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MEETING ABSTRACTS

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MEETING ABSTRACTS

Meeting abstracts:

Cardiovascular Pharmacology and Endocrinology

A1.1

Bioactivation of simvastatin by probiotic bacteria: *in vitro* study

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Background: Simvastatin is a lipid-regulating drug that acts as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor that reduces cholesterol production by the liver. It is administered perorally as a prodrug in the form of lactone (SVL) and is hydrolysed in the human body chemically or enzymatically to its open acid form, simvastatin acid (SVA). Due to great metabolic capacity of gut microflora and probiotic bacteria and their role in drug response, the aim of the study was to examine if selected probiotic bacteria may transform SVL into its active form.
Methods: The study was performed *in vitro*. A suspension of probiotic bacteria, *Lactobacillus acidophilus* and *Bifidobacterium longum* (10^8/ml), was incubated with SVL solution (50 µg/ml) for 24 h. In order to examine the intracellular, extracellular and total content of SVL and SVA after incubation, all samples were sonicated, centrifuged, processed and analysed by LC-MS/MS.
Results: The concentration of SVL decreased by 44% in total content (sum of extracellular and intracellular) after 24 h of incubation with selected probiotic bacteria. Semiquantitative and qualitative analysis of the incubation medium revealed a substantial amount of SVA. Regarding the distribution of compounds, the concentration of SVA after the incubation period was much higher in extracellular content while SVL accumulated to a greater extent intracellularly.
Discussion: The results of our study suggest that the selected bacterial strains, *L. acidophilus* and *B. longum*, lead to transformation of SVL into its active form SVA. This reaction may be mediated by hydrolytic enzymes that are present in selected bacterial strains. The distribution of compounds may be explained by their physico-chemical properties—the more hydrophilic properties of SVA do not make it a good candidate for transport through cell membrane compared to SVL, which is a highly lipophilic compound that accumulated predominantly intracellularly. Further *in vivo* studies are needed in order to provide more detailed insight into the effect of probiotic bacteria on the therapeutic response to simvastatin.
Acknowledgements: This research was supported by Horizon 2020 MEDLEM (project no. 690876), the Provincial Secretariat for Scientific and Technological Development of the Autonomous Province of Vojvodina (project no. 114-451-2072/2016-02) and the Ministry of Education, Science and Technological Development of the Republic of Serbia (project no. III41012).

A1.2

The association of NT-proBNP and asymmetrical dimethylarginine with all-cause mortality in long-term geriatric care patients

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Background: Increased asymmetrical dimethylarginine (ADMA), an endogenous NO synthase inhibitor, has been associated with increased mortality both in the population at cardiovascular (CV) risk and in the general population. The aim of the present study was to investigate the prognostic value of age, sex, BMI, and CV laboratory risk markers in long-term geriatric care residents.
Methods: In this prospective observational cohort study, the demographic data of all residents of "Haus der Barmherzigkeit", Vienna, including age, sex, admission diagnosis, height and weight, were collected. Routine blood samples were collected between 14.09.2009 and 16.12.2009. ADMA, its symmetric isomer SDMA, L-arginine, and NT-proBNP were determined at study entry.
Results: In total, 481 subjects were screened. Data from 449 subjects aged above 65 years were analyzed. From these, 381 subjects died during the observation period of 90 months. 320

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subjects had coronary heart disease, 409 subjects had peripheral artery disease, 335 subjects had a history of stroke, and 337 subjects had diabetes. From 449 subjects, data from 437 subjects were used for Cox regression analysis. Male gender, older age, lower BMI, and elevated plasma concentrations of ADMA, CRP and NT-proBNP were significant predictors of mortality.

Discussion: ADMA may be established as a risk marker for early overall mortality in geriatric care to enhance the prognostic value of plasma NT-proBNP in this group of elderly patients.

A1.3

Evidence from FDM-TIRF microscopy for endothelial ER–PM junctions harboring the Na^+/Ca^{2+} exchanger (NCX1) to serve privileged refilling of Ca^{2+} stores

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Background: The endoplasmic reticulum (ER)–plasma membrane (PM) junctions are of critical importance for many cellular processes such as lipid metabolism, protein synthesis and folding, and post-translation modification. These functions are tightly linked to the role of the ER as the main intracellular Ca^{2+} store and a pivotal regulatory element in cellular Ca^{2+} signaling. We have recently shown that privileged Ca^{2+} refilling of the endothelial ER via Ca^{2+} entry from the extracellular space involves a specialized junctional ER–PM contact [1]. We proposed that this process is critically dependent on junctional cooperation of NCX1 with the STIM–Orai signaling machinery in ER–PM regions. Taking advantage of a novel TIRF microscopy method that enables precise spatial delineation of subplasmalemmal organelle architecture, we set out to further analyze subcellular NCX1 localization in endothelial ER–PM junctions in detail.

