower class or leisure-time athletes.

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S4-E3

Effects of Darbepoetin-alpha treatment and TNF-alpha blockage on cardiovascular parameters, blood cCells, and body and kidney weights in L-NAME induced hypertensive rats

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Aim: We aimed to study effects of Darbepoetin-alpha (Da- a long acting erythropoietin analog) treatment and/or TNF- α inhibition by Infliximab (inf) on cardiovascular parameters, cell blood counts, body and kidney weights in L-NAME induced hypertensive rats.

Material and Methods: Total 64 Sprague-Dawley rats were randomly grouped within 8 groups and all injections were given for 30 days; A: daily 1ml/kg ip SF injection. B, C0.1, C0.25, C0.5, D and E groups all, given daily 20 mg/kg ip L-NAME injection. C0.1 0,1 μ g/kg, C.25 0,25 μ g/kg, C0.5 0,5 μ g/kg Da given once in 3 days; D; 5 mg/kg daily inf, E; 5 mg/kg daily inf and once in a 3 day 0.25 μ g/kg Da injections besides L-NAME. F group given once in a 3 day 0.25 μ g/kg Da injection. Biopac MP 100 data acquisition system and Abacus Junior Wet blood analysis sytem were used for cardiovascular and blood parameters. Data were analysed statistically (Grap Pad) and expressed as mean $\pm$ SD where P <0.05 accepted as significantly different.

Results: Given L- NAME injection induced hypertension. Da alone or together with inf didn't show any significant effect on systolic, diastolic or mean arterial blood pressure and also on heart rate in L-NAME induced hypertension. In group D, inf increased the pulse pressure significantly. There were no statistical difference on WBC, PLT, RBC, %Hct, %Hb values among the groups treated with 0.25 μ /kg Da but they were significantly high in C0.5, E and F groups. B and D groups lost but Da alone treated F group gain body weight during one

month injection period. There were no diffirence on kidney weight or % kidney weight between and among the groups.

Conclusion: It has been first time showed that one month treatment of 0.1 and 0.25 except 0.5 doses of Da treatment did not significantly change both cardiovascular and hematologic parameters in L-NAME induced hypertensive rats. Da treatment stops body weight lost seen in L-NAME induced hypertension. But TNF-alpha blokage has no effect on the weight while it is increasing pulse pressure. We are searching the kidney tissue histologies, blood EPO evaluations and molecular apoptotic mechanisms in our ongoing studies.

S4-E4

The effects of provinol on cardiodynamics and coronary flow in isolated rat heart

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Despite well-known contribution of wine polyphenols in the prevention of cardiovascular diseases, there are still lack of data about their potential direct effects on myocardial function and coronary circulation. This study was aimed to assess direct impact of Provinol® (an alcohol-free extract of red wine) on cardiodynamics and coronary flow in isolated rat hearts. Hearts (n=48, 12 for each experimental group) excised from male Wistar albino rats (age 10 weeks, BM=250 g) were retrogradely perfused according to the Langendorff technique at gradually increased coronary perfusion pressure (CPP=40-120 cmH₂O). After control sets of experiments, hearts were administered with 1) 1% DMSO, 2) 0.5 mg Provinol, 3) 1 mg Provinol, and 4) 5 mg Provinol. After the insertion of sensor in the left ventricle, the parameters of heart function: (maximum rate of left ventricular pressure development (dp/dt max), minimum rate of left ventricular pressure development (dp/dt min), systolic left ventricular pressure (SLVP), diastolic left ventricular pressure (DLVP), and heart rate (HR) were continuously registered. Coronary flow (CF) was measured flowmetrically. In comparison to the control conditions, the lowest dose of Provinol (0.5 mg) significantly reduced CF (at CPP= 40 cm H₂O). Medium dose of Provinol (1 mg) did not show any significant effects, except decrease in dp/dt max (again, at CPP= 40 cm H₂O). On the other hand, the highest dose of this compound (5 mg) induced significant increase of dp/dt max, dp/dt min, HR and CF. Our results suggest that direct effects of Provinol on the myocardium seems to be dose dependent. While in lower doses Provinol shows even slight impairment of cardiac function, and drop in CF, in the highest dose this wine polyphenol improved cardiac contractility and induced coronary vasodilation.

S4-E5

Evolution of spreading depolarizations in a rat cerebral microembolic stroke model

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Aging is the most important independent risk factor for the incidence of cerebral ischemic stroke. Recurrent waves of spreading depolarization (SD) spontaneously occur minutes after the onset of focal ischemia in the brain but whether SDs are elicited during microembolisation-induced ischemia has been unexplored. We set out to (i) visualize the focal area of SDs, (ii) determine the hemodynamic status of this region parallel with changes in membrane potential, and (iii) characterize hemodynamic responses associated with SDs propagating over the ischemic cortex.

Multifocal ischemia was produced in halothane-anesthetized rats (n=7) by infusion of polyethylene microspheres (d=45-53µm, 2000 particles/0.6 ml vehicle) into the left common carotid artery. Synchronous image sequences were taken at specific illuminations through a cranial window created above the ipsilateral frontoparietal cortex to visualize: Changes in membrane potential (voltage sensitive dye method); cerebral blood flow (CBF; laser speckle contrast imaging); cerebral blood volume (intrinsic optical signal, IOS at 540-550 nm); and hemoglobin (Hb) deoxygenation (red IOS at 620-640 nm). A total of 31 SD events were detected in 7 experiments. The foci of 5 SDs were identified in the cranial window, which were ignited at a site where CBF prior to SD elicitation was the lowest (56.9±9%). The CBF responses to most propagating SDs were hyperemic, but red IOS intensity changes indicated 3 different kinetics of Hb saturation: dominant Hb oxygenation; initial Hb oxygenation followed by Hb desaturation; and dominant Hb desaturation. More accentuated Hb desaturation was associated with larger initial drop in CBF shortly after ischemia induction.

We have shown that microsphere-induced embolization leads to SDs evolution in the rat cerebral cortex, which is clinically relevant for the pathophysiology of small embolic infarcts in patients. We propose that SD occurrence during the early phase of ischemia is not related to immediate infarct evolution. Hyperemic responses to SDs were shown to be coupled with various kinetics of Hb saturation, which may determine the metabolic consequences of individual SDs.

S4-E6

Reduced dietary zinc and selenium levels impairs vascular function via oxidative stress in Sprague-Dawley rats aortas

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Objective: Trace elements zinc (Zn) and selenium (Se) are important components of antioxidative enzymes. The aim of this

study was to determine if reduced dietary Zn and Se levels affect vascular function by increasing oxidative stress.

