

MEETING ABSTRACTS

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MEETING ABSTRACTS

A1

The anti-addictive drug ibogaine modulates voltage-gated ion channels and may trigger cardiac arrhythmias

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Background: Ibogaine is an alkaloid derived from the African shrub *Tabernanthe iboga*. Psychoactive properties of ibogaine have been known for decades, but more recently the drug has received much attention because of its promising "anti-addictive" actions. Thus, ibogaine and its derivatives are being studied as potential treatment for opioid and stimulant abuse, as well as for alcoholism and smoking. Because ibogaine has a complex pharmacology and is known to interact with numerous different cellular targets, its potential to generate adverse effects is significant. Besides the expected neurotoxic actions, ibogaine may e.g. also affect the heart. Thus, several cases of sudden death after ibogaine use were reported, which have been hypothesised to be related to cardiac arrhythmias. In accordance, a severely prolonged QT interval of the electrocardiogram and ventricular tachyarrhythmias were observed in a woman after she had taken ibogaine.

Methods: To study possible mechanisms by which ibogaine may trigger cardiac arrhythmias, we explored ibogaine's effects on the function of cardiac voltage-gated ion channels, by using the whole-cell patch-clamp technique. In addition, we also tested the ibogaine derivative 18-methoxycoronaridine (18-MC), which is considered less toxic.

Results: We found that currents through human ERG (hERG) potassium channels, heterologously expressed in tsA201 cells, were inhibited by ibogaine in low micromolar concentrations (IC_{50} : 3 μ M). In addition, ibogaine significantly altered the hERG channel gating properties. The IC_{50} of hERG current inhibition by 18-MC was 15 μ M. Heterologously expressed human $Na_v1.5$ sodium channels were also affected by ibogaine. For sodium current inhibition about 25-fold higher ibogaine concentrations were needed than for hERG. Finally, experiments on isolated adult mouse cardiomyocytes showed that ibogaine also affects currents through voltage-gated ion channels in their native environment.

Conclusions: Because the ibogaine concentrations in animal and human plasma after ibogaine uptake reach low micromolar concentrations which impair the function of cardiac ion channels, the drug must be considered a potential cardiac arrhythmia risk.

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A2

Neuropeptide Y Y₂ receptors modulate trace fear conditioning and spatial memory in the dorsal hippocampus

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Background: Neuropeptide Y (NPY), a highly conserved 36 amino acid peptide is widely distributed in the central nervous system. Besides its functions in various metabolic processes NPY has attracted considerable attention in modulating emotional-affective behavior. NPY exerts a pronounced anxiolytic effect most likely mediated by Y₁ receptors, whereas stimulation of predominantly pre-synaptic Y₂ receptors results in increased anxiety. The role of NPY Y₂ receptors in the processing of emotional learning, however, remains still elusive.

Methods: The current study aims to investigate the role of NPY Y₂ receptors in Pavlovian fear conditioning, a simple form of associative learning and in a spatial memory task, the Barnes maze. Y₂-KO mice were subjected to delay (amygdala-dependent) and trace (hippocampus-dependent) fear conditioning paradigms.

Results: While in delay fear conditioning Y₂-KO mice performed similar to wild-type controls, recall of a trace fear memory was significantly increased in Y₂-KO mice. Furthermore, Y₂-KO mice exhibited an improved long-term memory in the Barnes maze test, a paradigm investigating spatial learning. Trace fear conditioning and spatial memory are predominantly mediated by the dorsal hippocampus. For investigating the specific contribution of Y₂ receptors in the adult dorsal hippocampus in trace fear conditioning and spatial memory formation we locally deleted hippocampal Y₂ receptors in conditional Y₂-KO mice by injection of a rAAV-CreGFP vector. Moreover we over-expressed NPY₃₋₃₆, an Y₂ receptor preferring agonist, at the same brain sites.

Conclusions: Our data indicate that while Y₂ receptors are not involved in amygdala-dependent delay fear conditioning, they seem to play an inhibitory role on the acquisition of trace fear memories. Moreover, Y₂ receptors in the dorsal hippocampus are crucial for spatial memory formation. These actions are probably mediated by inhibition of glutamate release in dorsal hippocampal circuitries.

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A3

Reduced fear conditioning after viral vector mediated neuropeptide Y administration into the basolateral amygdala

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BMC Pharmacology 2011, **11(Suppl 2):A3**

Background: Neuropeptide Y (NPY) is a 36-amino-acid peptide that is abundantly expressed in the central nervous system. It is involved in various physiological and pathophysiological processes, including energy homeostasis, pain and epilepsy, but also in anxiety and depression. Consistent findings have demonstrated an anxiolytic effect of NPY. The presence of different NPY receptors in the amygdala and the effects of NPY on anxiety raise the question, whether NPY and its receptors may influence acquisition and extinction of conditioned fear. Therefore, we investigated NPY and NPY receptor knockout mice in Pavlovian fear conditioning.

Methods: Pavlovian fear conditioning is a simple form of associative learning. NPY knockout (NPY-KO) mice as well as Y receptor knockout mice (Y₁, Y₂, Y₄ and Y₁/Y₂ double KO) were subjected to a discriminative delay fear-conditioning paradigm. Extinction learning was performed the following day by repetitive exposure to the tone in the absence of a foot shock.

Results: In cued fear conditioning NPY-KO mice acquire higher freezing levels and show increased expression and impaired extinction of conditioned fear. Y₁-KO mice show faster conditioning and delayed extinction, whereas Y₂-KO mice are similar to wildtype mice. Compared to Y₁-KO mice, however, Y₁/Y₂ double KO mice exhibited enhanced fear acquisition and impaired between session extinction, indicating an important role of Y₂ receptors in these processes. Interestingly, Y₄-KO mice show normal fear conditioning but impaired extinction. Adeno-associated viral (AAV) vector-mediated over-expression of NPY in the basolateral amygdala (BLA) of NPY-KO mice significantly reduced the increased fear acquisition of NPY-KO mice. In addition, extinction was significantly improved after AAV-induced over-expression of recombinant NPY (rNPY) in the BLA of NPY-KO mice. No change was observed, however, after over-expression of rNPY in the central amygdala.

Conclusions: Our data indicate that NPY has an inhibitory role in the acquisition and facilitates extinction of conditioned fear. These effects seem to be mediated predominantly in the BLA. In particular, the Y₁ receptor may modulate the acquisition of fear, whereas for extinction a concerted action of Y₁ and Y₄ receptors seems to be conceivable.

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A4

Efficacy of systemic HS-198, an analogue of oxymorphone, on cancer pain-related behaviour in mice

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Background: Cancer pain is a significant clinical problem being one of the first symptoms of disease with 75–90% of the patients experiencing chronic pain syndromes in advanced stages [1]. The management of cancer pain is mainly based on the use of opioid drugs; however their clinical use is limited by high incidence of adverse effects. There is a continued search for highly efficacious opioid analgesics with reduced complications and improved patient compliance. An analogue of the clinically used

oxymorphone, 5-methyl-substituted 14-O-methyloxymorphone (HS-198), is a selective μ opioid agonist and a potent antinociceptive agent in animal models of nociceptive and inflammatory pain, while exhibiting a favourable dissociation between analgesia and the occurrence of side effects [2]. We report data on efficacy of this opioid agonist after subcutaneous administration (s.c.) in a murine model of cancer pain. The opioid receptor-mechanistic basis of the antinociceptive action was also investigated.

Methods: Cancer pain was induced in C57BL/6J mice by s.c. implantation of lung carcinoma cells, in the plantar and dorsal side of the right hindpaw [3]. Mechanical sensitivity was determined using von Frey monofilaments. Heat sensitivity was assessed using the Hargreaves test. *In vitro* biological activities were evaluated using binding and functional assays.

Results: On day 9 post-inoculation, s.c. HS-198 produced a dose-dependent inhibition with significant effects in attenuating cancer pain-related behaviour (thermal and mechanical hypersensitivity) on the tumour side. Pre-treatment with the opioid receptor antagonist naloxone reversed the antinociceptive effects induced by HS-198 in mice with cancer-induced pain. *In vitro*, HS-198 showed high affinity and selectivity for both mouse and rat μ opioid receptors, and it displayed potent μ -agonism through inhibition of G proteins.

Conclusions: Systemic s.c. administration of the μ opioid receptor agonist HS-198 induces potent antinociceptive effects in mice with cancer pain via opioid receptor-specific mechanisms.

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A5

Biological, pharmacological and immunological activities of novel 6-amino-acid-substituted 14-alkoxy-N-methylmorphinans

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BMC Pharmacology 2011, **11(Suppl 2):A5**

Background: Of the three opioid receptors, μ (MOP), δ (DOP) and κ (KOP), the MOP type is the most involved in the action of opioids in the gut. Experimental studies on models of intestinal inflammation and inflammatory bowel disease (IBD) support an anti-inflammatory role of peripheral MOP receptors in the gut, besides their involvement in pain control (analgesia) and gastrointestinal motility (anti-diarrheal effects). Research focuses increasingly on exploring the therapeutic potential of peripheral MOP receptors aiming for identification of peripheral ligands as improved treatment for debilitating conditions associated with bowel functions. One strategy to increase peripheral selectivity includes chemical modifications that enhance hydrophilicity [1,2]. Our work in the field of peripherally acting opioids has led to a series of opioids with zwitterionic moieties (i.e. amino acid residues) attached to the C-6 position of 14-O-methyloxymorphone, which may represent novel therapeutic molecules for IBD. These 14-alkoxymorphinans were pharmacologically and immunologically characterized.

Methods: Synthesis of novel zwitterionic 14-alkoxymorphinans was accomplished by multi-step syntheses. Binding, functional and immunomodulatory activities were determined *in vitro*. Antinociceptive activities were assessed using acetic acid-induced writhing and tail-flick tests. Physicochemical properties (logP and logD) were determined using the MarvinSketch software.

Results: *In vitro*, the new 6-amino-acid-substituted 14-alkoxymorphinans bound with high affinity and showed agonist activity towards the MOP receptor. They significantly inhibited the nuclear transcription factor kappaB (NF- κ B) activation in tumor-necrosis factor- α (TNF- α) and

lipopolysaccharide (LPS)-stimulated human monocytic THP-1 cells. *In vivo*, they produced dose-dependent antinociceptive effects in mice after subcutaneous administration, being several-fold more potent than morphine. Based on the calculated logP and logD values, an increase of hydrophilicity, and thus peripheral selectivity can be achieved by attachment of amino acid residues to the morphinan skeleton.

Conclusions: Novel MOP agonists acting in the periphery with combined immunosuppressive and analgesic properties may provide a new approach for the treatment of IBD.

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A6

Treprostinil stimulates the engraftment of haematopoietic stem cells

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Background: Successful transplantation of haematopoietic stem cells (HSC) is often limited by low transplantation efficiency. This may be enhanced by pharmacological means. In fact, HSCs require a $G\alpha_s$ -transduced signal to repopulate the bone marrow [1]. Pretreatment with prostaglandin E_2 (PGE_2) enhances engraftment via activation of $G\alpha_s$ -coupled EP_2 and EP_4 receptors [2]. Treprostinil is a stable analogue of prostacyclin/ PGI_2 . It predominantly acts via EP_2 and EP_4 receptors. Treprostinil is approved for treatment of pulmonary hypertension. Here we test the hypothesis that treprostinil may also be useful to promote engraftment of HSC.

Methods: Generation of murine bone marrow-derived HSCs: Undifferentiated HSCs (lineage-negative, Lin^- cells) were separated from bone marrow cells by MACS (magnetic-assisted cell sorting) and characterized by fluorescence-activated cell sorting (FACS) using cell surface markers. [3H]cAMP-accumulation assays: Lin^- cells were incubated in supplemented stem cell medium (StemSpanSFEM#09650). After 4 h at 37°C cells were stimulated with forskolin, treprostinil, other prostanoids and cholera toxin for 1 h. Bone marrow transplantation: Recipient mice were lethally irradiated. Lin^- cells were pretreated in absence/presence of 10 μM treprostinil, treprostinil plus 30 μM forskolin or 10 $\mu g/mL$ cholera toxin for 1 h at 37°C. 3×10^5 cells/mouse were injected via the tail vein. Transplantation efficiency was determined by the analysis of white blood cell counts. For competition assay, equal numbers of treated/untreated Lin^- cells, which can be distinguished according to surface expression of Ly5.1 and Ly5.2, were injected in one and the same recipient mouse.

Results: Successful MACS-purification of Lin^- cells was documented by FACS. Next, the cAMP-response of Lin^- cells to treprostinil was tested: Treprostinil elicited a concentration-dependent accumulation of cAMP in the range of 0.1–10 μM with an estimated EC_{50} in the range of 0.3 μM . A beneficial effect was also observed *in vivo*: mice injected with treprostinil-pretreated Lin^- cells had significantly higher levels of circulating white blood cells when compared to those receiving vehicle-treated Lin^- cells ($p < 0.05$, unpaired t test). In addition, when pretreated and untreated Lin^- cells were mixed to compete for bone marrow reconstitution, the white blood cells derived from the pretreated Lin^- cell population were 1.5–3-fold more abundant than those originating from the non-treated HSC.

Conclusions: The treprostinil-induced cAMP elevation translates into enhanced engraftment of haematopoietic stem cells. Because treprostinil is reasonably well tolerated, it may be of interest to explore its action in bone marrow transplantation in people.

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A7

PI3K δ is indispensable for CTL-mediated cytotoxicity

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Background: The expression of catalytic phosphoinositol-3-kinase isoform δ (PI3K δ) is restricted to the haematopoietic compartment. Accordingly, PI3K δ serves as a drug target to eliminate leukaemic cells. However, we previously showed that PI3K δ is indispensable for the function of natural killer (NK)-cells [1]. Thus, the therapeutic success of PI3K δ inhibitors is likely to be compromised by unintended side effects on the immune system. Besides NK-cells, CD8⁺ cytotoxic T-cells (CTLs) are well-known key players in natural host response against developing tumours and viral infections. In this study, we examine the role of PI3K δ for CTL function and CTL-mediated tumour surveillance.

Methods: PI3K $\delta^{-/-}$ animals have been described in [2]. Flow cytometric lymphocyte characterization, the *in vivo* CTL-assay and MC-38 tumour model were done as outlined in [3]. Membrane capacitance and degranulation were measured by patch-clamp recordings and flow cytometry, respectively [1]; the mixed lymphocyte reaction was monitored as in [4]. *In vitro*, expanded CTLs were generated by stimulation with an anti-CD3 ϵ antibody (0.5 $\mu g/\mu L$; BDP Pharmingen) and cultured for 3 days in T-cell medium containing 100 U/mL IL-2 prior to FACS analysis. For the *in vitro* cytotoxicity assay mice were immunized twice with the peptide SINFEKL. Peptide-reactive T-cells were generated by co-culturing splenocytes derived from immunized and control mice with SINFEKL-pulsed, irradiated splenocytes for 5 days. Peptide-reactive T-cells were co-cultured with CFSE-stained EL4 or EG7 cells in different effector:target-ratios. After 18 h peptide-specific killing was quantified via FACS.