Methods: We used the recently introduced FDM (fluorescence density mapping) TIRF microscopy approach to visualize ER junctional areas [2] in the subplasmalemmal space of cultured EA.hy926 (human umbilical vein endothelium-derived cells). Cells were genetically modified to express the FDM reporter RFP or the junctional marker MAPPER along with a GFP-NCX1 fusion protein.

Results: We demonstrate suitability of FDM-TIRF microscopy in endothelial cells. Our experiments identify a subpopulation of ER–PM junctions in EA.hy926 cells that hosts NCX1 and is likely to serve as the platform for STIM–Orai Ca^{2+} entry.

Discussion: Our results provide strong evidence for privileged ER Ca^{2+} refilling by a subplasmalemmal Ca^{2+} control unit that requires a specialized ER–PM junctional architecture harboring NCX1. This signaling element may be considered an attractive therapeutic target to preserve or restore endothelial functions.

Acknowledgements: This project has received funding from the European Union's Seventh Framework Programme for research and technological development under grant agreement no. PIIF-GA-2012-330657 (QuMoCa project) and the Austrian Science Fund FWF (grant W1226).

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A1.4

The unique characteristics of segment IS4 in voltage-gated calcium channel $Ca_V1.2$ inactivation

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Background: Voltage-dependent inactivation of the voltage-gated calcium channel $Ca_V1.2$ develops during the plateau phase of the cardiac action potential and enables timed repolarization and tuned calcium entry. Recently solved structures of $Ca_V1.1$ provided the first models for interpretation of opening/closing behavior in the Ca_V1 family on a structural basis. However, the molecular events during $Ca_V1.2$ inactivation remain unclear. There exists a correlation between the positions of the steady-state activation and inactivation curves in this channel; this may reflect two scenarios: either conformational changes in the pore trigger voltage-dependent inactivation by allosterically changing to the selectivity filter, or, both processes are coupled via conformational changes in the voltage-sensing domains (VSDs).

Methods: In order to investigate the above given hypotheses, we used $Ca_V1.2$ constructs with fully or partially neutralized charges in S4 segments to elucidate the role of VSD I–IV in voltage-dependent inactivation. Charged arginines or lysines were replaced by glutamines in down-stream direction using site-directed mutagenesis. Ionic currents were recorded using patch-clamp measurements. Furthermore, homology models of all four voltage-sensing domains were generated.

Results: (i) Compared to the lower impacts of IIS4 and IIIS4, neutralization of IS4 charges induced pronounced changes in voltage-dependence and kinetics of inactivation. (ii) Neutralization of IS4 charges gradually reduced the slope factors of the inactivation curves and accelerated the inactivation kinetics. (iii) Shifts of the inactivation curves induced by IS4 neutralization correlated with shifts of the activation curve.

Discussion: Bezanilla and Villalba-Galea [1] show that to estimate the effective charge using a Boltzmann fit is not precise, if the S4 movement occurs in multiple steps via sub-states. Given this assumption, the distribution between inactivated and open channels would be shallower than predicted by a two-state Boltzmann function. It is thus tempting to speculate that IS4 in $Ca_V1.2$ channel moves via sub-states that are themselves stabilized by interactions of arginines (and lysines) with their surrounding environment. Replacing these charged residues by neutral glutamines would consequently disrupt these interactions, thereby reducing the number of IS4 sub-states and decreasing the slope factor of the inactivation curve.

Acknowledgements: The research was funded by the Austrian Science Fund FWF (grant P27729). S.A. is a student of the FWF doctoral program "Molecular Drug Targets" (W1232).