Materials and Methods: Thirty three male Sprague Dawley rats were fed with 4 types of custom made rat chow (Faculty of Agriculture University of Osijek, Croatia) from weaning for 10 weeks: a) high Zn(70.81 mg/kg)-high Se (0.363 mg/kg) (N=5); b) high Zn(70.81 mg/kg)-low Se(0.043 mg/kg) group (N=10); c) low Zn(30.16 mg/kg)-high Se (0.363 mg/kg) group (N=10) and d) low Zn(28.56 mg/kg)-low Se(0.030 mg/kg) group (N=8). Prior to decapitation, rats were anesthetized with 75 mg/kg ketamine+2.5 mg/kg midazolam. Dose responses to ACh (10⁻⁹-10⁻⁵ M) and response to reduced pO₂ (bath gas mixture containing N₂ 95%, CO₂ 5%) were tested in noradrenaline-precontracted aortic rings in the absence/presence of the NOS inhibitor L-NAME, COX-1,2 inhibitor indomethacin (INDO) and superoxide scavenger Tempol in tissue bath. To test differences among groups Two-way ANOVA or One-way ANOVA was used when appropriate (SigmaPlot v11.2, Systat Software, Chicago, USA).

Results: ACh induced relaxation (AChIR) was reduced in low Zn-low Se group compared to other groups (P <0.001 for 10⁻⁸-10⁻⁵M ACh). L-NAME blocked AChIR in all groups of rats (P <0.01 for 10⁻⁸-10⁻⁵M ACh), while INDO reduced AChIR only in low Zn-high Se group (P <0.5 for 10⁻⁷-10⁻⁵M ACh). Tempol improved AChIR in low Zn-low Se group (P <0.01 for 10⁻⁷-10⁻⁵M ACh). Hypoxia induced relaxation (HIR) was increased in high Zn-low Se group compared to high Zn-high Se group and low Zn-low Se group (P <0.05). While INDO significantly blocked HIR in all groups of rats except high Zn-high Se group, L-NAME and TEMPOL did not have significant effect on HIR in any group of rats.

Conclusion: Zn and Se dietary deficiency affects vascular reactivity. Since AChIR (predominantly mediated by NO) is more affected than HIR (predominantly mediated by COX-1,2 metabolites), data suggest that that increased oxidative stress decreases NO bioavailability in rat aortas thus affecting NO-mediated response.

S4-E7

Different expression and localization pattern of MT1 melatonin receptor between conduit and resistant arteries can be involved in positive effects of melatonin on blood pressure control

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Background: Cardiovascular diseases are primarily cause of mortality and morbidity. Melatonin (MEL) may have hypotensive effects, but mechanisms how it can modulate blood pressure (BP) are unclear.

Aims: To evaluate expression and localization of specific MEL receptors in the thoracic aorta (TA) and mesenteric artery (MA) and effects of MEL treatment on BP in hypertensive SHR rats.

Methods: Tissues collected from normotensive Wistar rats (WKY) were analyzed by RT-PCR as well as RT-qPCR for the expression of MT1 and MT2 mRNA. Moreover, protein localization was investigated by immunofluorescence microscopy on 4µm paraffin-embedded tissue sections. Hypertensive SHR rats were exposed to physiological (D1: 2.5 mg/kg) and pharmacological (D2: 10 mg/kg) doses of MEL in drinking water during the dark phase and BP was measured by radiotelemetry. Sympathetic (LF) and parasympathetic (HF) markers of heart rate variability and spontaneous baroreflex sensitivity (BRS) were calculated from telemetry data.

Results: In WKY rats, we found mRNA expression of MT1 but not MT2 receptor with a significant difference ($p < 0.001$) between TA ($20.0 \pm 0.5 \Delta CT$) and MA ($12.5 \pm 0.3 \Delta CT$). In TA, MT1 was localized mainly in the endothelium and adventitia while in MA, the signal was prominent in the smooth muscle layer and endothelium. We observed a decrease ($p = 0.056$) of BP after MEL treatment: SHR (LD: 191 ± 2 mmHg; D1: 193 ± 2 mmHg; D2: 196 ± 2 mmHg) vs. SHR with MEL (LD: 187 ± 2 mmHg; D1: 187 ± 2 mmHg; D2: 185 ± 2 mmHg). On the other hand, MEL had no impact on relative changes of BRS ($p=0.56$) while LF and HF were improved mainly during the dark phase (LF: SHR -4.7%, SHR-MEL: -7.9%; HF: SHR: +2.1%, SHR-MEL: +4.3%) after D2 concentration. Locomotor activity was not affected by MEL.

Conclusions: Localization of MT1 receptors in different vessels supports the possibility that MEL can influence BP via specific membrane receptors mainly in resistant vessels, such as MA. Observed anti-hypertensive effects of MEL suggest a potential to develop a novel anti-hypertensive compounds with peripheral as well as central mechanisms of action on BP control.

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S4-E8

Angiogenic properties of human pluripotent stem cell-derived arterial and venous endothelial cells

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Endothelial derivatives of human pluripotent stem cells hold out for therapeutic angiogenesis in ischemic cardiovascular diseases. Our aim was to investigate the arterial and venous phenotype of stem cell derived endothelials and their angiogenic potential after in vivo engraftment.

H7 human embryonic stem cells (hESC) were differentiated via embryoid body (EB) formation in normoxygenic (20%) and hypoxic (5%) conditions as well as in monolayer. Human induced pluripotent stem cells (hiPSC) were differentiated under normoxygenic condition via EB formation. CD31-positive endothelial cells were sorted by FACS and characterized for morphology, immunocytochemistry, gene expression and proteome profiling pattern. For engineering 3D constructs human aortic wall samples were decellularised. Human ESC-EC and hiPSC-EC were seeded onto human

ECM matrix. Stem cell-derived endothelials were transplanted into three months old athymic nude rats.

As shown by immunocytochemistry, hESC-EC and hiPSC-EC were stained positive for anti-CD31, von Willebrand factor and ve-cadherin; cells formed capillary-like tubules on Matrigel and took up acetylated-LDL. Quantitative PCR showed expression of arterial (EphrinB2, Notch1-2) and venous (EphB4) endothelial markers. Wide range of angiogenesis-related proteins were detected in all endothelial types. The mRNA levels of angiopoietin2 increased significantly in hESC-EC when differentiated with EB method (EB normoxia 353.17 ± 86.29 ; EB hypoxia 323.89 ± 86.63 , $p < 0.001$ $n=3$, mRNA levels are normalized to those in hESC). Endothelial cells remained viable upon in vivo engraftment. Marked increase was found in mRNA levels of all arterial and venous marker genes in re-isolated cells. Endothelial cells seeded onto decellularised human extracellular matrix remained viable as shown by calcein AM staining.

Differentiating endothelial cells from human pluripotent stem cells via EB method increased the angiogenic potency in vitro. After in vivo conditioning endothelial markers increased, suggesting that endothelial cells linked to host microcirculation. These cells may be used as novel angiogenic therapies in the future.