Results: Antigen-specific cytotoxicity of PI3K $\delta^{-/-}$ CTLs was significantly reduced *in vivo* and *in vitro* as compared to wild-type. This defect translated into severely impaired CTL-mediated MC-38 tumour surveillance: tumours derived from PI3K $\delta^{-/-}$ recipients were significantly bigger. PI3K δ was required for full activation of CTLs and interfered with essential stages in the canonical killing pathway of CTL, e.g. with the endowment of their lytic machinery with key cytolytic molecules, with the production of interferon- γ and the fusion of lytic granules with the cellular membrane.

Conclusions: Our findings are of particular interest for the clinical development, because specific inhibitors of PI3K δ are entering clinical trials. Our observation shows that PI3K δ is indispensable for CTL effector functions. Accordingly, long-term drug safety monitoring ought to include adequate measures to identify side effects resulting from impaired surveillance of viral infections and tumour cells.

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A8

Pharmacoepidemiological aspects of outpatient pain medication

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Background: In Austria, 98% of the population is covered by (statutory) health insurance. The HVB (Main Association of Austrian Social Security Institutions) publishes the Reimbursement Code (EKO) for outpatient medicines, and if new medicines are submitted for inclusion, these are subjected to pharmacological, medical/therapeutic and health economic evaluation. Providing modern and comprehensive medical care is inevitably and causally linked to a constant rise in health care costs. Treatment of pain has a unique position in every health care system, because it is associated with many diseases of varying severity. Pain patients are confronted both with severe and disabling physical as well as psychological burdens.

Methods: Data were provided by the internal data warehouse of the HVB, which houses reimbursement data for ambulatory care from 2001 to 2009. In this study we concentrated on the analysis of prescribed DDDs (Defined Daily Doses). Pain medication (as available in the EKO) was grouped according to the WHO defined scheme for treatment of pain (1986) into 3 groups. Aims: First, we aimed to discover changes in prescribing habits. Next, we hoped to learn if and how either greater availability or the restriction of pain medication (e.g. the introduction of generics, the waiving of prescription fees, published severe safety concerns etc.) influence the rate of utilization of pain medication.

Results: We noticed a difference in the development of the 3 groups: prescribed DDDs of pain medication attributable to WHO stage 1 and 2 show a slight increase from 2003 to 2009 (19% and 22%, respectively). This is followed by a divergent development in the next period: for WHO stage 1, a decrease of prescribed DDDs was noted (-4.6%), whereas there was no marked change for WHO stage 2 (-0.3%). In clear contrast, there was a continuous and significant increase in prescribed DDDs of WHO stage 3 medications, with DDDs almost doubling. To summarize (2003 to 2010): WHO stage 1: +14% (127.5 mil. DDDs), WHO stage 2: +21% (13.8 mil. DDDs), WHO stage 3: +144% (13.4 mil. DDDs).

Conclusions: So far we can say that the analysis of prescribed DDDs and their allocation into 3 groups – as defined by the WHO scheme for pain treatment – is a useful tool to assess changes in prescribing habits. Next we will try to find out if it can be deduced from our data i) if and how prescribers follow current guidelines, ii) if safety concerns or iii) the introduction of innovative therapies are translated into changes in prescribing habits.

A9

Morphological characterization of large intercalated neurons provides novel insight on intrinsic networks of the amygdala

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Background: Although extinction-based therapies are effective treatments for anxiety disorders, the neural bases of fear extinction remain still largely

unclear. Recent evidence suggests that the intercalated cell masses of the amygdala (ITCs) are critical structures for fear expression and extinction. They consist of clusters of densely packed medium spiny GABAergic neurons surrounding the basolateral amygdaloid complex (BLA). Five percent of ITC neurons are large cells mostly present near the cluster borders. So far, no information is available regarding the neurochemical features, afferents and efferents of large ITC cells, preventing any elucidation of their functional role. Only recently we discovered that large ITC neurons display immunoreactivity for either neurokinin 1 or metabotropic glutamate 1 α (mGlu1 α) receptors. We also found that dendrites of these neurons receive inhibitory inputs from medial capsular projecting ITC cells [1]. The aim of our study consists in the characterization of the morphological features, as well as the afferent and efferent connectivity, of large ITC neurons in order to further clarify their potential participation in the neuronal processes underlying fear extinction.

Methods: The neurochemical phenotype of large ITC neurons and their afferent connectivity were investigated by confocal and pre-embedding electron microscopy performed on both rat amygdala slices and on three large mGlu1 α -positive ITC neurons, recorded and filled with neurobiotin in rat by means of the juxtacellular technique *in vivo*. In addition, by NeuroLucida, we could reconstruct the full dendritic and axonal arborization of one large filled ITC neuron.

Results: Immunofluorescence analysis demonstrated that large ITC mGlu1 α -positive neurons express the α 1 subunit of GABA_A receptors and the calcium-binding protein parvalbumin. The dendrites of these large ITC neurons were decorated by axon terminals enriched in presynaptic mGlu7 and/or mGlu8 receptors which, as shown by electron microscopy analysis, established both excitatory and inhibitory synapses. The full tridimensional reconstruction of one *in vivo*-recorded large ITC neuron showed a very wide axonal arborization predominantly innervating the BLA but also extending, rostrally, to the dorsal endopiriform nucleus and, caudally, to the entorhinal cortex.

Conclusions: These findings elucidate for the first time some of the key anatomical features of the large ITC neurons and shed new light on intrinsic microcircuits of the amygdala containing both pre- and post-synaptic mGlu receptors. Pharmacological manipulation of these receptors may thus influence extinction of fear conditioning and represent a new therapeutic avenue for anxiety disorders.

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A10

Activation of kappa opioid receptors reduces EEG seizure activity in a mouse model of temporal lobe epilepsy

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Background: Neuropsychiatric disorders are one of the main challenges of human medicine with epilepsy as one of the most common and serious disorders of the brain. Temporal lobe epilepsy represents the most common type of epilepsies and is often accompanied by marked neuronal degeneration. One main factor that causes neural loss is the excitotoxicity of glutamate, which is copiously released during seizures and hypoxia accompanying seizures. There is evidence that endogenous opioids, namely dynorphin (Dyn), act as modulators of neuronal excitability. It was also shown that the deletion of proDyn in mice and low expression in humans is associated with increased epilepsy vulnerability. Dyn targets opioid receptors and in particular the κ opioid receptor (KOP). The KOP receptors in the hippocampal formation are located in very strategically points for the control of the glutamate release and most important they are not altered under epileptic conditions. Interestingly, proDyn expression is reduced after an initial increase in most epilepsy models and activation of KOP receptors may be neuroprotective. Still, the functional background

of these neuroprotective effects is not fully understood. The aim of this study was to investigate the influence of KOP agonists and antagonists on EEG patterns of epileptic mice.

Methods: Kainic acid (KA; 3 nmol in 50 nL saline) was injected unilaterally into the dorsal hippocampus, causing acute and delayed behavioral and EEG effects. Four-channel EEG traces were recorded from ipsi- and contralateral hippocampi and motorcortices applying depth and surface electrodes, respectively. The KOP-specific agonist U-50,488H and antagonist GNTI were dissolved in saline (adjusted to pH 7.4) and applied i.p. or intracisternally, respectively.

Results: Sharp waves and paroxysmal discharges in the ipsilateral hippocampus were recorded about 2 weeks after KA injection. Paroxysmal discharges were accompanied by behavioral arrest and stereotypic behaviour like head nodding. Application of KOP agonists at different doses (2, 5, 10 mg/kg) markedly reduced paroxysmal discharges. In contrast, the KOP antagonist (3 nmol) prolonged the duration of such discharges. These data represent observations from preliminary experiments.

Conclusion: Data collected so far confirm the anticonvulsant action of KOP agonists in the subchronical phase of epilepsy, suggesting that neuroprotective effects are indeed due to reduced seizure activity.

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A11

Structural determinants of $\text{Ca}_v1.3$ L-type calcium channel gating

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Background: $\text{Ca}_v1.3$ channels, which belong to the family of voltage-gated L-type calcium channels (LTCCs), are involved in important physiological (e.g. hearing, hormone release and cardiac and neuronal pace making) and pathophysiological functions (e.g. Parkinson's disease). We have recently discovered that an intramolecular protein interaction within the C-terminus of $\text{Ca}_v1.3$ $\alpha 1$ subunits fine-tunes $\text{Ca}_v1.3$ channel function. This C-terminal modulatory mechanism (CTM) is present in the long ($\text{Ca}_v1.3_l$) but is absent in the short ($\text{Ca}_v1.3_{42A}$) splice variant. Its absence induces activation at a more negative voltage range and increases Ca^{2+} -dependent inactivation (CDI). Interestingly a functional CTM is present in the human [1] and rat $\text{Ca}_v1.3$ $\alpha 1$ subunit isolated from pancreatic islets (D38101, $\text{rCa}_v1.3_{pan}$) but not in a rat $\text{Ca}_v1.3$ $\alpha 1$ subunit cDNA clone isolated from superior cervical ganglion (scg) (AF370010; $\text{rCa}_v1.3_{scg}$). This causes substantial differences in the voltage- and Ca^{2+} -dependent gating of scg and pan.

Methods: We systematically compared scg and pan $\text{Ca}_v1.3$ $\alpha 1$ subunits by expression in tsA201 cells and analysis of their functional properties using the whole-cell patch-clamp technique, to determine the structural basis for this difference.

Results: $\text{rCa}_v1.3_{scg}$ differs from $\text{rCa}_v1.3_{pan}$ at three amino acid positions (S244G, V1104A, A2073V) and one alternatively spliced locus (absence of exon 31). Alternative splicing did not explain the functional differences between the two $\text{rCa}_v1.3$ $\alpha 1$ subunits. The amino acid difference A2073V is located within the recently identified distal part (DCRD) of a C-terminal modulatory domain. Mutation of A2073 in $\text{rCa}_v1.3_{scg}$ to the corresponding valine (A2073V) in $\text{rCa}_v1.3_{pan}$ fully restores the slower CDI of $\text{rCa}_v1.3_{pan}$. In contrast, A2073V only weakly affected the activation voltage range (rescue of only 5.3 mV of the 17.2 mV difference in the half-maximal voltage activation range ($V_{1/2}$)). Additional mutation of S244 to G in the $\text{rCa}_v1.3_{scg}$ S4-S5 linker of domain I caused a further shift to a more positive voltage close to the V_h of $\text{rCa}_v1.3_{pan}$.

Conclusions: Our data identify residues at proposed interfaces between voltage sensors and the intracellular channel gate controlling the voltage-dependence of $\text{Ca}_v1.3$ activation. We also show that the DCRD domain can moderate CDI independently of its effect on V_h , suggesting that these processes occur through different DCRD-dependent mechanisms.

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A12

Timothy mutation affects tightly sealing point of $\text{Ca}_v1.2$ activation gate

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Background: The Timothy syndrome (TS) mutations G402S and G406R abolish inactivation of $\text{Ca}_v1.2$ and cause multiorgan dysfunction and lethal arrhythmias.

Methods: In order to gain insights into the consequences of the G402S mutation on structure and function of the channel, we systematically mutated the corresponding G432 and the homologous S6 positions of the other three domains of the rabbit channel and applied homology modeling. **Results:** Homology modeling revealed that G432 forms part of a highly conserved structure motif (G/A/G/A) of small residues in homologous positions of all four domains (G432 (IS6), A780 (IIS6), G1193 (IIIS6), A1503 (IVS6)). In contrast, corresponding mutations in domains II, III and IV induced parallel shifts of activation and inactivation curves indicating a preserved coupling between both processes. Disruption between coupling of activation and inactivation was specific for mutations of G432 in domain I. Mutations of G432 removed inactivation irrespective of the changes in activation. In all four domains residues G/A/G/A are in close contact with larger bulky amino acids from neighboring S6 helices.

Conclusions: These interactions apparently provide adhesion points thereby tightly sealing the activation gate of $\text{Ca}_v1.2$ in the closed state. Such a structural hypothesis is supported by changes in activation gating induced by mutations of the G/A/G/A residues.

A13

Development of novel N-methyl and N-allyl-substituted oxazolomorphinans and their interaction with opioid receptors

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Background: The need for opioid analgesics with reduced undesirable side-effects has initiated a vast amount of scientific efforts, which have led to a number of new opioid ligands and significant expansion of knowledge in opioid pharmacology. The development of morphinans annelated with heterocycles gave rise to several potential therapeutic agents and useful pharmacological tools.

Methods: The chemistry involved the design and synthesis of two sets of oxazolomorphinans having the new heteroring annelated to the A-ring of the morphinan backbone. Binding affinities of the newly synthesized compounds at opioid receptors were determined by *in vitro* competition binding assays using rat brain (μ , δ) and guinea pig brain (κ) membranes and employing [³H]DAMGO (μ), [³H][Ile^{5,6}]deltorphin II (δ) and [³H]U-69,593 (κ) as specific opioid radioligands. The *in vitro* pharmacological activities were established using [³⁵S]GTP γ S functional assays in membranes from Chinese hamster ovary (CHO) cells expressing human opioid receptors.

Results: Binding studies on the newly synthesized N-methyl and N-allyl derivatives to opioid receptors revealed remarkable results for three compounds: the amino-substituted N-methylloxazolomorphinan showed high affinity and selectivity to the μ opioid receptor, while two N-allyloxazolomorphinans were found to interact with high affinity with μ and κ sites and moderate binding towards δ receptors. In ligand-stimulated [³⁵S]GTP γ S binding studies, the N-methyl congener acted as a potent and

full agonist at the μ receptor. The two *N*-allyl derivatives showed antagonistic effects at μ and κ receptors.

Conclusions: The design and synthesis of novel oxazolomorphinans led to an interesting alteration in opioid activity by influencing the biological and pharmacological profile of these compounds interacting with μ , δ and κ opioid receptors.

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A14

Bacterial peptidoglycan enhances sickness behaviour induced by bacterial lipopolysaccharide

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Background: Lipopolysaccharide (LPS) and peptidoglycan are microbial products recognized by Toll-like receptor-4 (TLR4) and nucleotide-binding oligomerization domain 1 (NOD1) and NOD2, respectively. LPS has been found to cause behavioural alterations indicative of sickness and depressed mood. The effect of peptidoglycan on exploratory and affective behaviour has not yet been explored, although it has been reported that it promotes sleep and anorexia. Since NOD1 and NOD2 are activated by two different peptidoglycan components, MurNAc-L-Ala- γ -D-Glu-meso-diaminopimelic acid (M-TriDAP) and muramyl dipeptide (MDP), respectively, the effects of both compounds, alone and in combination with LPS, on exploratory and affective behaviour were investigated.