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Immunopharmacology and Infection

A2.1

The pharmacodynamic effects of rituximab at very low doses in healthy volunteers

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Background: No dose-finding trials are available for rituximab that could guide dosing in non-malignant diseases. We hypothesized that currently used doses (≥ 375 mg/m^2) exceed several hundred-fold the half-maximal effective dose, which is most sensitive for detecting putative differences between biosimilars and important for dose-finding.
Methods: In an exploratory, dose-finding trial, healthy volunteers received single infusions of 0.1 ($n = 4$), 0.3 ($n = 4$) and 1.0 mg/m^2 ($n = 8$) rituximab. Subsequently, in a randomized, double-blind trial, healthy volunteers received single infusions of 0.1 ($n = 24$) or 0.3 mg/m^2 ($n = 12$) of two rituximab products. CD19/20$^+$ cell counts were measured, and pharmacokinetics and immunogenicity were assessed.
Results: Single infusions of 0.1, 0.3 and 1 mg/m^2 rituximab transiently depleted CD20$^+$ cells by 68% (95% CI: 24–100%), 74% (59–84%) and 97% (95–99%), respectively. In the randomized trial, infusion of 0.1 mg/m^2 or 0.3 mg/m^2 proposed biosimilar or reference rituximab decreased CD20$^+$ cells by 45% (32–58%) – 55% (45–66%), and 81% (73–87%) – 87% (74–100%), respectively. In the randomized trial, 26 of 36 patients developed human anti-chimeric antibodies, and 9 of 36 patients developed neutralizing anti-drug antibodies. Pharmacokinetic analyses were limited by the assay sensitivity and the very low rituximab doses. However, there was a clear pharmacokinetic signal during the first 24 hours in the 1 mg/m^2 group.
Discussion: It is important to understand that <1% of the authorized rituximab doses depletes all circulating B lymphocytes in healthy volunteers. This will particularly help countries struggling to meet the financial burden of therapy with biologics.
Acknowledgements: This trial was funded by Apotex Inc., Toronto, Ontario, Canada.

A2.2

Lysophospholipid-enriched HDL suppresses platelet activation

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Background: Secretory phospholipases A_2 (sPLA$_2$) are enzymes that hydrolyze the sn-2 ester bond in phospholipids generating nonesterified free fatty acids and lysophospholipids. sPLA$_2$ levels are highly increased under inflammatory conditions, and epidemiologic studies showed strong associations between elevated sPLA$_2$ levels and several inflammatory diseases. sPLA$_2$ is mainly associated with high-density lipoproteins (HDL), which are the principal plasma carriers of phospholipids and major substrates for sPLA$_2$. Surprisingly, clinical trials testing sPLA$_2$ inhibitors failed and sPLA$_2$ inhibition was associated with an unexpected 60% increased risk of myocardial infarction and stroke. Platelets are best known as main mediators of hemostasis and thrombosis. However, they are also potent immune modulators and can promote inflammation, which can be detrimental. HDL directly interacts with platelets and regulates their function. However, very little is known about the effects of inflammation-induced changes in HDL composition (especially enrichment with lysophospholipids) on HDL–platelet crosstalk and fundamental platelet responses and immune interactions. Therefore, we investigated the effects of sPLA$_2$-mediated modification of HDL on platelet function, a critical player in atherosclerosis and inflammation.
Methods: Platelets were isolated from peripheral blood from healthy human volunteers. Platelet aggregation was measured by light transmission aggregometry. P-selectin expression, GPIIb/IIIa activation, and Ca^{2+} flux were measured by flow cytometry. Kinase phosphorylation was assessed by western blot.
Results: Treatment of HDL with sPLA$_2$ (sPLA$_2$-HDL) resulted in the formation of palmitoyl-lysophosphatidylcholine (LPC 16:0) and stearoyl-lysophosphatidylcholine (LPC 18:0) as the most prominent LPC species. sPLA$_2$-HDL rapidly inhibited platelet aggregation, P-selectin expression and GPIIb/IIIa activation. Moreover, sPLA$_2$ treatment of HDL inhibited Ca^{2+} flux as well as ERK and Akt phosphorylation. Enrichment of native HDL with LPC 16:0 and LPC 18:0 mimicked sPLA$_2$-HDL effects.
Discussion: Overall, our studies suggest that the suppression of rise in intracellular Ca^{2+} levels and inhibition of kinase phosphorylation are likely mechanisms that counteract agonist-induced activation of platelets. Our results raise the possibility that sPLA$_2$-induced modification of HDL composition and function modulates platelet function during inflammation.
Acknowledgements: The work was supported by the Austrian Science Fund FWF (P22976 and P22521) and the Austrian National Bank (grant no. 14853).