S5-E

Micro RNA Networks and Potential Clinical Implications in Cancer, Cardiovascular and Renal Diseases

S5-E1

MiRNAs in renal glomerular disease: Novel insights into pathogenic Mechanisms and clues for treatment

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Focal segmental glomerulosclerosis (FSGS) is a frequent and severe glomerular disease characterized by destabilization of podocyte foot processes. We report that transgenic expression of the microRNA miR-193a in mice rapidly induces FSGS with extensive podocyte foot process effacement. Mechanistically, miR-193a inhibits the expression of the Wilms' tumor protein (WT1), a transcription factor and master regulator of podocyte differentiation and homeostasis. Decreased expression levels of WT1 lead to downregulation of its target genes PODXL (podocalyxin) and NPHS1 (nephrin), as well as several other genes crucial for the architecture of podocytes, initiating a catastrophic collapse of the entire podocyte-stabilizing system. We found upregulation of miR-193a in isolated glomeruli from individuals with FSGS

compared to normal kidneys or individuals with other glomerular diseases. Thus, upregulation of miR-193a provides a new pathogenic mechanism for FSGS and is a potential therapeutic target.

S5-E2

MicroRNAs in ischemia/reperfusion injury and cardioprotection by ischemic conditioning: ProtectomiRs

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Cardioprotection by ischemic conditioning leads to characteristic changes in gene expression profile. Cardioprotection by conditioning is attenuated by the presence of cardiovascular risk factors and co-morbidities including metabolic diseases such as hyperlipidemia and diabetes. These risk factors also lead to changes in the gene expression profile of the heart. Fine tuning regulators of mRNA expression, miRNAs may contribute to cardioprotective gene expression response of the heart.

Therefore, we aimed to characterize early changes in microRNA expression in acute cardioprotection by ischemic pre- and postconditioning in rat hearts. Hearts isolated from male Wistar rats were subjected to i) time-matched non-ischemic perfusion, ii) ischemia/reperfusion (30 min coronary occlusion and 120 min reperfusion), iii) preconditioning (3x5 min coronary occlusion) followed by ischemia/reperfusion, or iv) ischemia/reperfusion with postconditioning (6x10s global ischemia/reperfusion at the onset of reperfusion, respectively). Infarct size was significantly reduced by both interventions. Out of 350 different microRNAs assessed by microarray analysis, 147-160 showed detectable expression levels. As compared to microRNA alterations induced by ischemia/reperfusion vs. time-matched non-ischemic controls, 5 microRNAs were significantly affected by both pre- and postconditioning (microRNA-125b*, 139-3p, 320, 532-3p, 188), 4 microRNAs by preconditioning (microRNA-487b, 139-5p, 192, 212), and 9 by postconditioning (microRNA-1, let-7i, let-7e, let-7b, 181a, 208, 328, 335, 503), respectively. Expression of randomly selected microRNAs was validated by QRT-PCR. By a systematic comparison of the direction of microRNA expression changes in all groups, we identified microRNAs, specific mimics or antagomiRs of which may have pre- and postconditioning-like cardioprotective effect (protectomiRs). Transfection of selected protectomiRs (mimics of microRNA-139-5p, -125b*, let-7b, and inhibitor of microRNA-487b) into cardiac myocytes subjected to simulated ischemia/reperfusion showed significant cytoprotective effect. This is the first demonstration that

ischemia/reperfusion-induced microRNA expression profile is significantly influenced by both pre- and postconditioning, which shows the involvement of microRNAs in cardioprotective signaling. Moreover, by analysis of microRNA expression patterns in cardioprotection by pre- and postconditioning, specific protectomiRs can be revealed as potential therapeutic tools treating ischemia/reperfusion injury.

S5-E3

MicroRNA-25 regulates NOX4 in the hypercholesterolemic heart

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Diet-induced hypercholesterolemia leads to oxidative/nitrative stress and subsequent myocardial dysfunction. However, the regulatory role of microRNAs in this phenomenon is unknown. We aimed to investigate, whether hypercholesterolemia-induced myocardial microRNA alterations play a role in the development of oxidative/nitrative stress and in subsequent cardiac dysfunction. Male Wistar rats were fed with 2% cholesterol/0.25% cholate-enriched or standard diet for 12 weeks. Serum and tissue cholesterol levels were significantly elevated by cholesterol-enriched diet. Left ventricular end-diastolic pressure was significantly increased in cholesterol-fed rats both in vivo and in isolated perfused hearts, indicating diastolic dysfunction. Myocardial expression of microRNAs was affected by cholesterol-enriched diet as assessed by microarray analysis. MicroRNA-25 showed a significant down-regulation as detected by microarray analysis and QRT-PCR. In silico target prediction revealed NADPH oxidase 4 (NOX4) as a putative target of microRNA-25. NOX4 protein showed significant up-regulation in the hearts of cholesterol-fed rats, while NOX1 and NOX2 remained unaffected. Cholesterol-feeding significantly increased myocardial oxidative/nitrative stress as assessed by dihydroethidium staining, protein oxidation assay, and nitro-tyrosine ELISA, respectively. Direct binding of microRNA-25 mimic to the 3'UTR region of NOX4 was demonstrated using a luciferase reporter assay. Transfection of a microRNA-25 mimic into primary cardiomyocytes decreased superoxide production, while a microRNA-25 inhibitor resulted in an up-regulation of NOX4 protein and an increase in oxidative stress that was attenuated by the NADPH oxidase inhibitor diphenyleneiodonium. Here we demonstrated for the first time that hypercholesterolemia affects myocardial microRNA expression, and by down-regulating microRNA-25 increases NOX4 expression and consequently oxidative/nitrative stress in the heart.

We conclude that hypercholesterolemia-induced microRNA alterations play an important role in the regulation of oxidative/nitrative stress and in consequent myocardial dysfunction.

S5-E4

miR-200 in extracellular vesicles promotes metastasis of breast cancer cells

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Not all cancer cells in a tumor are capable of metastasizing. The miR-200 microRNA family, which regulates the mesenchymal-to-epithelial transition, is enriched in the serum of patients with metastatic cancers, and ectopic expression of miR-200 can confer metastatic ability to poorly metastatic tumor cells in some settings. Here we investigated whether metastatic capability could be transferred between metastatic and nonmetastatic cancer cells via extracellular vesicles. Metastatic breast cancer cell lines highly expressing miR-200 secrete miR-200 microRNAs in extracellular vesicles and transfer them to otherwise weakly metastatic cells either nearby or at distant sites and confer upon them the ability to colonize distant tissues in a miR-200-dependent manner. Thus, uptake of extracellular vesicles can transfer metastatic capability.

S5-E5

Targeting basal-like Triple Negative Breast Cancers and epithelial tumor-initiating cells with aptamer-siRNA chimeras

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RNA interference (RNAi) offers the exciting therapeutic possibility of selectively knocking down disease-causing genes. Recent Phase I and II clinical trials of small interfering RNAs (siRNAs) have shown promising gene knockdown and clinical benefit in a handful of diverse diseases caused by aberrant liver gene expression. In these studies siRNAs were targeted to the liver by encapsulation in lipoplexes or by conjugation with a sugar selectively recognized by hepatocytes. The major obstacle to harnessing RNAi for treating cancer is the difficulty delivering RNAs into disseminated cancer cells. Giangrande, McNamara and Gilboa developed a flexible and effective multifunctional RNA delivery platform using aptamer-siRNA chimeras (AsiC), to accomplish this goal. RNA aptamers, structured RNAs that bind with high affinity to a protein, covalently linked to siRNAs, cause knockdown selectively in cells bearing the receptor recognized by the aptamer. Although treatment for breast cancer has improved with the development of targeted therapeutics, there is still a need for new approaches for poor prognosis breast cancers. In particular, there is no targeted therapy for triple negative breast cancers (TNBC), which often relapse after current therapy; Her2+ breast cancers frequently develop resistance to targeted therapy with trastuzumab or lapatinib. We have developed AsiCs that target a receptor highly expressed on epithelial cancer cells and on the tumor-initiating cells (also known as cancer stem cells) within them.