Methods: Female C57BL/6 mice received intraperitoneal injections of M-TriDAP (100 μ g/mouse), MDP (10 mg/kg) or sterile saline (0.9% NaCl) and additional injections of LPS (0.83 mg/kg) or sterile saline 4 hours after the first injection. The weight of the animals was monitored throughout the study. Exploratory and anxiety-like behaviour was evaluated with the elevated plus-maze test (EPM) 1 day after treatment, while depression-related (stress coping) behaviour was assessed with the forced swim test (FST) 1 week after treatment.

Results: Mice receiving sterile saline plus LPS, M-TriDAP plus LPS and MDP plus LPS, respectively, lost body weight by 12% during the first day after treatment, which was reversed 1 week after treatment. When given alone, M-TriDAP and MDP failed to change the body weight. Likewise, M-TriDAP and MDP alone did not alter behaviour on the EPM as tested 1 day after treatment. LPS alone decreased exploratory behaviour in the EPM as displayed by a nonsignificant reduction of the total travelling distance and number of arm entries. When given in combination with LPS, especially MDP enhanced the effect of LPS. Specifically, the decrease of the total travelling distance and the total number of arm entries reached statistical significance. When tested in the FST, the animals tended to exhibit increased immobility and decreased activity (swimming and climbing), an effect that was again most pronounced in the MDP plus LPS group. However, this effect was not statistically significant.

Conclusions: The loss of body weight and decrease in exploratory behaviour following administration of bacterial immune stimulants reflect sickness behaviour associated with infection. The present results reveal that MDP, a NOD2 agonist, enhances the sickness behaviour caused by LPS. This observation indicates that the brain response to peripheral immune activation depends on both TLR4 and NOD2.

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A15

Identification of novel ligands interacting with kappa opioid receptors

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Background: The κ opioid (KOP) receptor belongs to the family of seven transmembrane G protein-coupled receptors (GPCRs) and it plays a significant role in a broad range of physiological functions. Stimulation of the KOP receptor results in analgesic actions, and KOP agonists appear to have some advantages over the μ opioid (MOP) receptor agonists. Inhibiting KOP receptors is proposed to be useful for treating addiction and stress-related conditions, such as depression and anxiety. The pharmacology of currently available KOP antagonists shows a delay in onset of action and an extremely long duration of action *in vivo*, which might limit their therapeutic application. The search for new ligands with potent biological activities, particularly as potential novel therapeutic agents, utilizing computational and synthetic approaches, is a key goal of life science research and drug development. Herein, we present the *in silico*, *in vitro* and *in vivo* profiles of new molecular scaffolds as novel KOP receptor ligands.

Methods: LigandScout and Catalyst softwares were used to generate and validate a merged feature ligand-based 3D pharmacophore model for KOP receptors. Biological activities were evaluated in *in vitro* opioid receptor binding and [³⁵S]GTP γ S functional assays. Antinociceptive properties were assessed in mice using the writhing test.

Results: The integrated computational screening strategy has led to the discovery of sewarine as KOP receptor ligand. This phenolic alkaloid from the plant *Rhazya stricta* binds with high selectivity to the KOP receptor and shows antagonist activity. A comprehensive SAR analysis on several analogues was pursued and primary chemical features responsible for KOP activity have been identified. Combining synthetic and pharmacological methodologies, two phenolic molecules were identified as novel highly selective ligands interacting with KOP receptors, and displaying full agonist or partial agonist properties, respectively. Dose-dependent and nor-BNI-sensitive antinociceptive effects were produced by the KOP agonist after subcutaneous administration to mice, exhibiting a potency comparable to that of U-50,488.

Conclusions: This study uncovers new classes of ligands interacting with KOP receptors and sharpens the understanding of ligand-receptor interactions, thus increasing the chance of developing useful clinical agents among KOP ligands.

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A16

Circular plant peptides as templates for GPCR drug discovery?

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Background: Cyclotides are disulfide-rich miniproteins with the unique structural features of a circular backbone and knotted arrangement of three conserved disulfide bonds. These features make them exceptionally stable and they have applications as plant defense (insecticidal) agents and stable drug frameworks. So far they have been found mainly in two plant families, including in every species of the violet family (*Violaceae*) so far examined, and in a few species of the coffee family (*Rubiaceae*).

Methods: We optimized the cyclotide screening protocol using MALDI-TOF/TOF and two dimensional nano LC-MS/MS followed by manual and automated peptide sequence assignments. Biological analysis was carried out by collagen uterine contractility assays and pharmacological data were generated by competitive displacement binding and functional GPCR luciferase assays.

Results: Using the rapid cyclotide discovery workflow, we analyzed >300 flowering plant species and confirmed the presence of cyclotides in several plant families. On the basis of the phylogeny of cyclotide-bearing plants and the analysis of precursor gene sequences, we have refined the current hypothesis of the evolution of circular plant peptides. Based on the traditional usage of the plant *Oldenlandia affinis* as an uterotonic agent, we have tested and identified cyclotides with oxytocin-like activity and identified the oxytocin receptor, as representative member of the GPCR family, as a molecular target of cyclotides.

Conclusions: Cyclotides are interesting targets for pharmaceutical applications due to their unique structural framework, their bioactivities and sequence diversity. Based on their predicted number of tens of thousands, which potentially makes them one of the largest protein families within the plant kingdom, they constitute an immense combinatorial library of natural peptides, which is accessible for drug discovery.

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A17

Neurodegeneration and histochemical plasticity in the rat subiculum after kainic acid-induced epilepsy

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Background: The subiculum, the main output region of the hippocampus, remains largely preserved in human temporal lobe epilepsy (TLE) and therefore may be importantly involved in the generation of epileptic activity arising from the hippocampal region. Our goal was to characterize histopathological and neurochemical changes in the rat subiculum using the kainic acid (KA) model of TLE and to correlate these alterations with data from EEG-recordings.

Methods: Rats (n = 35) were implanted with biopotential transmitters (EA-F20, DS1) for continuous EEG/video-monitoring. One week later, they were injected with KA (i.p., 10 mg/kg) and developed seizures and an initial status epilepticus (SE). Rats were killed 1, 8, 30 or 90 days after the initial SE and brain histopathology was investigated using immunohistochemistry and *in situ* hybridization for neuropeptides and calcium-binding proteins as markers for different neuronal subpopulations.

Results: Rats developed spontaneous seizures 3 to 36 days (15 ± 1.5 d) after the initial SE. Neurodegeneration and reactive gliosis were more pronounced in the proximal than in the distal part of the subiculum. The number of parvalbumin (PV)-ir GABAergic interneurons was significantly reduced in the pyramidal cell layer of the subiculum already 24 hrs after KA injection. The decrease in the number of PV-positive neurons in the subiculum correlated with the number of spontaneous seizures subsequently experienced by the rats. Increased (or even *de novo*) expression of neurokinin B (NKB) and NPY mRNA was observed in pyramidal neurons of the subiculum, and fiber labeling for NKB and NPY was increased at late intervals after SE.

Conclusions: Early degeneration of PV-ir GABAergic basket- and axo-axonic cells may result in decreased inhibition of pyramidal neurons and affects the numbers of spontaneous seizures occurring later. Since NKB has an excitatory action (activating phospholipase C) on NK₃ receptors, expression of the peptide in principal neurons of the subiculum may contribute to the generation of epileptic seizures. On the other hand, ectopic expression and subsequent release of NPY from axon terminals of pyramidal neurons may inhibit glutamate release by activating presynaptically located Y₂ receptors and thus exert an anticonvulsive action.

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A18

Novel pyrazole inhibitors for discrimination between receptor-operated and store-operated Ca²⁺ entry

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Background: Calcium governs a wide range of cellular processes. Specifically, control of gene transcription involves Ca²⁺ entry channels that are activated by either voltage, second messengers or depletion of intracellular stores. The family of classical transient receptor potential channels (TRPC) has been implicated in both the receptor/second

messenger as well as in store-operated Ca²⁺ entry pathway, and represents an attractive target for therapeutic intervention.

Methods: We tested a series of pyrazol compound structurally related to Pyr3 [1], a recently discovered TRPC3 inhibitor, for effects on receptor- as well as store-operated Ca²⁺ entry into RBL-2H3 mast cells and HEK293 cells overexpressing TRPC3.

Results and conclusions: We identified novel Ca²⁺ entry inhibitors, which are able to discriminate between the two tightly related pathways of receptor/second messenger-activated and store-operated calcium entry. These compounds appear suitable for selective modulation of Ca²⁺-dependent gene transcription in a variety of mammalian cells.

Acknowledgements: Supported by the Austrian Science Fund FWF project P21925-B19 and P21118-B09.

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A19

Amphetamine actions rely on the availability of phosphatidylinositol-4,5-bisphosphate

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BMC Pharmacology 2011, **11(Suppl 2)**:A19

Background: Neuronal functions, such as excitability or endo- and exocytosis, require phosphatidylinositol-4,5-bisphosphate (PIP₂) since ion channels and other proteins involved in these processes are regulated by PIP₂. Monoamine transporters control neurotransmission by removing monoamines from the extracellular space. They also display channel properties, but their regulation by PIP₂ has not been reported. The psychostimulant amphetamine acts on monoamine transporters to stimulate transporter-mediated currents and efflux and thereby increases the levels of extracellular monoamines.

Methods and results: Direct or receptor-mediated activation of phospholipase C (PLC) reduced membrane PIP₂ and amphetamine-evoked currents through recombinant serotonin transporters; extracellular application of a PIP₂-scavenging peptide mimicked this effect. PLC activation also diminished amphetamine-induced reverse transport without altering transmitter uptake. Inhibition of reverse transport by PLC activation was also observed in brain slices and with recombinant dopamine and noradrenaline, but not GABA transporters; rises in intracellular Ca²⁺ or activation of protein kinase C were not involved in these effects.

Conclusions: These data demonstrate for the first time PIP₂ dependence of reverse transport and current in monoamine transporters.

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A20

Truncations in the amino terminus reveal a region key to supporting amphetamine-induced efflux by the human serotonin transporter

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Background: The serotonin transporter (SERT) terminates neurotransmission via reuptake of serotonin from the synaptic cleft. Upon stimulation with amphetamines, SERT switches into an outward transport mode to rapidly release serotonin. We have previously shown that truncation of the first 64

residues of SERT amino terminus leads to loss of amphetamine-induced efflux [1]. This was comparable to the effects of a single point mutation of a juxtamembrane threonine residue at position 81 [1].

Methods: Truncation mutants of SERT amino terminus were generated by removing 22 ($\Delta 22$ -SERT), 32 ($\Delta 32$ -SERT) or 42 ($\Delta 42$ -SERT) amino terminal residues. In addition, alanine scanning mutagenesis was performed along a segment of amino acid residues 32–42. All mutants were pharmacologically characterised in uptake, binding and efflux studies.

Results: Cellular localisation of the mutants examined by confocal microscopy, revealed no differences compared to the wild-type SERT. Functional analysis showed only modest changes in their substrate uptake properties (no significant changes in the K_m values and a moderate decrease in the V_{max} value of $\Delta 42$ -SERT). Similarly, there were no marked alterations in the K_D and B_{max} values of imipramine or in the K_i values of *p*-chloroamphetamine and ibogaine, determined in radiolabelled imipramine binding assays. However, while amphetamine-induced efflux was unimpaired for $\Delta 22$ -SERT and to a slight extent decreased for $\Delta 32$ -SERT, it was completely abolished for $\Delta 42$ -SERT.

Conclusions: Our results shed new light on the functional role of the amino terminus and point to the segment encompassing residues 32–42 as a region of key importance to supporting amphetamine-induced efflux by SERT.

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A21

LRET-based intramolecular distance measurements in LeuT_{Aa}

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Background: LeuT_{Aa} is a bacterial orthologue of mammalian Solute Carrier Class 6 (SLC6) neurotransmitter transporters from *Aquifex aeolicus* which transports leucine and alanine. SLC6 transporters are of great pharmacological interests because of their crucial role in neurotransmitter clearance. These proteins are also targets of many clinically relevant drugs. The crystal structure of LeuT_{Aa} has been resolved at atomic resolution of ~1.5 Å. Although LeuT has a low overall sequence identity of about 20–25% to SLC6 members, the grade of conservation reaches around 55% in functionally critical transmembrane domains 1, 3, 6 and 8. For this very reason we are using LeuT_{Aa} as a good structural paradigm to explore the structural/functional information about SLC6-family members.

Methods and results: In order to explore structural/functional information about the SLC6 family, we initiated a study to measure intramolecular distance changes associated with substrate transport by Luminescence Resonance Energy Transfer (LRET). LRET is based on the Förster effect and relies on the non-radiative transfer of energy from a donor element to an acceptor fluorophore. We introduce Lanthanide-binding Tags (LBT) at selected positions into the LeuT to accommodate the excitable donor terbium along with cysteines, where acceptor fluorophores are attached. After expression and purification of these mutants, we measure the distances between donor and acceptor at atomic resolution. In order to screen the mutants of LeuT_{Aa} for their function, we have recently established the scintillation proximity assay (SPA). To date, we screened a number of LBT-mutants as well as cysteine-mutants for their function; several have been shown to be functional and will be tested now in the LRET setup.

Conclusion and future plan: Since we obtained functional LBT- and cysteine-mutants we are looking forward to measure distances between several terbium-bound LBTs and cysteine-bound fluorophores. We will

force the transporter into an outward- or inward-facing state and validate a 3D-model for LeuT.

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A22

The transcription factor STAT5 drives mutation and imatinib resistance in chronic myeloid leukemia via ROS production

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Background: Chronic myelogenous leukemia (CML) is a leukemic stem cell (LSC)-driven myeloproliferative disorder and is associated with a characteristic chromosomal translocation which generates a constitutively active tyrosine kinase, the BCR-ABL oncoprotein. The standard treatment therapy for CML patients is the BCR-ABL tyrosine kinase inhibitor (TKI) imatinib. It is a life-long treatment due to the fact that LSCs are resistant to TKIs. Since its introduction, imatinib has improved the 5-year survival rate up to 90%. An emerging problem is resistance to imatinib, which is mainly caused by mutations inside the BCR-ABL kinase domain, and its increasing incidence during disease progression. It has been reported that BCR-ABL drives its own mutation via upregulation of reactive oxygen species (ROS) causing oxidative DNA damage. Among the several dozens of intensively characterized mediators of BCR-ABL action, the transcription factor STAT5 is among the few ones that is critical for leukemia initiation and maintenance and it has been shown that STAT5 becomes upregulated during disease progression.

Methods and results: qPCR analysis of primary CML patient samples reveal a positive correlation of STAT5 mRNA levels and BCR-ABL mutations. Using BCR-ABL transformed murine cell lines retrovirally overexpressing STAT5A or STAT5B, we can show that STAT5 triggers ROS production leading to an increase in DNA double-strand breaks.