A2.3

Dose optimization of antibacterials: plasma *vs.* tissue concentrations

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Background: The outcome of antibiotic treatment depends on several complex interactions between the infectious agent, the antimicrobial drug and host defence mechanisms, and treatment outcome shows high variability of the dose–response relationship.
Methods: A thorough search of available databases using the terms “antibacterials”, “antibiotics”, “PK/PD”, “dose optimization”.
Results: Dosing regimens for antibacterials should be designed using integrated pharmacokinetic/pharmacodynamic approaches especially in case of serious systemic infections and in certain groups of patients whose present condition may influence the usual activity of antibacterial drugs. Knowledge of antibiotic pharmacokinetic and

pharmacodynamic properties can help clinicians in choosing antibiotic and dosing regimen that are linked to the highest likelihood of treatment success. Three measures of drug exposure are commonly used to link drug exposure with bacterial killing: the fraction of the dosing interval that the concentration of unbound (free) drug is greater than MIC, the ratio of the area under the unbound drug concentration–time curve to the MIC, and finally the ratio of the peak unbound-drug concentration during a dosing interval to the MIC. However, as the PK/PD indices are based on plasma concentration, for antibiotics that do not penetrate into site of the infection sufficiently, the pharmacokinetic/pharmacodynamic values may overestimate the expected efficacy. Microdialysis (μD) is an *in vivo* sampling technique that has been successfully applied to measure the distribution of antibiotics in the interstitial fluid of various tissue sites both in animal studies and clinical setting. μD enables continuous *in vivo* sampling and provides direct measurement of unbound concentration–time profile of antibacterials in the interstitial fluid.

Discussion: An antibacterial drug can exert its therapeutic action only with adequate penetration at the infection site. Multiple factors, such as rate of protein binding, drug liposolubility and organ blood flow all influence the ability of antibiotics to penetrate into target tissues. Clinical μD studies demonstrated that tissue concentrations for certain antibiotics and clinical conditions may be suboptimal despite adequate plasma levels. Standard tissue concentration measures based on tissue homogenates offer only values for total drug concertation (intracellular and extracellular) while for the majority of infections the interstitial free concentrations are relevant. Integration of μD-derived tissue pharmacokinetics with pharmacodynamic data offers crucial information for correlating exposure with antibacterial effect. Using μD-derived information for consecutive pharmacokinetic/pharmacodynamic models is increasingly becoming state-of-the-art for optimizing dosing regimens of antibiotics.

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A2.4

Pharmacokinetics of trimethoprim-sulfametrole during continuous haemofiltration

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Background: Trimethoprim-sulfametrole (TMP-SMT, Rokiprim®) is a combination with a broad antimicrobial spectrum comprising numerous Gram-positive and Gram-negative bacteria as well as *Pneumocystis jirovecii*. In critically ill patients, the most important indications are respiratory tract infections caused by highly resistant pathogens such as *Pneumocystis jirovecii* or *Stenotrophomonas maltophilia*. Renal failure with an indication for continuous haemofiltration (HF) is common in critically ill patients. The influence of HF on pharmacokinetic on SMT is unknown so far, and data on TMP is scarce. The chemical and pharmacokinetic properties of SMT and TMP, however, suggest relevant elimination by HF that may lead to subtherapeutic plasma levels.

Methods: Pharmacokinetics were determined in plasma and ultrafiltrate samples of patients on HF and of patients with approximately normal renal function off HF. In addition, the extracorporeal clearance by HF (CL_{HF}) was calculated using pre- and post-filter plasma levels, and from the sieving coefficient (*SC*). TMP-SMT was measured by high-pressure liquid chromatography and UV detection after sample preparation by solid phase extraction (SPE). SMT was detected at 250 nm and TMP at 306 nm. Quantification was validated according to the European Medicine Agency (EMA) guidelines. The lower limit of quantification was 0.5 mg/l (SMT) and 0.1 mg/l (TMP).

Results: So far, two patients requiring HF and four patients off HF have been enrolled. In one patient on HF, sampling was performed after the first dose. The other patient on HF as well as the patients off HF were at steady state. For SMT, $t_{½}$ was 7.4 h and 10.2 h, total *CL* amounted to 1.9 l/h and 2.2 l/h, and apparent volume of distribution during terminal phase (V_z) was 20.5 l and 7.7 l, after single and repeated dose, respectively, on HF. Off HF SMT, $t_{½}$ amounted to 9.7 ± 3.3 h (mean ± standard deviation), total *CL* was 0.8 ± 0.4 l/h and V_z was 4.35 ± 2.11 l. For TMP, $t_{½}$ was 10.4 h and 26.9 h, total *CL* was 5.9 l/h and 5.3 l/h, and V_z was 87.8 l and 20.0 l after single and repeated dose, respectively on HF. Off HF, a $t_{½}$ of TMP of 16.7 ± 7.9 h, a total *CL* of 4.0 ± 1.5 l/h and a V_z of 22.6 ± 9.3 l were observed. For SMT, CLHF was 1.6 l/h and 1.8 l/h (84% and 68% of total *CL*) after single and multiple doses, respectively. CL_{HF} of TMP amounted to 1.8 l/h and 1.9 l/h (31% and 30% of total *CL*), respectively. CL_{HF}, calculated from *SC*, was somewhat lower for both drugs, suggesting that moderate adsorption to the haemofilter takes place.