All basal-A TNBC and luminal breast cancer cell lines examined strongly expressed the receptor recognized by the aptamer, while a normal breast cancer epithelial line and mesenchymal TNBCs had close to background levels. RNA uptake, gene knockdown and the cytotoxic effect of these AsiCs were robust in basal-like TNBC and luminal breast cancer lines, but not in normal epithelial cells and mesenchymal cells. Treatment of mice bearing basal-like TNBCs on one flank and mesenchymal breast tumors on the other caused selective uptake and rapid (within a week) and sustained complete regression of the basal-like tumors, but had no effect on the mesenchymal tumors.

S5-E6

microRNA expression might predict prognosis of epithelial hepatoblastoma and sorafenib treated hepatocellular carcinoma

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Background and aims: The more differentiated fetal component of hepatoblastoma (HB) is suggested to associate with better survival when compared with embryonal component. Sorafenib represents the first effective targeted therapy for advanced stage hepatocellular carcinoma (HCC), however, adequate patient stratification regarding sorafenib-responsiveness is still missing. MicroRNA (miRNA) expression patterns were investigated in epithelial subtypes of HB and in pretreatment HCC under sorafenib treatment. and related to survival.

Material and methods: 20 cases of epithelial HBs subtyped as pure fetal components (n=12) and embryonal components (n=8), along with 15 non-tumorous surrounding liver (SL) samples were analysed. Further, diagnostic fine needle aspiration (FNA) cytology smears of 20 advanced stage HCC patients were investigated. Relative expressions of miR-17-5p, miR-18a, miR-21, miR-34a, miR-96, miR-122, miR-181a, miR-195, miR-210, miR-214, miR-221, miR-222, miR-223 and miR-224 were determined by TaqMan MicroRNA assay.

Results: No significant differences were revealed in overall survival between fetal and embryonal/fetal types of HBs. High miR-21, low miR-222 and low miR-224 levels proved to be independent prognostic factors for HB and were associated with significantly increased overall survival (p <0.03). Among the analysed miRNAs high expression of miR-214 was associated with smaller tumor size (p=0.019); high miR-17-5p expression correlated with better ECOG performance status (p=0.003). Survival analysis revealed that high miR-224 expression was associated with increased progression-free and overall survival (PFS p=0.029; OS p=0.012).

Conclusions: miR-21, miR-222 and miR-224 levels could serve as valuable tools to predict overall survival of HB patients regardless of epithelial subtype. Pretreatment microRNA profiling, especially miR-224 expression might serve as an ancillary tool for better assessment of expected survival under sorafenib treatment.

S5-E7

Exosomal microRNA in biofluids - Robust biomarkers for disease

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MicroRNAs constitute a class of small cellular RNAs (typically 19-23 nt) that function as post-transcriptional regulators of gene expression. Current estimates indicate that more than one third of the human cellular transcriptome is regulated by this small class of RNA (~2000 miRNA).

The high relative stability of microRNAs in common clinical source materials (FFPE blocks, plasma, serum, urine, saliva, etc.) and the ability of microRNA expression profiles to accurately classify discrete tissue types and specific disease states have positioned microRNAs as promising new biomarkers for diagnostic application in cancer. The study of extracellular microRNAs and their potential as pathophysiological markers has greatly expanded in the last couple of years. MicroRNAs have been shown to be actively exported from tissues into the circulation through a variety of mechanisms including complexing with RNA binding proteins or HDL and through exosome and microvesicle transport.

Exosomes are nanovesicles secreted into the extracellular environment by a wide range of mammalian cell types under normal and pathological conditions. As the profile of exosomal microRNAs may be a fingerprint of the releasing cell type and because they are released in easily accessible body fluids such as blood and urine, their microRNA content holds potential as biomarkers for early detection of malignancy. Using exosomal microRNA as a starting point for biomarker studies will reduce the risk of false negative results when the study involve detection of low abundance microRNAs.

We recently developed a new exosome enrichment method and will present a comparison of microRNA profiles obtained with this method to profiles obtained with different commercial available exosome isolation methods and to standard profiles of whole plasma and serum. We have applied our highly sensitive LNATM-based qPCR platform for detection of microRNAs, which has enabled microRNA profiling in biofluids where levels are extremely low. The platform uses a single universal RT reaction to conduct full miRNome profiling and allows high-throughput profiling of microRNAs without the need for pre-amplification.

Summary: Exosomal microRNAs have recently gained considerable interest as potential pathophysiological markers. We have developed a new miRNA purification method using exosome enrichment and compared microRNA profiles to profiles obtained with different commercial exosome isolation methods and standard profiles of whole plasma and serum.

S6-A

The Calcifying Vessel: New Genetic Findings for Future Pathophysiological Avenues

S6-A1

Mechanisms of arterial calcification: Lessons learned from rare monogenic disorders

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Arterial calcification significantly contributes to morbidity and mortality. Major insight into the pathophysiological mechanisms contributing to arterial calcification has been provided through three rare monogenic disorders associated with spontaneous, premature artery media calcification. Monogenic NPP1, ABCC6, and CD73 deficiencies each drive a molecular pathophysiology of the closely related, but phenotypically different human diseases Generalized Arterial Calcification of Infancy (GACI), Pseudoxanthoma Elasticum (PXE), and Calcification of Joints and Arteries (CALJA) respectively, in which premature onset of arterial calcification is a prominent but not sole feature. Based on the similarities of GACI, PXE and CALJA, it can be speculated that the underlying disease genes, ENPP1, ABCC6 and NTSE, drive a cohesive arterial molecular pathophysiology system modulated by ATP metabolism, inorganic pyrophosphate, adenosine, inorganic phosphate generation and functional activities.