Conclusions: We hypothesize that STAT5 is an important mediator of imatinib resistance in CML due to its ability to drive ROS production consequently leading to BCR-ABL mutations.

A23

Statins modulate the expression profile of ATP-binding cassette transporters in human neuroblastoma cells

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Background: The development of chemoresistance still is a major problem in cancer therapy. Mainly, impact of chemotherapy is based on the upregulation of ATP-binding cassette (ABC) transporters, which correlates with bad prognosis and less chemotherapeutic success. Previously, we could show that the HMG-CoA reductase inhibitor simvastatin is able to inhibit the most prominent ABC transporter ABCB1. Additionally, we demonstrated that simvastatin coadministered with the anthracycline doxorubicin led to increased apoptosis in neuroblastoma cells, and that these effects were comparable with the potential of verapamil, a first generation inhibitor of ABCB1 [1].

Methods: The neuroblastoma cell line SH-SY5Y was used for our analyses. Alterations in the protein level of ABC transporters were demonstrated by Western blot analyses and in more detail with FACS. RNA from SH-SY5Y cells treated with simvastatin for 6 and 72 hours was isolated, and the mRNA levels of various ABC transporters were quantified by real-time PCR.

Results: Simvastatin exposure led to a concentration-dependent decrease of ABCB1. Similarly, FACS analyses demonstrated a significant decrease of ABCB1 expression on the cell surface. Moreover, doxorubicin-induced elevations in ABCB1 cell surface expression were reversed by simvastatin. However, compensation of ABCB1 by other ABC transporters like ABC1 and ABC4 could not be detected. Conversely, ABCG2 showed upregulation on protein and mRNA level.

Conclusions: Here we show that simvastatin is able to modulate ABCB1 expression. This potential seems to be divided in immediate and long-term effects. Expression of ABCB1 was downregulated on protein as well as on mRNA level after long-term incubation and partially compensated by other ABC transporters. Moreover, we also observed short-term regulation of the cell surface expression without any changes in total cellular level which might be based on impact of simvastatin in ABCB1 turn-over. Based on our findings, we suggest that simvastatin is a promising candidate as an adjuvant chemotherapeutic drug to impair transporter-mediated multidrug resistance.

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A24

Interaction of V-type ATPase inhibitors and extracellular NAADP-triggered calcium release in skeletal muscle cells

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Background: Nicotinic acid adenine dinucleotide phosphate (NAADP) has been identified as a calcium-mobilizing second messenger. NAADP is regularly enzymatically synthesized by ADP-ribosyl cyclases, in particular under acidic conditions. In nanomolar concentrations NAADP targets selectively the ryanodine receptor type 1 on the sarcoplasmic reticulum, and the two pore channels localized in dense-core secretory vesicles and lysosomes.

Methods: Confocal microscopy was used to visualize calcium signalling and lysosomal movements within undifferentiated primary human skeletal muscle cells and C2C12 cells. Apoptosis and autophagy were analyzed by FACS, caspase 3 activity and Western blot analysis.

Results: The application of extracellular NAADP to skeletal muscle cells resulted in a dose-dependent increase in cytosolic calcium transients. Within 180 seconds approximately 30% of the cells responded. The V-type ATPase inhibitors, bafilomycin A1 (Baf) and concanamycin A1 (Con), are widely used to inhibit NAADP-triggered calcium signals. However, by preventing lysosomal acidification calcium loading of these organelles is also inhibited. Accordingly, we determined calcium transients triggered by 100 nM Baf or Con. Interestingly, by the co-administration of extracellular NAADP with Baf or Con calcium transients were suppressed to basal level. The kinetics of lysosomal destruction by Baf or Con were paralleled by "cell shrinking" and acidification. Beside these short-term effects, after 24 hours exposure caspase 3 activity and pre-G1 DNA fragmentation was already observed with 50 nM Baf. Conversely, autophagy was not induced.

Conclusions: Hence, extracellular NAADP triggers calcium transients which were sensitive to Baf and Con. However, local changes in cytosolic pH and calcium concentrations may also result from lysosomal destruction induced by Baf or Con. Interestingly, longer incubation of skeletal muscle cells with Baf induced apoptosis with high potency. Thus, the application of V-type ATPase inhibitors in biological assays has to be carefully evaluated.

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A25

Simvastatin targets the IL-6 signalling cascade in human melanoma cells

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Background: In the last years the number of melanoma patients has increased markedly. High plasma levels of interleukin-6 (IL-6) are

associated with bad prognosis and reduction in overall survival of these patients. Statins, HMG-CoA reductase inhibitors, are well-tolerated therapeutics for hypercholesterolemia. We have recently shown that simvastatin triggers apoptosis in 518A2 human melanoma cells which is paralleled by concentration-dependent changes in autocrine IL-6 secretion. Here, we investigated the impact of simvastatin on the IL-6 pathway.

Methods: We investigated the expression and distribution of the heteromeric IL-6 receptor (IL-6-R/gp130) and its down-stream pathway in 518A2 human melanoma cells by FACS, Western blot and real-time PCR analysis. Furthermore, we chose a fluorescent fusion protein (STAT3-YFP) plasmid construct to study the influence of simvastatin on signalling down-stream of the IL-6 receptor.

Results: Increasing concentrations of simvastatin led to enhanced surface expression of the IL-6-R and the gp130 subunit. Cells with higher IL-6 holoreceptor expression detached, but were still viable. In Western blot analysis the precursor level of the less glycosylated gp130 subunit was enriched in simvastatin samples, which was hand in hand with enrichment of mature gp130 receptor. The mRNA levels of IL-6-R and gp130 were not regulated by the statin treatment. Stimulation with IL-6 of the STAT3-YFP-transfected 518A2 cells resulted in the formation of cytosolic density spots of STAT3-YFP, which co-localized with lysosomal markers. Simvastatin significantly delayed the accumulation of STAT3 in these dots. The suppressor of cytokine signalling (SOCS) proteins (SOCS1, SOCS3), which may play a crucial role in the inhibition of the IL-6 pathway, were not regulated by simvastatin on protein or mRNA level.

Conclusions: These data indicate that statins like simvastatin are capable of interfering with the IL-6 pathway on the heteromeric IL-6 receptor, as expression is increased on the cell surface. Furthermore, simvastatin affected the dot formation of STAT3, which occurred significantly later compared to the untreated cells. However, simvastatin treatment is not able to impair the level of the negative feed-back protein SOCS3.

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A26

Introduction of a 6-cyano group in 14-oxygenated N-methylmorphinans influences *in vitro* and *in vivo* pharmacological activities

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BMC Pharmacology 2011, **11(Suppl 2)**:A26

Background: Being a disabling symptom of many medical conditions, effective pain control is one of the most important therapeutic priorities. Morphine and other opioid drugs produce analgesia primarily through μ opioid (MOP) receptors, which mediate beneficial but also the non-beneficial actions. Appropriate identification of novel opioid analgesics may reduce complications and improve patient compliance. It was reported that hydrazones, oximes, carbazones and semicarbazone derivatives of morphinan-6-ones, e.g. dihydromorphinone or oxymorphone, exhibit high affinity at the MOP receptor [1]. Since most of these structures show high antinociceptive potency while having less pronounced side effects, it remains a promising task to convert the carbonyl group of morphinan-6-ones into various functionalities. In this study, we aimed to investigate the effect of the replacement of the 6-keto function with a 6-cyano group on *in vitro* and *in vivo* pharmacological profiles.

Methods: Binding affinities at opioid receptors were determined using competition binding assays in rodent brain membranes. *In vitro* [³⁵S]GTP γ S functional assays were performed with Chinese hamster ovary (CHO) cell membranes expressing human opioid receptors. Antinociceptive activities were assessed in mice using tail-flick, hot-plate and writhing tests.

Results: Replacement of the 6-keto group by a 6-cyano substituent in N-methylmorphinan-6-ones leads to qualitative and quantitative differences in the interaction with opioid receptors. Consequently,

we have conducted a comparison of the biological activities of the 6-cyanomorphinans to those of structurally-related opioids, oxycodone, oxymorphone and of the clinically relevant morphine. The 6-cyanomorphinans displayed high affinity and behaved as agonists at the MOP receptor. When tested *in vivo*, they acted as potent antinociceptive agents after subcutaneous administration, being more active than the 6-keto analogues. The presence of a 14-methoxy or a 14-cinnamyl group instead of a hydroxy group not only increased *in vitro* opioid activity at the MOP receptor, but also enhanced the antinociceptive potency.

Conclusions: Our findings revealed that targeting position 6 in the morphinan skeleton represents a viable approach for tuning the pharmacological properties of this class of opioids. Appropriate molecular manipulations could afford ligands that, besides their scientific value as pharmacological tools, may also have the potential of emerging as novel analgesics with fewer side effects compared to currently available treatments.

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A27

Expression and differential distribution of the shaker-related voltage-gated potassium channel family ($K_v1.x$) in human hippocampus and neocortex

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Background: All excitable cells express voltage-gated potassium (K_v) channels; in neurons they play an essential role in setting the resting membrane potential, controlling the firing frequency and duration of action potentials, and modulate neurotransmitter release. Due to this critical function as regulators of neuronal excitability, mutations and/or deletions in potassium channel subunit genes are associated with diverse clinical phenotypes (channelopathies), including seizure or movement disorders in both humans and animals. Additionally, voltage-gated potassium channels might play a crucial role in neurodegenerative and psychiatric disorders. For this reason, a better understanding of the occurrence and specific distribution of voltage-gated potassium channels would be highly necessary. Several members of the K_v1 subfamily have been found, but only $K_v1.1$, $K_v1.2$, $K_v1.4$ and $K_v1.6$ are widely expressed in the CNS in both human and rodent brain. However, unlike to rodents, little is known regarding the regional localization of these four members of the K_v1 subfamily in human brain. Therefore we investigated, for the first time, the distribution of these four K_v1 channel subtypes in human neocortex and hippocampus, which are known for their vulnerability to epilepsy and their importance for learning, memory and cognitive processes.

Methods and results: To examine the relative expression levels of the $K_v1.1$, $K_v1.2$, $K_v1.4$ and $K_v1.6$ proteins in human as well in mouse brain and to determine the specificity of each individual α -subunit antibody for human brain tissue, Western blots were conducted. Individual expression patterns for each K_v1 channel subtype were established by immunohistochemistry using polyclonal anti- $K_v1.1$, $K_v1.2$, $K_v1.4$, and $K_v1.6$ as primary antibodies. We found that the staining patterns of these four K_v1 channel subunits overlap in some areas but each K_v channel subunit shows a unique pattern of distribution in human cortex and hippocampus. The pyramidal cell bodies of cornu Ammonis (CA) 1–3 areas and the granule cell bodies of the dentate gyrus were strongly immunoreactive for $K_v1.1$, $K_v1.2$, $K_v1.4$ and $K_v1.6$. Varying degrees of immunoreactivity were also found in other layers, such the inner and outer molecular layer, stratum lacunosum and stratum oriens.

Conclusions: Precise knowledge of the differential distribution of K_v1 channels in human brain may provide useful data for future

investigations on common pathological conditions such as epilepsy and neurodegenerative disorders.

A28

Current-clamp experiments on primary hippocampal neurons shed light on the role of L-type voltage-gated calcium channels in depolarization shifts

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Background: Paroxysmal depolarization shifts are the cellular representations of interictal spikes. Interictal spikes (IIS) have a long history in the diagnosis of epilepsy. Previously, it was thought that IIS are largely asymptomatic, but there is growing evidence that beside being involved in epileptogenesis, they are also involved in the pathogenesis of various other neurological diseases. However, the pathomechanisms leading to the formation of IIS and how IIS may lead to functional neuronal impairment are poorly understood. In a previous study [1] we showed that L-type voltage-gated calcium channels (LTCCs) are capable of augmenting brief neuronal depolarizations. Hence, we were interested in the question whether LTCCs contribute to neuronal depolarization shifts (DS).

Methods: 1 mM caffeine was applied as an epileptogenic agent, and LTCC activity was modulated by Bay K8644 (BayK) and isradipine, respectively.

Results: In contrast to earlier studies on hippocampal slices [2], caffeine alone failed to induce DS in all but one out of 12 neurons tested. However, when BayK (3 μ M) was co-administered, DS were readily evoked in about 60% of 21 neurons tested. DS were abolished by subsequent addition of isradipine (3 μ M). Since oxidative stress has been shown to augment LTCC currents, we tested hydrogen peroxide in our DS assay, again employing caffeine co-administration. Within 5 min of exposure to 3 mM H_2O_2 , DS appeared in a subpopulation of neurons. Testing both BayK and H_2O_2 on the same neurons we found that H_2O_2 evoked DS only in those neurons where BayK showed a clear DS-inducing effect. In those cells, where BayK failed to cause DS, H_2O_2 also had no effect.

Conclusions: Our data suggest that potentiation of LTCCs promotes the formation of depolarization shifts. Oxidative stress appears to be a pathogenic initiator of DS, and this activity may require LTCCs. Since LTCC augmentation is being considered as a pathological mechanism in neurodegenerative diseases, our data point to the possibility that the initiation of IIS may be a precipitating factor and that LTCC inhibition may provide a means to counteract neuropathogenic mechanisms.

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A29

Characterization of CPCA-induced action on isolated rat femoral artery

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Background: Adenosine is a purine nucleoside, which modifies different physiological functions, including vascular tone in numerous blood vessels. This effect is a consequence of interaction between adenosine and specific adenosine A_1 , A_2 or A_3 receptors. Still, the relaxant effect of this endogenous nucleoside has been shown on some blood vessels to

be mainly dependent on activation of adenosine A_2 receptors that can be located on endothelial or smooth muscle cells. To examine this assumption the aim of this study was to determine the effects of CPCA (a selective adenosine A_2 receptor agonist) on the isolated rat femoral artery and to establish whether potassium channels are involved in this action.

Methods: Experiments were conducted on isolated femoral arteries of male rats. Circular vascular segments were placed in an organ bath with Krebs-Ringer's solution. Concentration-response curves for CPCA were obtained in a cumulative fashion on precontracted artery rings. Tension alterations induced by CPCA were continuously recorded.

Results: CPCA (0.1–100 μ M) produced endothelium-dependent relaxation. Incubation of DPCPX (a selective antagonist of A_1 receptors, 10 nM) did not influence the control effect of the examined agonist, while SCH 58261 (a selective antagonist of A_{2A} receptors, 1 μ M) significantly reduced CPCA-induced vasodilatation. The maximal vascular response to CPCA was comparable after denudation and incubation of SCH 58261. In the presence of high K^+ (100 mM) a significant inhibition of the control CPCA-induced relaxation was recorded. This was not the case after the application of glibenclamide, a blocker of ATP-sensitive K^+ channels.

Conclusions: CPCA induced an endothelium-dependent vasodilatation of the examined blood vessel by activation of adenosine A_{2A} receptors, most probably located on the endothelial cells. It can be assumed that the CPCA-evoked action was most likely mediated via some endothelium-derived hyperpolarizing factor. However, ATP-sensitive K^+ channels did not contribute to the overall femoral artery response to CPCA.