Discussion: Considerable amounts of SMT and TMP are eliminated by HF resulting in an enhanced total *CL* in comparison with patients off HF. CL_{HF} was similar for SMT and TMP. If this is confirmed by data from a larger number of patients, higher doses have to be considered for this clinical condition.

Acknowledgements: The study was supported by Rokitan, Vienna.

A2.5

Quantification of the antifungal anidulafungin in human brain tissue

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Background: The echinocandin anidulafungin shows fungistatic activity against *Aspergillus* spp. and fungicidal activity against *Candida* spp. It is recommended for treatment of invasive candidiasis in critically ill patients. Treatment of *Candida* infections of the central nervous system (CNS) with echinocandins, however, is discouraged because of their poor CNS penetration in animal models. Data on anidulafungin penetration into human brain are lacking so far. We therefore addressed the question whether effective anidulafungin concentrations can be achieved in human brain by standard doses.

Methods: Human brain tissue samples were taken at routine autopsies of deceased who had been treated with standard dose of anidulafungin for proven or suspected invasive candidiasis. Anidulafungin was measured by high-pressure liquid chromatography and UV detection. Sample preparation was performed by mechanical homogenisation, followed by solid-phase extraction. Anidulafungin quantification was done by triplet measurements ($n = 3$). Quantification was validated according to the European Medicines Agency (EMA). The lower limit of quantification (LLOQ) was 0.05 µg/g.
Results: So far, autopsy samples have been obtained from four deceased. The time between the last anidulafungin infusion and death ranged from 11.5 h to 299 h. Treatment duration was between 6 and 17 days. The cumulative doses ranged from 700 to 1,800 mg. The highest concentration (0.55 µg/g) was detected in patient 3 who died 11.5 h after the last infusion. Anidulafungin brain concentration in patient 2 was below LLOQ. She had received her last anidulafungin infusion 299 h before death.
Discussion: Anidulafungin brain concentrations, however, were low in all autopsy samples. They were comparable to the levels previously reported from animal studies. Anidulafungin was detectable even 11 days after the last administration, although its concentration was below 0.05 µg/g at this time. Thus, our preliminary results do not support the use of anidulafungin in CNS infections.
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A2.6

Impact of erythrocytes and thrombocytes on bacterial growth and antimicrobial activity of selected antibiotics