S6-A2

The role of ABCC6 in chronic and acute calcification, a tale of 3 diseases

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Abnormal mineralization occurs in the context of several common conditions, including advanced age, diabetes, hypercholesterolemia, chronic renal failure and certain genetic conditions. Metabolic, mechanical, infectious, and inflammatory injuries promote ectopic mineralization through overlapping yet distinct molecular mechanisms of initiation and progression. The ABCC6 protein is an ATP-dependent transporter primarily found in the plasma membrane of hepatocytes and appears to prevent ectopic mineralization by inducing the cellular release of nucleotides, including nucleotides triphosphate (NTPs). Surface ectonucleotidases hydrolyze NTPs into pyrophosphate, a potent inhibitor of calcification, which is then released for systemic circulation. ABCC6 deficiency is the primary cause for chronic and acute

forms of ectopic mineralization described in diseases such as pseudoxanthoma elasticum (PXE), beta-thalassemia, and generalized arterial calcification of infancy (GACI) in humans and dystrophic cardiac calcification (DCC) in mice. These pathologies are characterized by mineralization of cardiovascular, ocular, and dermal tissues. PXE and some cases of GACI are caused by inactivating ABCC6 mutations, whereas the mineralization associated with beta-thalassemia patients derives from a liver-specific change in ABCC6 expression. DCC is an acquired phenotype resulting from cardiovascular insults (ischemic injury or hyperlipidemia) and secondary to ABCC6 insufficiency. Abcc6-deficient mice develop ectopic calcifications similar to both the human PXE and mouse DCC phenotypes. Although, we have acquired a better understanding of the physiological role of hepatic ABCC6, its precise molecular and cellular functions still remain unknown. In addition, new evidences are now emerging suggesting that ABCC6 physiological function is not limited to preventing ectopic calcification.

S6-A3

Tissue-wide mineralization: What can we learn from the vasculature

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Soft tissue mineralization is a complex process, the molecular and cellular mechanisms of which are not fully understood. Mineralization is necessary for the formation of skeletal tissues but it needs to be rigorously controlled and restricted to specific regions. In many cases, ectopic mineralization results from a disturbance of the complex interplay between mineralization propagators and antagonists set out to regulate this process. Although most soft tissues can undergo calcification, certain ones - including the skin, the kidneys, the cartilage and tendons, the eyes and the vasculature - are considerably more prone. Pathologic mineralization in one of those tissues may result in disease which can be very debilitating, painful and typically destructive for the compromised tissue.

The knowledge on the molecular background of soft tissue mineralization largely comes from insights in vascular calcification, with involvement of the osteoinductive Transforming Growth Factor beta (TGF β) family (TGF β 1-3 and Bone Morphogenetic Proteins [BMP]), together with ectonucleotides (ENPP1), Wnt signalling and a variety of local and systemic calcification inhibitors. Questions which arise are whether the cellular pathways and mechanisms documented to induce vascular calcification can simply be extrapolated to other soft tissues that are prone to mineralization and how relevant these pathways are for diseases which are characterized by a more generalized ectopic mineralization. In this presentation, concepts will be presented on the relevance of cardiovascular mineralization mechanisms for soft tissues in general and how these can contribute to our understanding of rare and more common ectopic mineralization disorders and to the identification of genetic modifiers which can be used to foresee the phenotype of an individual patient more accurately.

S6-A4

Arterial calcifications and cardiovascular diseases: Clinical and therapeutic issues

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Calcification is an actively regulated biological process that is normally restrained to bony structures in physiological conditions. Ectopic calcifications in soft tissues, such as the arterial wall, is part of normal aging, but can also occur in acquired metabolic diseases e.g. diabetes and chronic renal insufficiency and in several inherited diseases. Arterial calcification is currently recognized as an independent risk factor for cardiovascular diseases. In atherosclerosis, calcification localizes mainly within the intimal layer whereas the accumulation of hydroxyapatite within the media layer (i.e. medial calcification) is observed mainly in arteriosclerosis. Arterial calcification within the media lead to arterial wall stiffness thus contributing to the development of a systolic hypertension. Intimal calcification, however, leads to plaque rupture leading to thrombosis and ischemic lesions. Based on existing literature and our data, we will review the different methods to evaluate the impact of the calcifying phenotype on the arterial function. Data obtained from studies of inherited genetic calcifying diseases will be also presented since they represent excellent models for the understanding of the mechanisms linking calcification to the arterial dysfunction and possible therapeutic applications.

S6-A5

Conformation correction therapy in arterial calcification disorders, PXE and GACI

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Arterial calcification is a leading cause of death, especially in the Western countries. Little is known about the mechanisms regulating the calcification process, however, some rare monogenic disorders gave insight into the pathophysiological mechanisms contributing to arterial calcification.

Mutations in the gene of the ABCC6 transporter, a member of the ABC protein family, are responsible for the development of two genetic diseases: Pseudoxanthoma elasticum (PXE, OMIM 26480) and Generalized Arterial Calcification of Infancy (GACI, OMIM 208000). Furthermore, a missing allele of ABCC6 is a genetic risk factor in coronary arterial disease (CAD).

In order to better understand how mutations in the ABCC6 gene lead to development of abnormal calcification symptoms, and to establish potential therapeutic treatment for patients carrying ABCC6 mutations, we have set up a complex experimental

strategy to determine the structural and functional consequences of disease-causing mutations in the human ABCC6 transporter. The transport activity of the protein was determined by biochemical transport assays, and the subcellular localization of wt and mutant proteins was investigated both in vitro and in vivo. The major aim of our study was to identify mutants with preserved transport activity but failure in intracellular targeting, as these mutants are candidates for functional rescue. Sodium 4-phenylbutyrate (4PBA), an FDA-approved drug, has been shown to act as a chemical chaperon and to restore the reduced cell surface expression of certain mutated plasma membrane proteins.

To analyze the effect of 4-PBA, not only to the subcellular localization but also to the function of ABCC6 mutants, an in vivo functional assay was set up, using the cardiac freeze-thaw injury method to induce an acute Abcc6-dependent phenotype referred to as dystrophic cardiac calcification (DCC). The expression of the human WT ABCC6 in mouse liver reduced significantly the DCC phenotype. On this line we showed that while disease-associated missense ABCC6 mutants expressed in the liver of Abcc6-/- mice do not reduce calcification, administration of 4-PBA attenuates in vivo calcification by directing the mutants to the plasma membrane.

These results indicate that 4-PBA treatment restored both the localization and the physiological function of certain disease-causing ABCC6 mutants.

S6-B

Physiology of Interaction Between RAS, IRAP and Glucose Metabolism

S6-B1

Does insulin-regulated aminopeptidase play a role in regulating glucose uptake in neurones?

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IRAP (insulin-regulated aminopeptidase) is a type II transmembrane protein with two functional domains. Its N-terminal cytoplasmic domain contains trafficking motifs and this protein was originally identified in fat cells, co-localized with the insulin-regulated glucose transporter, GLUT4, in intracellular vesicles. IRAP is translocated together with GLUT4 to the cell surface following insulin stimulation. Its larger extracellular domain contains classical aminopeptidase motifs and has been shown to cleave a number of substrates including vasopressin, oxytocin, and neurokinins A & B. IRAP is also known as oxytocinase because of its role in regulating circulating oxytocin levels during pregnancy. Our group has demonstrated that peptide inhibitors of IRAP, Angiotensin IV

(Ang IV) and LVV-hemorphin 7 (LVV-H7), elicit robust effects on accelerating spatial learning and facilitating memory consolidation in rodents. More importantly, these peptides restore memory in animals with experimental amnesia. We propose that IRAP is an exciting target for the development of a novel class of cognitive enhancers. We have developed a series of small molecular weight compounds targetting the catalytic domain of IRAP that have memory-enhancing properties, offering proof-of-principle that inhibition of the enzyme have beneficial effects on memory. We are currently elucidating the mechanisms by which these IRAP inhibitors facilitate learning and memory. In neurons in the hippocampus and entorhinal cortex, IRAP is found almost completely co-localized with GLUT4 as is the case in insulin-responsive tissues. We also demonstrated that IRAP inhibitor treatment results in enhanced glucose uptake into neurons via a GLUT4 mediated mechanism. Exogenous glucose administration has been shown to improve memory and increased glucose uptake has been detected in the hippocampus of rats during the performance of memory tasks. Therefore we hypothesize that one of the mechanisms by which IRAP inhibitors enhance memory is via facilitation of glucose uptake into neurons.