A30

Differential modulation of rNa_v1.4 channel inactivated states by lidocaine and its charged analogue QX222

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Background: The local anesthetic lidocaine is generally believed to reach its binding site in the intracellular vestibule of the voltage-gated sodium channel via the cell membrane. QX222 is a permanently charged, quaternary amine analogue of lidocaine, which can access this binding site via a hydrophilic route across the channel protein. The mutation I1575E of the adult rat muscle-type sodium channel (rNa_v1.4) opens such a hydrophilic pathway. When bound to the internal vestibule, 500 μ M lidocaine stabilize both fast and slow inactivated states. We have tested if QX222, once bound to the internal vestibule of I1575E mutant channel, exerts a similar modulatory action on inactivated states as lidocaine.

Methods and results: The construct I1575E was transiently expressed in tsA201 cells and studied by means of the whole-cell patch-clamp technique. When applied from the extracellular side, 500 μ M QX222 stabilized the slow but not the fast inactivated state in I1575E. When applied internally, QX222 entered the channel, but stabilization of inactivated states could not be observed. Position F1579 is the most important residue in local anaesthetic binding. Therefore we have tested I1575E/F1579A double mutant channels to see if QX222 modulates inactivation via this site. However, this mutant was insensitive to QX222.

Conclusions: For both lidocaine and QX222 the binding site is in the inner vestibule of the channel, at position F1579. The hydrophilic form of lidocaine is responsible for block and stabilization of slow inactivation, whereas fast inactivation can only be stabilized by the hydrophobic form of lidocaine.

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A31

Characterising the interaction between the COPII component SEC24C and the human serotonin transporter

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Background: The serotonin transporter (SERT) belongs to the SLC6 family of neurotransmitter transporters, which mediate reuptake of previously released neurotransmitters from the synapse. Mutation of C-terminus residues R607–608 to alanine results in intracellular retention of SERT [1]. We subsequently showed that SERT depends on the COPII component SEC24C for its ER export and proposed R607–608 as a putative interaction site on SERT for SEC24 proteins [2]. The aim of our current study is to characterise the nature of ER export of monoamine transporters.

Methods: Using siRNAs to knock down SEC24 isoforms A–D in HeLa cells, we screened a series of double and truncation mutants generated along the C-terminus of SERT. HeLa cells were transfected with Sec24 siRNAs and, after 48 h, with YFP-tagged transporter plasmids. Functional effects of SEC24A–D knockdowns were determined by substrate uptake assays.

Results: Export of the IK(609,610)AA-SERT mutant was not sensitive to knockdown of Sec24C. Remarkably, the closely related transporters for dopamine (DAT) and noradrenaline (NET), rely on Sec24D, and not C, for their ER export [2]. Accordingly, we replaced K610 by a tyrosine residue (Y) to switch the SERT export motif to a NET/DAT motif. The resulting K610Y-SERT mutant was more sensitive to the knockdown of SEC24D than of SEC24C. These observations predicted that SLC6 family members with a K-residue at the pertinent position ought to be clients of Sec24C. This prediction was verified by examining mGAT4.

Conclusions: The data imply that residue K610 and the equivalent residues in other transporters specify which SEC24 paralogue is recruited for ER export. These export signals work independently because a concatenation of SERT and GAT-1 is affected by depletion of both SEC24C and SEC24D.

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A32

Uremic albumin blocks reverse cholesterol transfer: role of lysine modifications

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BMC Pharmacology 2011, **11(Suppl 2)**:A32

Background: Due to a lack of antioxidant enzymes in plasma, plasma proteins are preferential targets of oxidant injury. Reaction of chlorinated oxidants (e.g. hypochlorous acid), generated from activated neutrophils, with plasma albumin gives rise to formation of an oxidized protein fraction, termed advanced oxidation protein products (AOPPs). AOPP-albumin is a potent high-density lipoprotein (HDL) receptor antagonist, blocking HDL association and reverse cholesterol transport (RCT). However, it is not known whether structural alterations and/or an increase in negative charge (through oxidation of positively charged lysine residues on albumin) are required for high affinity binding to SR-BI.

Methods and results: T-lymphocytes were incubated with AF 488-TFP-labelled HDL in the presence of different AOPP-albumin preparations (albumin oxidized *in vitro* by the myeloperoxidase product hypochlorous acid). HDL association to SR-BI was measured by flow cytometry. Our data show that already mild oxidation of albumin generates a high affinity ligand to SR-BI that effectively displaces HDL from the receptor. Oxidation/decomposition of lysine residues are required for binding of AOPP-albumin to SR-BI since masking lysine residues prior to oxidation as well as regeneration of lysine oxidation products completely averted binding. Interestingly, modification of albumin-located lysine residues with reactive carbonyls only moderately increased binding affinity of albumin to SR-BI.

Conclusions: Structural alterations induced by lysine oxidation rather than an increased negative charge determine binding affinity of AOPP-albumin to SR-BI.

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A33

Screening for serine/threonine kinases phosphorylating Stat5

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Background: Stat transcription factors are highly conserved key regulators of cellular processes such as proliferation, differentiation, growth and apoptosis. Stats have been frequently found to be deregulated in cancer. One example is the constitutive activation of Stat5 in chronic myelogenous leukaemia (CML). The disease is caused by a chromosomal translocation resulting in the expression of the Bcr/Abl fusion kinase. The transcription factor Stat5 is required for Bcr/Abl-induced leukaemic initiation and disease progression. Furthermore, serine phosphorylation of a constitutive active form of Stat5 is crucial for haematopoietic transformation. These findings put Stat5 into the spotlight of new therapeutic tactics. Here, we screen for kinases phosphorylating Stat5 by employing a cell viability-based screening approach.

Methods: Stable leukemic cell lines expressing variants of the *bcr/abl* oncogene and different levels of the Stat5 protein were screened for the induction of apoptosis with purchased kinase inhibitor libraries. Additionally, cells over-expressing phospho-mimetic mutants were included in the screening and served as controls for specificity.

Results: We showed that serine phosphorylation of Stat5 is required to prevent apoptosis in Bcr/Abl-dependent cell lines. Therefore, we used a cell viability-based screening assay showing that 42 of 300 kinase inhibitors induced apoptosis in Bcr/Abl-dependent cell lines. Predominately, those hit compounds were inhibiting members of the CMGC kinase family, which are primarily proline-directed serine/threonine kinases. Furthermore, cell lines with endogenous levels of Stat5 and over-expressing wild-type Stat5 were compared in an additional round of screening. The resulting 6 hit compounds, which inhibit CDKs, GSK3, PKC and p38 MAP kinase, are currently being validated by Western blot analysis with phospho-Stat5 specific antisera.

Conclusions: These findings point towards the importance of CMGC kinase family members for survival of Bcr/Abl-dependent leukaemic cell lines.

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A34

Urea induces intercellular adhesion molecule-1 expression

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BMC Pharmacology 2011, **11(Suppl 2):A34**

Background: The dramatically increased cardiovascular risk in patients with chronic renal disease cannot be explained entirely by traditional cardiovascular risk factors. The continuous exposure of renal patients to elevated levels of urea may contribute to increased inflammation and oxidative stress. While urea may not be directly toxic, many molecules can be carbamylated through cyanate, a reactive decomposition product of urea. Cyanate irreversibly transforms lysine to ϵ -amino-carbamyl lysine.

This pathway may be of particular relevance since clinical studies have shown that carbamylated proteins are independent risk factors for the development of coronary artery disease and stroke.

Methods and results: Here we show that urea potently induces intracellular cell adhesion molecule-1 (ICAM-1) expression with subsequently enhanced neutrophil adhesion in human coronary artery endothelial cells. ICAM-1 expression is triggered through a mechanism depending upon activation of the mitogen-activated protein kinase p38 and nuclear factor κ B. Interestingly, ICAM-1 expression was not induced when low-molecular weight substances were removed from cell culture medium, ruling out a role of carbamylated (lipo)proteins in ICAM-1 induction. Moreover, in end-stage renal disease patients the extent of plasma protein carbamylation (a marker for cyanate formation) correlated significantly with plasma levels of soluble ICAM-1.

Conclusions: Collectively, our data raise the possibility that urea amplifies vascular inflammation in renal patients.

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A35

Teaching therapy-oriented pharmacology within a medical curriculum

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BMC Pharmacology 2011, **11(Suppl 2):A35**

Background: Pharmacology has been known to medical students as a hard-to-study subject that includes learning by heart hundreds of receptors and pathways, mechanisms of action, substances and side effects in a very limited time. Once graduated, young MDs are confronted with applied pharmacology without being properly prepared choosing and prescribing the right drug in the proper dose to individual patients.

Methods and results: My new approach in teaching pharmacology focuses on the application of pharmacology in up-to-date therapy, stressing information relevant for decision-making and prescription. Exam questions contain patient cases to test prescribing and decision-making abilities. My approach in teaching pharmacology and clinical pharmacology within the curriculum with special focus in therapy was honoured by the students with two "Professor of the Term" awards and excellent ranking by the students and with outstanding results in the evaluation programme conducted by Innsbruck Medical University. The interest of the students in pharmacology grew, 116 students chose to attend extra classes in pharmacology beyond the curriculum during the last semester.

A36

ABC transporters of *Fasciola hepatica* as putative drug targets

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BMC Pharmacology 2011, **11(Suppl 2):A36**

Background: The liver fluke *Fasciola hepatica* is one of the most important parasites affecting animal health all over the world, causing the so called liver fluke disease (fascioliosis). Although infections of humans are rather rare in most western countries, several million people worldwide are infected by this trematode. Beside its threat to humans the infection of animal stock leads to large financial losses. As vaccinations against this parasite are not available yet, anthelmintic drugs, like triclabendazole, are the treatment of choice. During the last decades more and more flukes resistant to these drugs have been found. One possible mechanism for these resistances seems to be the expression of so called ABC transporters. Inhibitors of P-glycoprotein (ABCB1) can change the status of flukes from resistant to susceptible. Up to date little is known about proteins expressed by the fluke. In addition almost no information on the fluke's genome is available. We therefore

try to identify and characterize yet unknown proteins of the fluke to investigate their potency as putative new drug targets.

Methods: Starting from a previously published sequence of an ABC transporter of *Fasciola hepatica* we used RACE (rapid amplification of cDNA ends) to generate a full length ABC transporter. Heterologous expression of the protein allows basic analysis of this transporter. Other proteins will be identified by screening a cDNA library, prepared from flukes isolated from the liver of infected cows.

Results and conclusions: Comparison of the published sequence of a previously identified ABC transporter from *Fasciola hepatica* with other transporters of this family revealed that the sequence was lacking the first six transmembrane regions of the transporter. After cloning of the missing part, it became clear that this transporter is highly homologous not only to ABC transporters of the evolutionary close *Schistosoma mansoni*, but also to transporters of mammals. Expression of this transporter in eukaryotic cell lines should therefore allow testing the transporter's properties, identifying substrates and blockers, and therefore getting a glance on future approaches that can be used to treat fasciolosis in animals and humans.

A37

Autocrine signalling as cause of sensitized cAMP formation

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Background: Cyclic adenosine monophosphate (cAMP) is a second messenger essential for neural functions. The propensity of a nerve cell to mount a cAMP response may be conditional, e.g. depending on a preceding exposure to stimuli (as in long term potentiation).

Methods: We measured cAMP formation in SH-SY5Y neuroblastoma cells at various stages of differentiation to a neuronal phenotype. We analyzed expression pattern of enzymes involved in regulated cAMP production, differentiation markers and protein composition of culture supernatant.

Results: SH-SY5Y cells (a model nerve cell) required differentiation to produce cAMP in substantial amounts; in undifferentiated proliferating cells, forskolin or activation of G_s-coupled receptors barely stimulated cAMP formation. A cell-autonomous process induced sensitization. The process relied on an autocrine factor, which we identified as Dickkopf1 protein. Serum protein quenched the activity of Dickkopf1; conversely, serum deprivation allowed for sensitization to unfold. The effect of Dickkopf1 was mediated by a high-affinity receptor activated at concentrations of ≤1 nM. In accordance with its cognate function as Wnt antagonist, sensitization was a consequence of suppressing the canonical Wnt signaling pathway; the inhibitors of glycogen-synthase kinase-3β, lithium chloride and, in addition, valproic acid mimicked Wnt signals and diminished the extent of sensitized cAMP formation. We found that in differentiated cells, expression of the α-subunit of G_s (Gα_s) increased due to activation of the GNAS gene. Although sufficient to support G_s-coupling of the A_{2A} adenosine receptor, increased Gα_s alone failed to enhance receptor-stimulated cAMP formation. We infer that sensitized cAMP formation reflected increased responsiveness of the catalyst, adenylyl cyclase, to stimuli.

Conclusions: SH-SY5Y provide for a nerve cell model to study the effect of Wnt signaling on regulated cAMP formation. Our data suggest that mood stabilizing agents act by reducing the ability of nerve cells to produce cAMP.

A38

Differential regulation of amphetamine-induced serotonergic and dopaminergic efflux by syntaxin 1A

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Background: The plasma membrane serotonin transporter (SERT) is a key regulator of synaptic serotonergic neurotransmission and is a major target of both antidepressants and psychostimulant drugs of abuse. The pre-synaptic soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein syntaxin 1A has been reported to modulate the intrinsic activity of multiple monoamine neurotransmitter transporters both *in vitro* and *ex vivo*. However, in contrast to the dopamine transporter (DAT) little is known of its effect on SERT-dependent amphetamine-mediated efflux in neuronal cells. Thus, the purpose of this study was to examine the specific effects of syntaxin 1A on both SERT function and regulation by common drugs of abuse.

Methods: Murine catecholaminergic cells (CAD cells) were transiently transfected with either DAT or SERT in the presence and absence of syntaxin 1A. Transporter function was assessed by [³H]MPP⁺ and [³H]5-HT uptake, respectively. The cells were pre-loaded with [³H]MPP⁺ and superfused with amphetamines in order to determine the effect of syntaxin 1A co-expression on amphetamine-mediated transporter-dependent efflux. Mutagenesis was performed using the QuikChange II site-directed mutagenesis kit from Stratagene. Transporter and syntaxin 1A co-localisation was confirmed using confocal microscopy.

Results: The co-expression of SERT and syntaxin 1A led to a significant reduction in the V_{max}, but not the K_m, for [³H]5-HT uptake. Similarly, syntaxin 1A co-expression greatly reduced parachloroamphetamine-induced SERT-dependent efflux. Neither the pharmacological inhibition of CaMKII, nor the mutation of a CaMKII-binding motif in the N-terminal tail of SERT had any effect on the down-regulation of SERT activity by syntaxin 1A in neuronal cell lines. In contrast to SERT, the co-expression of syntaxin 1A and DAT had no effect on [³H]MPP⁺ uptake via DAT. Moreover, D-amphetamine-induced efflux via DAT was increased by the co-expression of syntaxin 1A.