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Background: The efficacy of antibiotics is often predicted by *in vitro* pharmacodynamic (PD) models. Host factors such as protein binding, temperature and pH have already shown that they influence the reliability of these existing models. However, the impact of corpuscular blood compounds is not well understood and therefore often neglected.
Methods: We set out to investigate if addition of human erythrocytes or thrombocytes to standard growth media (Mueller-Hinton Broth, MHB) has an influence on bacterial growth behavior and on the efficacy of antibiotics by using bacterial growth assays and time–kill curves of selected strains; *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853. Experiments were performed in triplicates over 24 hours. The final concentration of the corpuscular blood components in our experiments was set to physiological concentrations in blood of healthy human, *i.e.* an erythrocyte concentration of 3×10^6 cells/µl in cation-adjusted MHB and 2.5×10^5 thrombocytes/µl in MHB; 20 mM Ca^{2+} were used. At an infection site, thrombocytes are found to be activated. Calcium is capable to activate thrombocytes; therefore, an adjusted calcium level was set in the MHB to resemble accurate conditions. Meropenem, ciprofloxacin and tigecycline were tested as representative of broad-spectrum antibiotics with very different chemical and pharmacokinetic characteristics. Concentrations several-fold above and below the minimal inhibitory concentration (MIC) were simulated.
Results: We could confirm that erythrocytes slightly promoted bacterial growth (between 0.32 and 0.80 $\log_{10}$) and decreased antibiotic efficacy in dependence on bacterial species and the type of antibiotic in most assays. Addition of erythrocytes to MHB decreased bacterial killing after 24 hours (ratio of 24 h *vs.* baseline) for most bacterial species and tested antibiotics (delta 0.46 to 4.76 $\log_{10}$). *P. aeruginosa* tested with meropenem is an exception and showed the opposite effect with a delta of −1.27 $\log_{10}$. Tests with thrombocytes revealed no difference in bacterial growth assays. Likewise, addition of 20 mM Ca^{2+} to MHB did not influence bacterial growth. Addition of thrombocytes led to a reduction of antimicrobial activity of meropenem and ciprofloxacin for all bacterial strains. Addition of thrombocytes to MHB decreased bacterial killing after 24 hours in all bacterial species tested with meropenem and Ciprofloxacin (delta 1.30 to 2.37 $\log_{10}$). Experiments with tigecycline showed delta values around zero. Tests with *P. aeruginosa* followed the trend of a decrease in bacterial killing with a delta of 0.42 $\log_{10}$. A slightly positive effect on bacterial killing was seen with *E. coli* (delta −0.2 $\log_{10}$) and *S. aureus* (delta −0.94 $\log_{10}$).
Discussion: It can be hypothesized that decreased antimicrobial activity of antibiotics in the presence of erythrocytes may be caused by binding or intracellular accumulation in erythrocytes. The reduced activity caused by thrombocytes might also be due to binding and thereby reduced free levels of antibiotics. Whether activation and the consecutive change in confirmation or agglutination enhances this impact remains to be investigated. In conclusion, we have demonstrated that corpuscular blood components influence antimicrobial activity, which might have an impact with regard to extrapolation of *in vitro* activity testing to *in vivo* efficacy in patients.

Molecular Pharmacology, Oncology, Toxicology

A3.1

Identification and characterization of the *Fasciola hepatica* taurine transporter

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Background: The liver fluke (*Fasciola hepatica*) is a parasite infecting mainly livestock animals world-wide; metacercariae are ingested and the developing juvenile flukes invade the liver via the peritonel cavity. The drug of choice to treat fascioliasis is triclabendazole (TBZ), which has been on the market for several decades. Predictably, resistant fluke strains have emerged. Here, we focused on the taurine transporter of *F. hepatica* (*Fh* TauT) as a candidate drug target. The working hypothesis underlying our approach posits that, because the vast majority of the fluke surface is exposed to bile, *F. hepatica* requires protective defense mechanisms against bile acids. We surmised that taurine uptake served as one of these defense mechanisms; thus, a taurine transporter supplies a substarte to conjugate free bile acids, which reduces their toxicity.
Methods: The full-length cDNA encoding *Fh* TauT was cloned from reverse-transcribed mRNA of flukes (from Lower Austria) and inserted into a mammalian expression vector: *Fh* TauT and its human counterpart (*Hs* TauT) were tagged on their N-terminus with YFP (yellow fluorescent protein) and expressed heterologously in HEK 293 cells. The celular uptake of [^{3}H]taurine was analysed to K_M values of the substrate and the co-transported ions (Na^+ and Cl^-). Antibodies recognizing the transporters were generated by immunizing rabbits with unique peptides. Finally, flukes were exposed to a medium containing bile acids in the absence and presence of taurine and the TauT inhibitor guanidino ethane sulfonic acid (GES) to detremine their survival rate.

Results: A phylogenetic analysis showed that *Fh* TauT is more closely related to human GABA transporter-2 than to *Hs* TauT. Nevertheless, *Fh* TauT transports negligible amounts of [^{3}H]GABA and its affinity for taurine (K_M = 12.0 ± 0.5 µM) is higher than that of *Hs* TauT (K_M = 41.0 ± 10.4 µM). As expected for a eukaryoric SLC6 transporter, *Fh* TauT requires two Na^+ and one Cl^- to support substrate uptake. GES is a competitive inhibitor of *Hs* TauT. Interestingly, we observed that GES was a non-competitive inhibitor of *Fh* TauT. An antibody raised against the C-terminus of *Fh* TauT recognized a prominent band on immunoblots. This antibody was used for immunohistochemistry to locate the transporter in the tegumental cells consistent with a role of *Fh* TauT in mediating defense against bile acids. This conjecture was further verified by incubating flukes with unconjugated bile acids in the presence and absence of taurine and of GES: taurine promoted survival of flukes and this was abolished by GES.
Discussion: Our observations indicate that *Fh* TauT has evolved from the putative common ancestor of GABA and taurine transporters to become a high-affinity taurine transporter, which requires only low Cl^- concentrations. Thus, *Fh* TauT is adapted to conditions that prevail in bile. Our observations are also consistent with the assumption that *Fh* TauT is required to protect flukes against bile acids. Selective inhibitors of *Fh* TauT should therefore be of interest as an alternative to triclabendazole.
Acknowledgements: This work was supported by a grant from NÖ Forschungs- und Bildungsges.m.b.H. (NFB project LS12-019) and the Gaston H. Glock Foundation.