S6-B2

Presence, regulation and function of insulin-regulated aminopeptidase in macrophages

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Angiotensin II-(3-8) is a fragment of the cardiovascular hormone Angiotensin II that incited the interest of several research groups because of its ability to facilitate memory as well as reversal of memory deficits in animal behavior models. These effects are mediated by its interaction with the membrane-bound insulin-regulated aminopeptidase (IRAP) which was formerly denoted as AT4 receptor. Using our recently developed IRAP selective analogue [3H]-IVDE77 as well as by western blot analysis and real-time PCR we demonstrated the presence and regulation of IRAP in mouse as well as human macrophages. Macrophages are first-line defense cells that can adopt distinct activation states in response to particular cytokines. Interestingly IRAP mRNA expression in mouse thioglycolate-elicited primary peritoneal macrophages was increased by the pro-inflammatory mediators interferon- γ (IFN- γ) and lipopolysaccharide (LPS) but not by anti-inflammatory cytokines (IL-4, IL-10, TGF- α). In agreement the corresponding [3H]-IVDE77 binding to intact macrophages was increased after IFN- γ pretreatment during increasing periods of time. On the other hand only a relatively short pre-incubation (2 hours) with LPS enhanced [3H]-IVDE77 binding which then declined after longer pre-incubations. Subsequently we investigated whether Angiotensin IV and/or IRAP selective analogues have an influence on the expression of pro- and/or anti-inflammatory genes, macrophage mediated phagocytosis, antigen cross-presentation and glucose uptake. Human peripheral blood mononuclear cells were isolated and differentiated into mature macrophages with colony-stimulating-factor. The presence of IRAP in these cells was shown by the specific binding of [3H]-AL11, a former analogue of IVDE77. Similar as in mouse cells, IFN- γ and LPS upregulated mRNA levels of IRAP in human macrophages.

Further work is needed to elucidate whether IRAP is involved in the normal and/or pathological functions of macrophages. These findings may extend our knowledge of RAS components beyond the classical cardiovascular functions.

S6-B3

Ex vivo assessment of tissue angiotensin metabolism. Focus on Ang I/Ang IV/IRAP axis in various kinds of fat tissue in rat model of obesity and insulin resistance

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Next to angiotensin II (Ang II), several “new” biologically active peptides [e.g. Ang-(1-7), Ang-(1-12), Ang IV, Ang-(2-10)] and enzymes [e.g. angiotensin converting enzyme 2 - ACE2, IRAP/oxitocinase] have been described; at least one of them - Ang-(1-7) - has been shown to oppose actions of Ang II. Several lines of evidence suggest that adipose tissue could be a rich source of various angiotensins, which may play a multiple roles in regulation of adipose tissue physiology and constitute a link between obesity and cardiovascular disorders.

Based on LC-ESI-MS method we established ex vivo system for assessment of angiotensin metabolites. Here, we present the short summary of our results regarding angiotensin I metabolism in various animal tissues, with focus on of fat tissue from different depots (epididymal, subcutaneous, periaortic and retroperitoneal) in rat model of obesity and insulin resistance - in young (12 w/o) and old (33 w/o) Zucker rats. For comprehensive identification and quantitation of production of angiotensin peptides LC-ESI-MS method was used. Additionally, qPCR measurements of mRNA expression of main enzymes involved in Ang I metabolism as well as determination of IRAP activity were performed.

In every type of fat, there was prevalence of production of Ang-(1-7) over Ang II and Ang IV; the generation of Ang IV was most efficient in subcutaneous and periaortic fat. The use of ACE inhibitor (perindoprilat) resulted in inhibition of Ang II and Ang IV and increase of Ang-(1-7) formation. In all kinds of fat the ability to generate Ang IV tended to decrease with age of animals. In both lean and obese animals plasma IRAP activity decreased with age. Interestingly, in epididymal fat of old obese Zucker rats there was a significant increase in IRAP expression as compared to old lean animals.

The quantitative and qualitative description of Ang I metabolism in various kinds of fat tissue in Zucker rats poses the questions about the biological roles of particular peptides [esp. Ang-(1-7)/MAS and Ang IV/IRAP axes] both in local regulation of fat tissue functions and in cardiovascular complications of obesity and diabetes.

S6-B4

Activity assays for proteolytic enzymes in complex biological samples - Technical aspects and novel methods for measuring angiotensinase and oxytocinase activities

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Peptide hormones are crucial regulators of multiple physiological functions resulting in a major scientific interest in the enzymes involved in their metabolism. Despite the fact that the chemical nature of a proteolytic cleavage is well defined, the accurate determination of a certain protease activity in a biological sample remains a significant analytical challenge. The availability of a variety of artificial chromogenic and fluorogenic substrates suggests the easy and rapid measurement of different protease activities in tissue homogenates and body fluids. However, biochemical considerations regarding substrate specificity and sample complexity put a question mark over the use of artificial substrates for protease activity measurements. The availability of mass spectrometry as a novel sensitive and highly specific tool for the quantification of peptide hormones opens up new possibilities for the analysis of peptide hormone cascades in tissue homogenates and body fluids. The utilization of natural substrates for the quantification of enzymatic activities abolishes the presence of false positive and unspecific signals, focusing on the metabolism of physiologically relevant substrates. Mass spectrometry based characterization of peptide hormone metabolism will be discussed by reference to the renin angiotensin system and IRAP aiming to create awareness about methodological limitations and frequent pitfalls coming along with the use of artificial substrates in the measurement of proteolytic enzyme activities.

S6-B5

Effect of high fat diets on angiotensinase and IRAP activities. Their role in blood pressure and glucose homeostasis control

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High fat diets have been related to the development of hypertension and other metabolic dysfunctions such as obesity or type 2 diabetes. However, these effects depend on the specific profile of the fatty acids in the diet. Extra virgin olive oil (EVOO), the main fat source of Mediterranean diet and specially rich in monounsaturated fatty acids, has demonstrated a positive role in prevention of hypertension and cardiovascular diseases, but the physiological and molecular mechanisms of these effects are not totally understood.