Conclusions: In contrast to DAT, syntaxin 1A is a negative regulator of amphetamine-induced SERT-mediated efflux, an effect which occurs independently of CaMKII activation. The significance of this differential regulation is currently being investigated using endogenous expression systems.

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A39

129S1/SvlmJ mice display impaired contextual fear extinction, enhanced fear incubation and deficit extinction consolidation phenotypes: rescue via pharmacological and non-pharmacological treatments

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Background: We recently revealed that 129S1/SvlmJ (S1) mice exhibit deficits in engaging associative mechanisms to extinguish cued emotional responses. Here, we aimed to further characterise emotional responding in S1 and determine the effect of novel treatments on aberrant emotional responding.

Methods and results: The present experiments firstly tested whether deficits in extinguishing emotional responses in S1 extends to contextual extinction. No reduction in emotional responses to the context was observed during an extinction training session which indicates generalised inability of S1 to engage associative fear extinction mechanisms. This was in contrast to mice of another 129 strain, 129S6/SvEvTac (S6), which showed contextual (and cued) extinction of emotional responding. Importantly, S1 and S6 showed identical responses on the flinch/jump test, which indicated that the results obtained were not an artefact of altered sensitivity to the unconditioned stimulus (US). Evidence that S1 mice can engage non-associative mechanisms to extinguish emotional responses was revealed

as complete abolishment of contextual emotional expression was observed following US habituation (devaluing emotional responding to the US). We next investigated whether impaired cued emotional extinction persisted following also very "weak" conditioning paradigms. Results revealed that under these conditions, S1 mice displayed cued emotional (within) extinction when extinction training was performed using massed-CS presentations. This result indicates that the strength of the conditioning determines the ability of S1 to engage associative mechanisms to extinguish emotional responding. Using this paradigm it was possible to reveal impaired extinction consolidation in S1, as no between-session extinction was observed. Post extinction training application of D-cycloserine (an NMDA receptor agonist) and MS-275 (an HDAC inhibitor) rescued impaired extinction consolidation in S1. Finally, using "weak" conditioning, an enhanced sensitivity in S1 to incubate emotional responses was observed. During extinction training, temporally spaced CS presentations increased emotional responding in S1, but not the comparator strain C57BL6, revealing that S1 display an increased propensity over "normally" behaving mice to incubate emotional responses (via non-associative mechanisms) following weak trauma.

Conclusions: Taken together, the present results suggest the utility of S1 to explore mechanisms underlying impaired contextual extinction, impaired extinction consolidation and enhanced emotional incubation.

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A40

Modulation of magnesium deficiency-induced anxiety and HPA axis dysregulation by therapeutic drug treatment

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Background: Preclinical and some clinical studies suggest a relationship between perturbation in magnesium homeostasis and pathological anxiety, although the underlying mechanisms remain largely unknown. Since there is evidence that Mg²⁺ modulates the hypothalamic-pituitary-adrenal (HPA) axis, we tested whether enhanced anxiety-like behaviour can be reliably elicited by dietary Mg²⁺ restriction and whether Mg²⁺ deficiency is associated with altered HPA axis function.

Methods: Mice assigned to Mg²⁺-deficient groups were allowed to freely access a 0.005% Mg²⁺-containing diet while control mice were fed a normal, 0.2% Mg²⁺-containing diet. The emotional behaviour of Mg²⁺-deficient mice was assessed in a battery of anxiety tests including the open field test, the light/dark test, the stress-induced hypothermia test, and the hyponeophagia test. Markers of HPA axis function including CRH gene expression and plasma ACTH levels were quantified. Neuronal activation patterns in the HPA system were investigated using mapping of the immediate early gene c-Fos as a marker of neuronal activation in response to an anxiety-provoking situation.

Results: Compared to controls, Mg²⁺-deficient mice did indeed display enhanced anxiety-related behaviour in numerous anxiety tests. The enhanced anxiety-related behaviour of Mg²⁺-deficient mice was sensitive to chronic desipramine treatment in the hyponeophagia test and to acute diazepam treatment in the open arm exposure test. Mg²⁺ deficiency caused an increase in the transcription of corticotropin releasing hormone in the paraventricular hypothalamic nucleus (PVN), which coincided with elevated ACTH plasma levels, pointing to an enhanced set-point of the HPA axis. Chronic treatment with desipramine reversed the identified abnormalities of the stress axis. Functional mapping of neuronal activity revealed hyper-excitability in the PVN of anxious Mg²⁺-deficient mice and its normalisation through diazepam treatment.

Conclusions: Overall, the present findings demonstrate the robustness and validity of the Mg²⁺ deficiency model as a mouse model of enhanced anxiety, showing sensitivity to treatment with anxiolytics and antidepressants. It is further suggested that dysregulations in the HPA axis may contribute to the hyper-emotionality in response to dietary induced hypomagnesaemia.

A41

The folding interactome of GPCRs

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Background: The A_{2A} adenosine receptor is a prototypical G protein-coupled receptor. It is expressed in a wide variety of cells including as different types as neurons, platelets, cells of the immune system and muscle. The A_{2A} receptor has an unusually long C-terminus (of >120 residues), which for the most part is dispensable for coupling to G_s. This C-terminus turned out to be the docking site for other proteins. Using a yeast-2-hybrid screen we have previously identified proteins interacting with the C-terminus including ARNO/cytohesin2, SAP102 and USP4.

Methods: To verify these interactions *in vivo* and to identify new interacting proteins of the A_{2A} adenosine receptor we chose a two-step proteomics approach: we first expressed tagged receptors in HEK293 fibroblasts using various TAP (tandem affinity purification)-tag variants; the differently tagged receptors were analyzed for expression, localization and their pharmacological properties (ligand binding and cAMP accumulation) to identify tags suitable to further analyze the receptor's interactome. These tagged receptors were then used to optimize the purification and to make the first initial screens using 2D-nano-LC-MS/MS approach. To prove the interaction of the A_{2A} receptor with promising targets found in our screens, biochemical approaches, e.g. co-immunoprecipitation and whole-cell binding, were performed.

Results and conclusions: We could identify two tags suitable for further analysis of the A_{2A} adenosine receptor interactome. Pharmacological properties of the tagged receptors were comparable to the native receptor. However, the tags seemed to retain the receptor to a large extent in the endoplasmic reticulum (ER) and hence we used this system to study the ER/folding interactome of the receptor. LC-MS/MS analysis of the purified ER-trapped version of the receptor revealed proteins putatively involved in the folding of the receptor, such as chaperones. We are currently generating a transgenic mouse-model expressing the TAP-tagged version of the A_{2A} adenosine receptor under the control of its endogenous promoters (homologous knock-in). This will allow us to examine tissue- and development-specific interaction partners of the A_{2A} adenosine receptor utilizing the optimized proteomics approach.

A42

Fear learning induces structural and functional plasticity at GABAergic synapses in the basolateral amygdala

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Background: Previous work has suggested that alterations in GABAergic function within the amygdala underlie fear learning. In particular, it has been shown that Pavlovian fear conditioning induces a downregulation of benzodiazepine binding sites as well as transcripts for gephyrin and some GABA_A receptor subunits in the basal nucleus of the amygdala (BA), which were restored to control levels after fear extinction.

Methods: We have undertaken a combined anatomical and physiological approach to examine whether these alterations distinctively involve GABA_A receptors in synaptic or extrasynaptic areas. Specifically, we analyzed – in the BA of mice that underwent fear conditioning as well as

extinction – miniature inhibitory postsynaptic currents (mIPSCs), mRNA levels by *in situ* hybridization, and the density for the GABA_A γ 2 subunit by means of the freeze-fracture replica immunolabelling technique (SDS-FRL). SDS-FRL also allowed to precisely measure the size of GABAergic synapses.

Results: A significant decrease in labelling density for the GABA_A γ 2 subunit could be detected in the synaptic area in fear-conditioned mice as compared to the control group and mice that had undergone extinction ($p < 0.01$; Kruskal-Wallis and Dunn's multiple comparison tests). Conversely, GABA_A γ 2 extrasynaptic density was lower in the extinction group when compared to both the control and fear-conditioned mice ($p < 0.005$). The average size of GABAergic synapses in control mice was $0.034 \pm 0.001 \mu\text{m}^2$ ($n = 227$ full synapses from 3 animals; $CV = 0.62$). Fear-conditioned animals showed a significantly ($p < 0.01$) larger average synaptic size ($0.040 \pm 0.001 \mu\text{m}^2$; $n = 249$; $CV = 0.59$), whereas in fear extinction mice it was similar to controls ($0.031 \pm 0.001 \mu\text{m}^2$; $n = 290$; $CV = 0.59$). Alterations in synapse size upon fear conditioning and extinction were associated with functional changes. In neurons recorded from acute slices obtained from fear-conditioned animals mIPSCs were larger (increased charge transfer/mIPSC) compared to recordings obtained from slices of control mice and animals subjected to extinction training. *In situ* hybridization analysis of the mRNA content for GABA_A γ 2 subunits revealed highly similar levels among the 3 groups in the BA ($p = 31$, one-way ANOVA) and central nucleus ($p = 41$).

Conclusions: Our results indicate that, in the BA, fear conditioning produces a reversible enlargement of GABAergic synapses and an increase in mIPSC charge transfer with no change in the overall number of synaptic GABA_A receptors.

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A43

Septal urocortin 3 modulates stress-coping behaviour but not hypothalamic-pituitary-adrenal axis activity during forced swimming

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Background: The lateral septum (LS) has been shown to play an important role in the generation and modulation of behavioural and neuroendocrine stress responses. However, the exact neurochemical mechanisms mediating these effects are not well studied so far. Several lines of evidence suggest a robust contribution of septal urocortin 3 (UCN3) and its preferred receptor, the corticotropin-releasing factor type 2 (CRF2) receptor in mediating these effects. Therefore, the aim of the present study was to examine the role of septal CRF2 receptors in neuroendocrine and behavioural stress responses.

Methods: Male Sprague-Dawley rats implanted with a jugular venous catheter and a microinjection cannula aimed at the LS received bilateral injections of either UCN3, the selective CRF2 receptor antagonist anti-sauvagine-30 (ASV-30) or vehicle, and were exposed to forced swim stress.

Results: Our data show that intraseptal UCN3 infusion reduced active coping and increased immobility during the forced swim exposure as UCN3 treated animals showed less struggling and swimming behaviour and increased floating behaviour compared to controls. Conversely, the administration of ASV-30 had the opposite effects, an increase in struggling and swimming and a reduction in floating. In contrast to the behavioural stress response, the administration of UCN3 or ASV-30 directly into the LS had no effect on either basal or stress-induced increase of plasma ACTH levels, indicating that septal CRF2 receptors are not involved in hypothalamic-pituitary-adrenal (HPA) axis regulation.

Conclusions: Taken together, our data suggest that CRF2 receptors in the LS are critically involved in the regulation of behavioural stress reactivity but not in HPA axis regulation.

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A44

Identification of a new C-terminal splice variant of Ca_v1.3 L-type calcium channels with unique functional properties

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Background: In L-type voltage-gated calcium channels (VGCCs) the long C-terminal tail contains several sites for modulation by protein-protein interaction. Ca_v1.3 VGCCs (Ca_v1.3_L) activate at negative voltages and support sinoatrial node pacemaking and hearing, and shape neuronal excitability. In Ca_v1.3_L an intermolecular automodulatory C-terminal interaction (CTM) has been described which affects channel gating. CTM is characterized by interaction of a distal C-terminal regulatory domain (DCRD) with a more proximal regulatory domain (PCRD). If this CTM is absent as in previously described short Ca_v1.3_{42A}, calcium-dependent inactivation (CDI) increases and the channel activation range shifts to more negative voltages (i.e. "short" gating properties). Here we show that alternative splicing in exon 43 creates a new short splice variant Ca_v1.3_{43S} found in human and mouse brain. It lacks CTM, but still contains the PCRD motif, in contrast to previously described Ca_v1.3_{42A}. Semiquantitative PCR experiments showed that in mouse brain 39% of Ca_v1.3 channels contain exon 43S contrary to heart (6% 43S).

Methods and results: Biophysical analysis showed "short" gating properties for Ca_v1.3_{43S} in both 15 mM and 2 mM external Ca²⁺ when co-expressed with β 3 and α 2 δ -1 subunits in tsA-201 cells. In 2 mM Ca²⁺ the inactivation rate of Ca_v1.3_{43S} was faster for Ca_v1.3_L, but slower for Ca_v1.3_{42A} (% inactivation after 100 ms at V_{max} : Ca_v1.3_{42A}: $64.5 \pm 3.5\%$; Ca_v1.3_{43S}: $52 \pm 4.5\%$, Ca_v1.3_L: $37.4 \pm 3\%$, $p < 0.001$) by affecting the extent of CDI. Due to a presence of PCRD, DCRD-containing C-terminal fragments from Ca_v1.3 or Ca_v1.2 channels could restore Ca_v1.3_L gating behaviour. Indeed, co-expression of GFP-Ca_v1.2_{C349} fully restored long channel gating properties in Ca_v1.3_{43S} ($V_{0.5}$: Ca_v1.3_{43S}+GFP-Ca_v1.2_{C349}: 1.37 ± 1.3 mV; Ca_v1.3_L: -2.4 ± 0.6 mV). C-terminal splicing also changed I_{Ca} kinetics during stimuli mimicking trains of action potential waveforms, revealing a lower total I_{Ca} during AP bursts in short splice variants.

Conclusions: Taken together, our data indicate that Ca_v1.3 C-terminal splicing can serve as an important mechanism to fine-tune the dynamics of calcium entry in neurons in an activity dependent manner.

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A45

Effect of orally administered moxaverine on ocular haemodynamics

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Background: Reduced ocular perfusion is related to several common eye diseases, like age-related macular degeneration, diabetic retinopathy or glaucoma. Moxaverine, a phosphodiesterase inhibitor, has a vasodilating effect on peripheral vessels and is therefore clinically used to increase blood flow. It was recently shown that a dose of 150 mg moxaverine administered intravenously increases retinal blood flow in healthy subjects and in patients with ocular diseases. The aim of this study was to investigate the effect of oral moxaverine on ocular blood flow.

Methods: Sixteen healthy subjects were included in this randomized, double-blinded, placebo-controlled, two-way crossover study and two study days were scheduled. The subjects were randomized to receive either 900 mg moxaverine (p.o. in three equal doses at two-hour intervals) or placebo on the first study day. On the second study day the subjects were crossed over to the alternative treatment. Ocular haemodynamics were measured at baseline and 5 hours after the study drug administration. A Laser Doppler Flowmeter was used to measure the choroidal and optic nerve head blood flow. The blood velocities in the retrobulbar vessels were assessed using the Color Doppler Imaging.