A3.2

Hepatoprotective and antioxidant potential of apigenin in paracetamol-induced hepatotoxicity in rats

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Background: Apigenin is known to have various pharmacological properties without causing significant toxicity. However, hepatoprotective effects of apigenin are not often reported. The aim of our study was to investigate whether the alterations in lipid peroxidation and antioxidant status are in favor to prove the efficacy of apigenin against paracetamol-induced hepatotoxicity.
Methods: The effect of apigenin on paracetamol-induced hepatotoxicity was examined in rats by determining biochemical parameters, histological assessment and oxidative status in liver homogenates.
Results: Treatment of animals with apigenin attenuated the parameters of paracetamol-induced hepatotoxicity, especially for ALT and ALP activity, which was significantly lower compared to animals treated with saline and instead of paracetamol. Hepatotoxicity induced by a toxic dose of paracetamol was revealed also by notable histopathological alterations, which were not observed in the group treated with paracetamol together with apigenin. Apigenin also prevented paracetamol-induced increases in malondialdehyde (MDA) levels. The activities of both catalase (CAT) and glutathione reductase (GR) in liver homogenates were significantly increased after a toxic dose of paracetamol compared to the control group. Apigenin reversed these parameters to values near to those of the control group.
Discussion: According to the results of our study, hepatotoxicity induced by a toxic dose of paracetamol was revealed not only by a noticeable elevation of AST and ALT activities, but also by notable histopathological alterations. As expected, these alterations were not detected in the group treated with paracetamol together with apigenin. Our study is comparable with the results of an earlier study using a model of *N*-nitrosodiethylamine (NDEA)-induced liver damage, where necrosis of hepatocytes was confirmed histologically. Apigenin showed an ability to prevent paracetamol-induced increases in MDA levels, which suggests that apigenin can preserve cellular integrity. Previous findings also showed that the administration of apigenin in NDEA-induced and phenobarbital-promoted hepatocarcinogenesis in rats could decrease lipid peroxidation. In our study, the activities of antioxidant enzymes were significantly changed after the administration of paracetamol. The antioxidant enzymes GPx and CAT catalyze the reduction of peroxides to alcohols or water, whilst GR reduces glutathione disulfide (GSSG), generated during the reduction of peroxides, to the sulfhydryl form of glutathione (GSH). A toxic dose of paracetamol induced a significant reduction of GR activity, which might be explained by inactivation induced by extreme creation of free radicals. Apigenin exhibited an ability to reverse GR activity to values near those of the control group. CAT activity was increased significantly after paracetamol, while apigenin restored the changes near to values of the control group. On the contrary, in the early study enzymatic antioxidant CAT was decreased significantly in carcinogen-administered animals; however, apigenin restored the changes to near normal by its antioxidant activity.
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Neuropharmacology and Neurosciences

A4.1

The mechanisms of folding and trafficking of the human dopamine transporter (hDAT) and its disease-causing mutants

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Background: The dopamine transporter (DAT) is responsible for sequestering released dopamine from the synaptic cleft. DAT is a target of therapeutically relevant and illicit recreational drugs. Point mutations within the coding sequences of human membrane proteins can result in their retention in the endoplasmic reticulum (ER) and thus may lead to clinically relevant phenotypes. Folding-defective mutants of the human dopamine transporter (hDAT) cause a syndrome of deficiency, infantile parkinsonism-dystonia (IPD).
Methods: We created 13 mutations responsible for IPD by site-directed mutagenesis. HEK 293 cells were transiently co-transfected with plasmids encoding the wild-type dopamine transporter and IPD mutants using jetPRIME (Polyplus). Radioligand dopamine uptake, confocal laser scanning microscopy and immunoprecipitation experiments were performed 48 h after transfection to study the pharmacochaperonig effect on folding of wild-type and mutant DATs.
Results: Confocal microscopy experiments indicated that all 13 mutated DATs were retained in intracellular compartments, namely in the ER, since they co-localized with an ER-resident chaperone, calnexin. Regarding their functional activity, none of the mutants showed any appreciable dopamine uptake compared to the wild-type DAT. Interestingly, 3 of the mutants could be functionally rescued, *i.e.* they responded to treatment by pharmacological chaperones, noribogaine (a non-competitive DAT inhibitor) and pifithrin-µ (an