All the components of the renin-angiotensin system (RAS) are present locally in tissues such as kidney, brain or white adipose tissue. They play a critical role not only during development of hypertension but also in the progression of insulin resistance and type 2 diabetes. In the RAS, AngII is hydrolyzed by aminopeptidase A to generate Ang III. Subsequently this peptide is metabolized to Ang IV by aminopeptidase N, being

these enzymes called angiotensinases. The specific receptor for Ang IV (AT4) identified as the insulin-regulated aminopeptidase (IRAP), is associated with the Glut4 transporter in intracytoplasmatic vesicles, which suggests a possible link between RAS, blood pressure control and glucose uptake. These activities have demonstrated significant local changes between normotensive and hypertensive animal models, after anti-hypertensive drug treatments and compared with healthy controls, in plasma of patients with type 2 diabetes and metabolic syndrome.

Results obtained in our laboratory by measuring blood pressure, body weight and fasting insulin and leptin levels, demonstrated a possible beneficial effect when diets enriched with EVOO were compared with a high fat diet enriched with saturated fatty acids and cholesterol; and some of these effects have also been observed in spontaneously hypertensive rats. This effect was related to specific changes of angiotensinase and IRAP activities in the above-mentioned tissues.

Taking together, these results indicate a role of dietary fat in the regulation of angiotensinase and IRAP activities, and open a new approach to the study and treatment of metabolic dysfunctions.

S6-B6

Chronic treatment of rats with oxytocin upregulates renin and (pro)renin receptor expression in kidney

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Oxytocin (OT) has been shown to induce renin release by kidney during acute peptide infusion in rats^{1,2}. The aim of our study was to evaluate whether the low dose peripheral OT chronic administration leads to renal renin-angiotensin system (RAS) activation in the kidney of lean and obese Zucker rats. We administered OT by osmotic minipumps into male lean (+/?) and obese (fa/fa) 10-week-old Zucker rats for 14 days at a dose rate of 3.6 µg/100 g/day. Rats were classified into four groups: control lean group, OT-treated lean group, control obese group and OT-treated obese group. Controls received isotonic saline. Animals were given ad libitum access to standard diet and water. The water intake had been monitored daily during OT treatment period. Gene expression of renin, (pro)renin receptor, angiotensinogen, ACE, ACE2, AT1 receptor, AT2 receptor, Mas receptor and neutral endopeptidase in kidney was determined. Data were analyzed by two-way ANOVA test with variables genotype and treatment. We found significant main effect of OT treatment on kidney weight ($p < 0.05$), relative kidney mass ($p < 0.001$) and the gene expression of the renin ($p < 0.001$) and (pro)renin receptor ($p < 0.05$) and we observed an increase in all of these parameters. The gene expression of other RAS components was not changed. OT administration had no significant effect on the water intake. No interaction of genotype x OT treatment effect on all measured parameters was revealed. Present study demonstrates stimulatory effect of OT administration on renin expression in kidney in lean and obese Zucker rats. Increased renin production in our experiment may

contribute to regulation of cardiovascular homeostasis and/or, considering elevated (pro)renin receptor expression, may have impact on intrarenal angiotensin II formation and thus physiology of the kidney. Observed alterations at mRNA level prompt to determine protein expression, renin activity and renal function test.

- 1 Huang W. et al. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R226, 2000.
- 2 Loichot C. et al. Eur. J. Pharmacol. 454: 241, 2002 The study was supported by grants VEGA 2/0174/14, MZ 2012/51-SAV-1 and APVV-0028-10.

S6-C

Basic Research Meets Clinical Endocrinology

S6-C1

Organogenesis in a petridish - How to generate functional thyroid tissue from mouse embryonic stem cells

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Congenital hypothyroidism (CH), the most common congenital endocrine disorder in humans, affects approximately 1/3000 live births. Most CH cases are due to thyroid dysgenesis (TD). However, the molecular mechanisms underlying TD remain poorly understood; in part, due to our limited understanding on how intrinsic factors and extrinsic signaling cues orchestrate thyroid follicular cell (TFC) differentiation and thyroid morphogenesis. Embryonic stem cells (ESC) emerged as a powerful model system to define signaling pathways and gene networks controlling cell differentiation and tissue morphogenesis. Until recently, the toolkit to interrogate thyroid organogenesis was lacking a protocol for efficient generation of TFC from ESC. Directed differentiation of ESC into a given cell type relies on step-wise adjustments of culture conditions in order to reproduce in vitro the sequential events that characterize the normal ontogenesis of a given cell type. However, given the lack of knowledge on the in vivo signals regulating TFC differentiation, we employed an alternative differentiation strategy (direct programming) which relies on forced expression of key transcription factors to promote TFC differentiation. For this purpose, we generated recombinant mESC lines that permit inducible overexpression of Nkx2.1 and Pax8 upon doxycycline (Dox) treatment. Using this model system, a transient overexpression of Nkx2.1 and Pax8 for 3 days was sufficient to generate cell cultures displaying a TFC-like gene expression profile. Application of recombinant hTSH to cell cultures immediately after Dox treatment further stimulated the formation of follicular organoids displaying characteristics reminiscent of thyroid follicles in intact animals. Analyses of iodide uptake and organification in such cell cultures demonstrated the generation of functional thyroid tissue. Importantly, when follicular organoids were grafted in

vivo into athyroid mice, substantial reconstitution of functional thyroid tissue was observed at the grafting site. Moreover, athyroid mice grafted with follicular organoids displayed normalization of thyroid hormone plasma levels as well as symptomatic recovery.

S6-C2

Central regulation of hypothalamic-pituitary-thyroid axis under physiological and pathophysiological conditions

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The primary central regulator of the hypothalamic-pituitary-thyroid (HPT) axis is the thyrotropin-releasing hormone (TRH), a tripeptide amide that functions as a neurotransmitter but also serves as a neurohormone. Hypophysiotropic TRH neurons involved in this neuroendocrine process are located in the hypothalamic paraventricular nucleus and secrete TRH into the pericapillary space of the external zone of the median eminence for conveyance to anterior pituitary thyrotrophs. The released TRH controls the activity of thyroid gland through the regulation of TSH synthesis, glucosylation and release in the anterior pituitary. The activity of hypophysiotropic TRH neurons is regulated by the negative feedback effects of thyroid hormone to ensure stable concentration of circulating level of thyroid hormone. This mechanism involves complex interactions between hypophysiotropic TRH neurons and the vascular system, cerebrospinal fluid, and specialized glial cells called tanycytes. The set point of this feedback regulation, however, can be altered under certain conditions to adjust the circulating thyroid hormone concentration to the actual demand. This mechanism facilitates adaptation of the organism to changing environmental conditions, including the shortage of food and a cold environment. Circulating hormones, neuronal inputs and the tanycytes play critical role in this adaptation. Adverse conditions such as infection, also alter the feedback regulation of this axis, however, it is not completely understood whether the illness induced central hypothyroidism is an adaptive or maladaptive response.

S6-C3

Regulation of cell cycle by microRNAs and its implication in the pathogenesis of endocrine tumors

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Dysregulation of cell cycle has been implicated in the pathogenesis of various tumors including tumors originating from endocrine glands. MicroRNAs (miRNAs) are small

noncoding RNAs that negatively regulate gene expression. Their role has been demonstrated in the regulation of numerous physiological and pathophysiological processes including regulation of cell cycle.