Results: No difference was found in the haemodynamic parameters (choroidal, optic nerve head and retrobulbar blood flow) between moxaverine and placebo group. The p values for the choroidal and optic nerve head blood flow were $p = 0.52$ and $p = 0.54$, respectively. Similarly, the values assessed with the Color Doppler Imaging also showed no significant difference. Additionally, moxaverine had no effect on choroidal, optic nerve head and retrobulbar blood flow.

Conclusions: Our results show that orally administered moxaverine, in contrast to intravenous moxaverine, does not alter the ocular blood flow. This may be related to the low bioavailability of moxaverine after oral administration.

A46 [¹¹C]Elacridar as a novel P-glycoprotein PET tracer, assessment of whole-body distribution and radiation dosimetry in humans

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Background: The ATP-binding cassette transporter P-glycoprotein (P-gp) is expressed at the blood-brain barrier (BBB) where it protects the brain from toxic substances and xenobiotics by active efflux transport. The ¹¹C-labelled third-generation P-gp inhibitor [¹¹C]elacridar was developed as a positron emission tomography (PET) tracer for the *in vivo* quantification of P-gp expression levels in different organs. The aim of this study was to provide human dosimetry estimates for [¹¹C]elacridar based on whole-body PET.

Methods: Whole-body low dose computed tomography (CT) and dynamic and static whole-body PET scans were acquired in 4 healthy subjects for a total of 100 min after i.v. injection of 400 ± 8 MBq of [¹¹C]elacridar using a Siemens Biograph scanner. Volumes of interest were placed in the brain, liver, pancreas, gallbladder, kidneys, lung, muscle, heart, spleen, bone marrow and bile by using ROVER (v. 2.0.31, ABX, Germany) software. Residence times were derived by spreadsheet calculation and adapted to the standard human model. Organ doses and effective dose were calculated utilizing the OLINDA (v. 1.1, Vanderbilt University) dosimetry program.

Results: Organs with highest radiation burden included pancreas, spleen, liver and gallbladder wall. Furthermore, lungs, heart wall and kidneys received above average organ doses. As excretory organ the gallbladder was identified. Monoexponential fitting of activity overlying the gallbladder suggested that >95% of activity was excreted via the bile. The calculated effective dose was 7.0×10^{-3} mSv/MBq yielding 2.8 mSv for an injected amount of 400 MBq of [¹¹C]elacridar.

Conclusions: The estimated radiation burden of [¹¹C]elacridar is in the range of other ¹¹C-labeled PET tracers and would allow multiple PET examinations of the same subject per year.

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A47

Role of nitric oxide in optic nerve head blood flow regulation while experimental increase of ocular perfusion pressure

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Background: The involvement of nitric oxide (NO) in choroidal blood flow regulation during experimental increase of ocular perfusion pressure (OPP) has been shown in previous studies. It is also known that during isometric exercise, the inhibition of NO synthase (NOS) leads to a rightward shift of pressure-flow curves. In this study the influence of inhibited NOS on optic nerve head (ONH) blood flow during isometric exercise was investigated.

Methods: A randomized, double-blinded, placebo-controlled, three-way crossover design was chosen for the present study. In order to increase systemic perfusion pressure during application of either a NOS inhibitor (L-NMMA), an α -receptor agonist (phenylephrine) or placebo, 18 healthy subjects were asked to squat for 6 minutes. Laser Doppler flowmetry (LDF) was used for continuous assessment of ONH blood flow and OPP was calculated as 2/3 mean arterial pressure minus intraocular pressure (IOP).

Results: L-NMMA and phenylephrine both significantly increased OPP at rest ($p < 0.001$ vs. baseline). However, only L-NMMA significantly decreased ONH blood flow at rest compared to baseline ($p = 0.02$). While isometric exercise was performed and using all three drugs administered, no difference in ONH blood flow and OPP response was recorded ($p = 0.43$ and $p = 0.69$, respectively).

Conclusions: The findings of this study indicate that NO seems to be involved in basal regulation of ONH blood flow. However, this was not the case during isometric exercise. Whether different regulatory systems gain importance after increase of OPP and blood flow has to be the focus of further studies.

A48

Inhibition of breast cancer resistance protein at the murine blood-brain barrier by Ko143 studied with [¹¹C]tariquidar and PET

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Background: The ATP-binding cassette (ABC) transporters breast cancer resistance protein (BCRP) and P-glycoprotein (P-gp) are expressed in the blood-brain barrier (BBB), where they impede brain uptake of their substrates by active efflux transport. BCRP has recently been shown to be the quantitatively most important ABC transporter at the human BBB. Inhibition of BCRP by inhibitors such as the fumitremorgin C derivative Ko143 [1] may be an interesting strategy to improve brain uptake of BCRP substrates. The aim of this study was to assess the dose-response relationship of Ko143 for inhibition of Bcrp1 at the murine BBB using small-animal positron emission tomography (PET) together with the dual P-gp/BCRP substrate radiotracer [¹¹C]tariquidar.

Methods: [^{11}C]Tariquidar PET scans were performed in female wild-type (FVB), *Bcrp1*^{-/-} and *Mdr1a/b*^{-/-} mice before and 60 min after i.v. injection of Ko143 (Axon Medchem BV, The Netherlands) at a dose of 5 mg/kg. Additionally, in *Mdr1a/b*^{-/-} mice scans were performed after i.v. administration of vehicle (n = 2), 1 mg/kg (n = 2), 3 mg/kg (n = 1), 10 mg/kg (n = 3) and 15 mg/kg (n = 2) of Ko143. After the 60-min PET scans a venous blood sample was taken by retro-orbital puncture. Brains were manually outlined on the reconstructed PET images and time-activity curves expressed as percent injected dose per gram (%ID/g) were generated, for which areas under the curve (AUC) were calculated.

Results: Wild-type and *Bcrp1*^{-/-} mice showed no increase in brain AUCs after administration of 5 mg/kg Ko143 as compared to baseline scans, whereas in *Mdr1a/b*^{-/-} mice brain AUC was 4.5-fold increased. In *Mdr1a/b*^{-/-} mice, the half-maximum effect dose of Ko143 to increase brain AUC of [^{11}C]tariquidar was 5.6 ± 2.3 mg/kg. Maximum increase in brain AUC was 8.2-fold after the 15 mg/kg dose. No changes in blood activity concentrations of [^{11}C]tariquidar were found after administration of different Ko143 doses.

Conclusions: Performing PET scans in *Mdr1a/b*^{-/-} mice in combination with the dual P-gp/BCRP substrate [^{11}C]tariquidar allowed individual assessment of *Bcrp1* inhibition at the BBB. Our data demonstrate that Ko143 is a potent inhibitor of cerebral *Bcrp1* *in vivo*, which apparently does not inhibit P-gp at the studied doses.

Acknowledgements: The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement number 201380 (Euripides) and from the Austrian Science Fund (FWF) project 'Transmembrane Transporters in Health and Disease' (SFB F35).

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A49

Salmide down-regulates sirtuin proteins to induce human cancer cell apoptosis

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Background: The NAD⁺-dependent family of sirtuin proteins (SIRT1-7), is involved in cell apoptosis and senescence. Salmide is a potent inhibitor of SIRT1 and SIRT2 and can induce tumor-specific cell death in selected human cell lines. In this study we investigated salmide's apoptotic effect in a wide range of other human cancer cell lines and its antiproliferative potential in combination with cisplatin.

Methods: Seven different cancer cell lines (SKOV-3, MKN45, MKN28, N87, FaDu, NuLi1, Jurkat) were treated with salmide (1 μM - 0.1 nM) for 24, 48, and 72 hours and assessed for cell viability. Three cell lines (SKOV-3, N87, Jurkat) were selected for combination therapy with salmide and cisplatin (30 μM). In order to characterize salmide's proapoptotic pathway SIRT1, SIRT2, pAKT, p53, acetyl-p53 and Nampt (nicotinamide phosphoribosyltransferase) were determined in SKOV-3 and Jurkat cells by Western blotting.

Results: Salmide yielded greater dose-dependent apoptotic effects in Jurkat, SKOV-3 and N87 cells than in the other cell lines, with most potent effect after 48 h of incubation. The anti-proliferative activity was associated with a G₀-G₁ cell cycle arrest. SIRT1 and SIRT2 protein were down-regulated after 48 h and 72 h. This was accompanied by a down-regulation of pAKT, p53 and Nampt. Acetyl-p53 levels were not consistent across cell types. Cisplatin exerted synergistic effects with salmide in all cell lines and reduced cell viability up to 50%.

Conclusions: Salmide-induced apoptosis is cell line-dependent and more effective in slow-proliferating (SKOV-3) and hematologic (Jurkat) cancer cells. The synergism with cisplatin implies a potentiating effect of this sirtuin inhibitor as add-on in clinical cancer therapy.

A50

Capsaicin-sensitive sensory nerves, TRPV1 receptors and tachykinins play important roles in mast cell tryptase-induced arthritis and hyperalgesia

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Background: Protease-activated receptors (PARs) are G protein-coupled receptors activated through proteolytic cleavage. They are localized on epithelial, endothelial and inflammatory cells, as well as on Transient Receptor Potential Vanilloid 1 (TRPV1) receptor-expressing capsaicin-sensitive sensory nerves. Tachykinins, such as substance P (SP) and neurokinin A (NKA) encoded by the TAC1 gene are released from these fibres and play an important role in inflammatory and nociceptive processes. We investigated the involvement of capsaicin-sensitive peptidergic afferents, TRPV1 ion channels and TAC1-encoded tachykinins in mast cell tryptase (MCT)-induced joint swelling, hyperalgesia and synovial microcirculation.

Methods: The natural PAR2 activator MCT (20 μl , 12 $\mu\text{g/ml}$) was injected into the right tibiotarsal joint of mice. Pretreatment with high doses of the TRPV1 receptor agonist resiniferatoxin (RTX) was used to selectively inactivate capsaicin-sensitive peptidergic sensory nerves. TRPV1, TAC1 and neurokinin 1 receptor (NK₁) gene-deficient animals were also studied compared to their wild-type (WT) C57Bl/6 counterparts. Knee diameter was measured with a digital micrometer, mechanonociceptive threshold with dynamic plantar aesthesiometry and spontaneous weight distribution with incapitance tester throughout a 6-hour period. Synovial bloodflow in urethane-anaesthetized animals was determined by laser Doppler imaging. In these studies, MCT was applied topically on the joints.

Results: MCT-induced joint swelling and secondary hyperalgesia were significantly reduced in TAC1^{-/-} and NK₁^{-/-} mice, but not in the other groups compared to WTs. Spontaneous weight distribution decreased by 10% on the injection site in response to MCT in WT mice, but not in any other groups. Synovial vasodilatation in response to topical MCT application was significantly smaller not only after the destruction of the capsaicin-sensitive afferents by RTX pretreatment, but also by the selective genetic deletion of the TRPV1 ion channels, but was not altered in TAC1 and NK₁-deficient mice.

Conclusions: These data provide evidence that MCT-evoked acute oedema and hyperalgesia are mediated by tachykinins through NK₁ receptor activation. The lack of difference observed in RTX-desensitized and TRPV1^{-/-} mice is likely to be explained by a counteracting effect of simultaneously released inhibitory peptides (e.g. somatostatin, endomorphins) from the same capsaicin-sensitive fibres. In contrast, these afferents and the TRPV1 receptors are essential in acute synovial vasodilatation, but tachykinins are not involved in this response.

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A51

Penetration of polar organic compounds through the blood-brain barrier

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Background: The effect of polar organic xenobiotics in the central nervous system depends on blood-brain barrier (BBB) penetration of these compounds. Newly synthesized pyridinium aldoximes (K-compounds) are promising antidotes for organophosphate intoxications. However, being

highly polar, their BBB penetration is questionable. Using an *in vivo* model we aimed to characterize the BBB penetration of K-compounds.

Methods: Male Wistar rats were injected intramuscularly with various doses of pyridinium aldoximes, blood, cerebrospinal fluid (CSF), and brain samples were collected after 5, 15, 30, 60 and 180 min. A recently developed and optimized RP-HPLC method was used for analysis. Samples of brain homogenate, blood serum and CSF were subjected to clean-up using precipitation by perchloric acid ($\text{pH} < 1$) and centrifugation at 14,000 rpm at 4°C for 20 min. Before load onto Zorbax Rx-C18 stationary phase, the pH of the supernatants was adjusted to 2. As mobile phase a mixture of acetonitrile and aqueous buffer pH 4.5, also containing ion-pairing agent, was used.

Results: Dose- and time-dependent BBB penetration of pyridinium aldoximes was experimentally found.

Conclusions: Dose- and time-dependent brain and CSF levels of these highly polar K-compounds following intramuscular administration suggest contribution of active transport or specific transporters in their BBB penetration. The BBB transport may also depend on the size and charge of the solutes.

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A52

Stability monitoring of some acetylcholinesterase reactivating drugs

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Background: Widespread use of organophosphorous compounds (OPs) in agriculture and as nerve agents as well as a lack of clinically effective antidotes initiated the synthesis of new pyridinium bis-aldoximes (K-compounds) with high potency in reactivating acetylcholinesterase irreversibly inhibited by OPs [1,2]. We aimed to optimize an HPLC method sensitive enough to determine K-compounds from different biological matrices (blood, brain and cerebrospinal fluid) [3,4].

Methods: Samples of biological origin needed proper clean-up. An RP-HPLC method using either UV and amperometric detector was used following separation on a Zorbax Rx-C18 octadecyl silica column with a mobile phase of phosphate buffer with 20% acetonitrile (pH 3.7). 1-Octane sulphonic acid sodium salt (OSA) was used as ion-pairing agent. Calculation of theoretical plate number, asymmetry of peaks, limit of quantitation (LOQ), lower limit of detection (LLOD) and determination of pH, temperature and OSA concentration dependence was done.

Results: Elution characteristics of bis-pyridinium mono-aldoximes were depending on the OSA concentration, however, to a lesser extent than the bis-pyridinium bis-aldoximes. A double bond in the alkyl chain decreased the dependence from the ion-pairing agent concentration only to a minor extent. When the samples were kept at a pH under 1.5 a peak of degradation product was generated. The time course of degradation in an acidic milieu was calculated.

Conclusions: Appropriate clean-up, optimal concentration of the ion-pairing agent and a well-selected mode of detection are the key factors for optimal determinations. We point out decomposition of pyridinium aldoximes at acidic pH.

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A53

Electrophysiological effects of rosiglitazone on heart ventricular papillary muscles of control and diabetic histidine decarboxylase knock-out and wild-type mice

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Background: Rosiglitazone is a thiazolidinedione derivative oral hypoglycemic agent active in both diabetic animal models and type 2 diabetic patients. Rosiglitazone is a high affinity ligand for the peroxisome proliferator-activated receptor gamma, which is responsible for the insulin-sensitizing action of the compound. Recent large clinical trials found an association between the antidiabetic drug rosiglitazone therapy and increased risk of cardiovascular adverse events.