Our group studied the pathogenesis of adrenal and pituitary tumors. Multi-omics approach was used for integrating data obtained from various high-throughput technologies including whole comparative genomic hybridization, gene, miRNA and protein expression data.

Our data revealed that both in adrenal and pituitary gland tumors a dysregulation of cell cycle by aberrant miRNA expression might have an important role in the pathogenesis of these tumors. In adrenocortical cancer dysregulation of the G2/M transition of cell cycle by aberrant mRNA and miRNA expression was confirmed. In pituitary tumors underexpression of Wee1 kinase and overexpression of CDC25 phosphatases (both key regulators of cell cycle G2/M transition) together with the opposite expression of their 3' UTR targeting miRNAs were demonstrated. In addition, CDC25A targeting miRNAs showed a strong negative correlation with tumor size suggesting that these miRNAs may have an important role in tumor growth.

Our data suggest that tumors of endocrine glands have differentially expressed miRNAs compared to their normal counterparts and these miRNAs are involved in the regulation of G2/M checkpoint of cell cycle.

S6-C4

The roll of microRNAs in preimplantation embryos and pluripotent stem cells

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MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate multiple biological processes. Increasing experimental evidence implies an important regulatory role of miRNAs during early embryonic development and in embryonic stem cell biology.

We analyzed the expression profile of pluripotency-associated miRNAs in rabbit embryos and embryonic stem-like (ES-like) cells. The rabbit specific ocu-miR-302, ocu-miR-290 clusters and three homologs of human C19MC cluster (ocu-miR-512, ocu-miR-520e and ocu-miR-498) were identified in rabbit preimplantation embryos and ES-like cells. Ocu-miR-498, ocu-miR-520e and ocu-miR-512-5p exhibited an interesting expression pattern, because gene cluster members are extensively expressed in extra-embryonic tissues.

The ocu-miR-302 cluster was highly similar to its human homolog, while ocu-miR-290 revealed a low level of evolutionary conservation with its mouse homologous cluster. The expression of ocu-miR-302 cluster members began at 3.5 dpc early blastocyst stage and they stayed highly expressed in rabbit ES-like cells. In contrast, high expression level of ocu-miR-290 cluster members was detected during preimplantation embryonic development, but low level of expression was found in rabbit ES-like cells. This results show that the expression of ocu-miR-302 cluster is characteristic for the rabbit ES-like cell, while the ocu-miR-290 cluster may play a crucial role during early embryonic development.

It has been recently demonstrated that miR-99a modulates TGF- β induced epithelial to mesenchymal transition. Therefore expression of ocu-miR-99a-5p and ocu-miR-125b-5p in epiblast cells of 6 dpc blastocysts might have a direct correlation with epithelial-mesenchymal transition of epiblast cells and formation of mesoderm cells prior to implantation. In the future we would like to examine the expression of ocu-miR-99a-5p and ocu-miR-125b-5p in epiblast cells compared to hypoblast and trophoblast cells in 6 day old rabbit embryos.

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S6-C5

Novel insight in the regulation of the brainrenin-angiotensin system and its connection with hypertension

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The hyperactivity of the brainrenin-angiotensin system (RAS) has been implicated in the development and maintenance of arterial hypertension (HTA). We first demonstrated that aminopeptidase A (APA) and aminopeptidase N (APN), two membrane-bound zinc metalloproteases, are involved in the formation and metabolism of brainangiotensin III (AngIII), respectively. Then, we designed the specific and selective APA and APN inhibitors, EC33 and PC18. By blocking in vivo brain APA and APN with these compounds, we showed that AngIII and not AngII, as established in the periphery is one of the main effector peptides of the brain RAS, exerting a tonic stimulatory control over blood pressure (BP) in hypertensive rats. Central injection of EC33 blocks the formation of brainAngIII and normalizes BP in hypertensive rats, suggesting that brain APA may be potential therapeutic target for HTA treatment

For a clinical use of APA inhibitors, we, therefore, designed 4,4-dithio[bis[(3S)-3-aminobutyl sulfonic acid]] (RB150), a systemically active prodrug of EC33 obtained by disulfide bridge mediated dimerization. RB150, given orally in conscious DOCA-salt rats or spontaneously hypertensive rats (SHRs), crosses the intestinal, hepatic and blood-brain barriers, enters the brain, where it is cleaved by brain reductases, generating two active molecules of EC33 which inhibit brain APA activity, block the formation of brainAngIII and induce a marked and sustained decrease in BP. The RB150-induced BP decrease is due to a decreased vasopressin release in the blood circulation, which results in increased diuresis, thus reducing extracellular volume and to a decrease in sympathetic tone, leading to a reduction of vascular resistances. Furthermore, in SHRs, concomitant RB150 oral administration with enalapril, a systemic RAS blocker, potentiates the RB150-induced BP decrease. RB150 also named QGC001 was then evaluated in humans and Phase I clinical trials have shown that this compound is clinically and biologically well-tolerated in

healthy normotensive male subjects after a single oral dose up to 2,000 mg or after repeated doses up to 750 mg b.i.d. for 7 days.

RB150 is the first drug candidate of a new class of antihypertensive agents targeting the brain RAS, the clinical efficacy of which will be soon evaluated in hypertensive patients.

S6-C6

Mutation of the palmitoylation site of Estrogen Receptor ER α in vivo reveals tissue-specific roles for membrane versus nuclear actions

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Estrogen Receptor alpha (ER α) activation functions AF-1 and AF-2 classically mediate gene transcription in response to estradiol (E2). A fraction of ER α is targeted to plasma membrane and elicits membrane-initiated steroid signalling (MISS), but the physiological roles of MISS in vivo are poorly understood. We therefore generated a mouse with a point mutation of the palmitoylation site of ER α (C451A-ER α) to obtain membrane-specific loss-of-function of ER α .

The abrogation of membrane localization of ER α in vivo was confirmed in primary hepatocytes, and it resulted in female infertility with abnormal ovaries lacking corpora lutea and increase in luteinizing hormone levels. In contrast, E2 action in the uterus was preserved in C451A-ER α mice and endometrial epithelial proliferation was similar to wild-type. However, E2 vascular actions such as rapid dilatation, the acceleration of endothelial repair and endothelial NO synthase phosphorylation were abrogated in C451A-ER α mice. A complementary mutant mouse lacking the transactivation function AF-2 of ER α (ER α -AF2^o) provided selective loss-of-function of nuclear ER α actions. In ER α -AF2^o, the acceleration of endothelial repair in response to estrogen-dendrimer conjugate, which is a membrane-selective ER ligand, was unaltered, demonstrating integrity of MISS actions. In genome-wide analysis of uterine gene expression, the vast majority of E2-dependent gene regulation was abrogated in ER α -AF2^o whereas in C451A-ER α it was nearly fully preserved, indicating that membrane-to-nuclear receptor crosstalk in vivo is modest in the uterus.

Thus, this work is the first to genetically segregate membrane versus nuclear actions of a steroid hormone receptor and to demonstrate their in vivo tissue-specific roles.