Methods: The aim of this report is to elucidate the cardiac electrophysiological properties of rosiglitazone on control and diabetic murine ventricular papillary muscles using conventional microelectrode technique.

Results: In control histidine-decarboxylase knock-out mice (HDC-KO) as well as in their wild-types (WT) rosiglitazone (1–30 μM) shortened AP duration at the 90% level of repolarization (APD_{90}) and increased the AP amplitude (APA) in a concentration-dependent manner. Moreover, rosiglitazone reduced the maximum velocity of depolarization (V_{max}). In diabetic animals we detected very similar effects.

Conclusions: The action potential changes caused by rosiglitazone probably can be explained by ion channel effects. The observed alterations may carry a serious proarrhythmic risk in case of overdose intoxication with rosiglitazone, especially in patients having multiple cardiovascular risk factors, like elderly diabetic patients.

A54

Can the electrophysiological action of rosiglitazone explain its cardiac side effects?

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BMC Pharmacology 2011, 11(Suppl 2):A54

Background: Several recent clinical trials showed that the antidiabetic drug rosiglitazone is associated with an excess risk of cardiovascular adverse events. In spite of its widespread clinical application there is little information on its cellular cardiac effects in larger mammals. In the present study, therefore, concentration-dependent effects of rosiglitazone on action potential morphology and the underlying ion currents were studied in canine hearts.

Methods: Standard microelectrode techniques, conventional whole cell patch-clamp, and action potential voltage-clamp techniques were applied in enzymatically dispersed ventricular cells.

Results: At concentrations $\geq 10 \mu\text{M}$ rosiglitazone decreased the amplitude of phase-1 repolarization, reduced the maximum velocity of depolarization and caused depression of the plateau potential. These effects developed rapidly and were readily reversible upon washout. Rosiglitazone suppressed several transmembrane ion currents in a concentration-dependent manner under conventional voltage-clamp conditions and altered their kinetic properties. The EC_{50} value for this inhibition was $25.2 \pm 2.7 \mu\text{M}$ for the transient outward K^+ current (I_{to}), $72.3 \pm 9.3 \mu\text{M}$ for the rapid delayed rectifier K^+ current (I_{Kr}), and $82.5 \pm 9.4 \mu\text{M}$ for the L-type Ca^{2+} current (I_{Ca}) with Hill coefficients close to unity. The inward rectifier K^+ current (I_{K1}) was not affected by rosiglitazone up to concentrations of 100 μM . Suppression of I_{to} , I_{Kr} , and I_{Ca} was confirmed under action potential voltage-clamp conditions as well.

Conclusions: The observed alterations in the densities and kinetic properties of ion currents may carry serious proarrhythmic risk in case of overdose intoxication with rosiglitazone, especially in older diabetic patients having multiple cardiovascular risk factors.

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A55

The rise and fall of CB₁ receptor antagonists: possible future perspectives

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Abstract: Cannabinoid type-1 (CB₁) receptor antagonists were among the most promising drug targets in the last decade. They have been explored and found to be effective as therapeutic agents for obesity and related cardiometabolic problems, including e.g. dyslipidaemias, diabetes, and metabolic syndrome. However, the use of rimonabant, the first marketed CB₁ receptor antagonist, has been suspended due to its anxiogenic and depressogenic side effects, which were present in about 20–30% of the patients, i.e. a 2.5–3-fold increase compared to placebo. Since some other anti-obesity drugs like dexfenfluramine or sibutramine were also suspended, the unmet need for drugs that reduce body weight became enormous. One approach that emerged was the development of peripheral CB₁ receptor antagonists that poorly cross the blood brain barrier, the second, the development of neutral antagonists instead of inverse agonists, and the third, the selection of the patient population with reduced risk for psychiatric side effects. An analysis regarding peripheral and central mechanisms involved in the effects of CB₁ receptor antagonists strongly suggest that central mechanisms are more or less involved in most cardiometabolic therapeutic effects and thus, among patients with unsatisfactory therapeutic response to compounds with peripheral action, centrally acting antagonists may be needed. Based on our existing knowledge concerning the role of genetic, phenotypic and environmental factors the selection of persons who are at no or low risk for psychiatric adverse effects may be possible. Molecular mechanisms and receptors involved in the effects of stress and anxiety-related neurocircuits sensitive to CB₁ receptor antagonists, like the serotonergic and noradrenergic systems which regulate the synthesis of the endocannabinoid 2-arachidonoyl-glycerol mediated by 5-HT_{2C} and α_1 receptors can be identified [1]. Furthermore, variants of the serotonin transporter and the CB₁ receptor genes have been shown to modulate stress-induced anxiety in human studies [1]. In conclusion, development of peripherally acting, or the use of personalised medicine for centrally acting CB₁ receptor antagonists are promising approaches with diverse advantages.

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A56

The double-faced role of P2X₇ receptors in toxin-induced animal models of Parkinson's disease

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Background: Previous studies indicate a role of P2X₇ receptors in processes that lead to neuronal death. The main objective of our study was to examine whether genetic deletion or pharmacological blockade of P2X₇ receptors influenced dopaminergic cell death in various models of Parkinson's disease (PD).

Methods: PC12 cells and primary mesencephalic neurons were used in culture, and the striatum and the substantia nigra were prepared from wild-type and P2X₇ receptor knockout mice. Rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatments were applied *in vitro*

and *in vivo* to reproduce neurochemical hallmarks of PD. Receptor expression, cell survival indicators, and endogenous biogenic amine, amino acid, adenine nucleotide and endocannabinoid contents were analyzed.

Results: mRNA encoding P2X₇ and P2X₄ receptors was up-regulated after treatment of PC12 cells with MPTP. P2X₇ antagonists protected against MPTP- and rotenone-induced toxicity in the LDH assay, but failed to protect after rotenone treatment in the MTT assay in PC12 cells and in primary midbrain culture. *In vivo* MPTP and *in vitro* rotenone pretreatments increased the mRNA expression of P2X₇ receptors in the striatum and substantia nigra of wild-type mice. Basal mRNA expression of P2X₄ receptors was higher in P2X₇ knockout mice and was further up-regulated by MPTP treatment. Genetic deletion or pharmacological inhibition of P2X₇ receptors did not change survival rate or depletion of striatal endogenous dopamine (DA) content after *in vivo* MPTP or *in vitro* rotenone treatment. However, depletion of norepinephrine was significant after MPTP treatment only in P2X₇ knockout mice. The basal ATP content was higher in the substantia nigra of wild-type mice, but the ADP level was lower. Rotenone treatment elicited a similar reduction in ATP content in the substantia nigra of both genotypes, whereas reduction of ATP was more pronounced after rotenone treatment in striatal slices of P2X₇-deficient mice. Although the endogenous amino acid content remained unchanged, the level of the endocannabinoid, 2-arachidonoyl glycerol (2-AG), was elevated by rotenone in the striatum of wild-type mice, an effect that was absent in mice deficient in P2X₇ receptors.

Conclusions: We conclude that P2X₇ receptor deficiency or inhibition does not support the survival of dopaminergic neurons in *in vivo* or *in vitro* models of PD.

A57

The G protein-coupled receptor-associated protein 1 (GASP-1) regulates rimonabant-induced downregulation of GPR55

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Background: The G protein-coupled receptor 55 (GPR55) has recently been suggested to be responsible for those cannabinoid responses that could not be attributed to either the cannabinoid 1 (CB₁) or cannabinoid 2 (CB₂) receptor. Several potent GPR55 agonists were identified such as lysophosphatidylinositol (LPI) and several synthetic cannabinoids: One of these is rimonabant (SR141716A), an antagonist at the CB₁ receptor, which showed clinical promise, but approval was revoked due to adverse events. Generally, the activity of G protein coupled receptors (GPCRs) is coordinated by receptor signalling, receptor desensitization and receptor resensitization. One regulatory mechanism to guarantee appropriate GPCR expression levels in physiological conditions is that of downregulating GPCRs via the G protein-coupled receptor-associated sorting protein 1 (GASP-1), thus leading to an attenuation of cellular signalling events. GASP-1 was originally found to target δ opioid receptors to lysosomes and, hence, to the degradative pathway. It was shown that GASP-1 is a key determinant in the development of analgesic tolerance to cannabinoids via its role in facilitating downregulation of the CB₁ receptor.

Methods and results: By a variety of approaches we demonstrated that rimonabant promotes downregulation of GPR55 via GASP-1 *in vitro* and *in vivo*. We show that GPR55 interacts with GASP-1 *in vitro* and that disrupting the GPR55/GASP-1 interaction prevents post-endocytic receptor degradation, and thereby allows receptor recycling. Together, these data implicate GASP-1 as an important regulator of rimonabant-mediated downregulation of GPR55.

Conclusions: This work provides tangible evidence that GPR55 is degraded after prolonged agonist stimulation and that this mechanism is regulated by GASP-1.

A58

The D-type prostanoid (DP) receptor enhances the signaling of chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2)

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Background: Prostaglandin (PG) D₂ is substantially involved in allergic responses and signals via the seven-transmembrane-spanning/G protein-coupled receptors, chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) and D-type prostanoid (DP) receptor. While the proinflammatory function of CRTH2 is well recognized and CRTH2 is hence considered as an important emerging pharmacotherapeutic target, the role of the DP receptor in mediating the biological effects of PGD₂ in allergic inflammation has remained unclear.

Methods: The cross-talk of CRTH2 and DP receptors was investigated using both a recombinant HEK293 cell model and human eosinophils in Ca²⁺ mobilization assays, co-immunoprecipitation and radioligand binding assays.

Results: We show that CRTH2 and DP receptors modulate each other's signalling properties and form CRTH2/DP heteromers without altering their ligand-binding capacities. We find that the DP receptor amplifies the CRTH2-induced Ca²⁺ release from intracellular stores and, coincidentally, forfeits its own signalling potency. Moreover, desensitization or pharmacological blockade of the DP receptor hinders CRTH2-mediated signal transduction. Pharmacological blockade of G_{αq/11} proteins abolishes the Ca²⁺ response to both CRTH2 and DP agonists, while inhibition of G_{αi} proteins selectively attenuates the CRTH2-mediated response but not the DP signal.

Conclusions: Our data demonstrate the capacity of DP receptors to amplify the biological response to CRTH2 activation. Therefore, the CRTH2/DP heteromer may not only represent a functional signalling unit for PGD₂ but also a potential target for development of heteromer-directed therapies to treat allergic diseases.

A59

Effects of diclofenac on ventricular muscle repolarization: proarrhythmic implications

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BMC Pharmacology 2011, **11(Suppl 2)**:A59

Background: The aim of the present work was to characterize the electrophysiological effects of the non-steroidal anti-inflammatory drug diclofenac and to study the possible proarrhythmic potency of the drug in ventricular muscle.

Methods: Ion currents were recorded using the voltage clamp technique in canine ventricular cells, and action potentials (AP) were recorded from canine ventricular preparations using microelectrodes. The proarrhythmic potency of diclofenac was investigated in an anaesthetized rabbit proarrhythmia model.

Results: Diclofenac (30 μM) decreased the amplitude of rapid (I_{Kr}) and slow (I_{Ks}) delayed rectifier and L-type calcium currents (I_{Ca}) without influencing transient outward (I_{to}) and inward rectifier (I_{K1}) potassium currents. The action potential was slightly lengthened in ventricular muscle but shortened in Purkinje fibres by diclofenac (20 μM). The maximum upstroke velocity (V_{max}) was decreased in both preparations. Larger repolarization lengthening was observed when repolarization reserve was impaired by previous BaCl₂ application. Diclofenac (3 mg/kg) did not prolong the QT_c interval, while the potassium channel blocker dofetilide (25 μg/kg) significantly lengthened QT_c in anaesthetized rabbits. The combination of diclofenac and dofetilide

significantly prolonged QT_c. Diclofenac alone did not induce torsades de pointes ventricular tachycardia (TdP) while TdP incidence following dofetilide was 20%. However, the combination of diclofenac and dofetilide led to a significant increase in the incidence of TdP.

Conclusions: The results indicate that diclofenac, at therapeutic concentration and even at high dose, does not increase the risk of arrhythmia in normal heart. However, high dose drug treatment may enhance the proarrhythmic risk in the heart when the repolarization reserve is reduced.

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A60

Properties of the transient outward, ultra-rapid delayed rectifier and acetylcholine-sensitive potassium currents in isolated atrial myocytes from dogs: sinus rhythm and tachypaced model of permanent atrial fibrillation

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BMC Pharmacology 2011, **11(Suppl 2)**:A60

Background: Atrial fibrillation (AF) is a common and severe arrhythmia, which largely affects quality of life. State-of-the-art treatment of AF still relies heavily on pharmacological modalities. Therefore, the aim of the present study was to investigate and compare the properties of three repolarizing currents which contribute to AF-induced remodeling, i.e. the transient outward (I_{to}), ultra-rapid delayed rectifier (I_{Kur}) and acetylcholine-sensitive potassium currents (I_{K,ACh}) in isolated atrial myocytes obtained from normal (SR) and tachypaced model of permanent atrial fibrillation (ATR) dogs.

Methods: The tachypaced atrial fibrillation model was performed in dogs. Transmembrane ionic currents were investigated by applying the whole-cell patch clamp technique at 37°C, and ECG was recorded in conscious dogs.

Results: In all atrial canine myocytes, we have identified an I_{to} current sensitive to 4-aminopyridine (4-AP; 3 mM). The current inactivation was best fitted by two exponentials. The I_{to} current was slightly downregulated in ATR cells when compared with that recorded in SR cells. The I_{Kur} current, measured as sustained current (I_{ssus}), was upregulated in ATR dogs. However, the selective I_{Kur} blocker 4-AP (50 μM) did not block either I_{ssus} or I_{Kur} "like tail" currents, which questions the reliability of these results. I_{K,ACh} was activated by the cholinergic agonist carbachol (CCh; 2 μM). In SR, CCh activated a large current either at inward or outward directions. The selective I_{K,ACh} blocker tertiapin (10 nM) blocked the CCh-induced current by 57%. In atrial myocytes from ATR dogs we could measure the presence of a constitutively active I_{K,ACh}, which could be blocked by 26% with 10 nM tertiapin. However, in ATR atrial myocytes, CCh in addition could also activate a significant ligand-dependent and tertiapine-sensitive I_{K,ACh} current. Tertiapin effectively prevented burst-induced AF in conscious ATR dogs.

Conclusions: The presence of the constitutively activated I_{K,ACh} in atrial myocytes from ATR dogs shows that electrical remodeling developed in our model; this was further supported by the inducibility of AF by rapid atrial bursts in these dogs. The I_{K,ACh} current (both ligand-dependent and constitutively active currents) seems to play a significant role in the canine atrial electrical remodelling, and may be a promising drug target for suppressing AF.

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