inhibitor of the heat shock protein HSP70). The combination of noribogaine and pifithrin-μ produced the largest increase in the mature core-glycosylated form of hDAT. Pretreatment of cells with noribogaine and pifithrin-μ is predicted to reduce the association of hDAT mutants with calnexin and HSP70-1A. In addition, experiments were also done to examine these effects in flies (*Drosophila melanogaster*) carrying the IPD hDAT mutations. Flies harboring specific mutations in DAT are known to exhibit sleep deficiency, resembling a DAT knock-out phenotype. We rescued two mutants (V158F and G327R) *in vivo*. In the case of L368Q, flies were not able to hatch due to unknown reasons. We also found that DAT relied on the exocyst complex to reach the cell surface. After studying the effect of different components of the exocyst complex we came to the conclusion that three components of the exocyst complex, Sec6, Sec8 and Exo70, individually control trafficking of DAT. The same will be examined for the IPD mutants after pharmacological rescue with noribogaine and pifithrin-μ.

Discussion: The current research work not only provides a systematic *in vitro* and *in vivo* approach for screening IPD-causing DAT mutants, but also consolidates the fact that pharmaco-chaperoning can be used to remedy disease-causing folding deficiencies in SLC6 transporters. We also studied that exocyst mediates DAT targeting to the presynaptic membrane. Identification of proteins as DAT interactors along with the molecular bases and physiological significance of such interactions will result in a better understanding the role DAT plays in regulating dopamine homeostasis in the brain.

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A4.2

129S1/SvlmJ mouse model of impaired fear extinction shows abnormal sleep architecture

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Background: Post-traumatic stress disorder (PTSD) is a psychiatric disorder that may develop after exposure to a traumatic event. However, it remains unclear why only about 10–20% of trauma victims develop the disease in the later course. Sleep disturbances in particular constitute a hallmark of PTSD, but it is not known whether impaired sleep is a secondary symptom or a core feature. Sleep has been strongly implicated in memory consolidation, and emerging evidence suggests that in PTSD sleep disturbances interfere with fear memory processing, most likely by impairing fear memory extinction.

Methods: To address the assumption that disturbed sleep *per se* contributes to the development of PTSD we evaluated circadian sleep/wake behavior of 129S1/SvlmJ (S1) mice, which have a well-documented deficit in fear extinction, and a C57BL/6N (BL6) control group. Electroencephalogram (EEG) and electromyogram (EMG) activities were recorded chronically prior to and following contextual fear conditioning. Animals were also assessed for fear expression during exposure to the aversive unconditioned stimulus (US) consisting of a footshock, and during a recall session.

Results: Our results revealed that baseline sleep/wake behavior of S1 mice differed significantly from that of BL6 mice, especially in the active period. Here, S1 animals spent significantly more time sleeping with increased amounts of non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS). In line with these findings also sleep fragmentation was distinct from that in BL6 mice. Contextual fear conditioning seemed to affect sleep/wake behavior in both groups differently. Most interestingly, after exposure to the aversive stimulus BL6 animals showed an elevation in REMS in the inactive period, while REMS amount in S1 mice appeared to be unaffected. Moreover, also changes in spectral power densities following the fear conditioning protocol were significantly different in S1 and BL6 mice, pointing to a poorer sleep quality in S1 mice.

Discussion: Our findings strongly suggest that altered baseline sleep and an impaired adaptation of sleep behavior after exposure to an aversive stimulus might interfere with fear memory processing in the S1 mouse model. This in turn may predispose these animals towards the development of a pathological PTSD-like phenotype. Generally, our data are in line with the recent theory that impaired sleep, and in particular altered REMS behavior, prior to and in the early aftermath of a trauma may represent a risk factor for PTSD. Therefore, we propose that further evaluation of such sleep disturbances in humans may provide a vantage point for the development of prognostic and early diagnostic biomarkers of PTSD to identify high-risk individuals and facilitate immediate treatment of trauma-exposed persons.

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A4.3

K_V7 and TRPV1 channels: neuronal targets for the analgesic action of paracetamol

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Background: Paracetamol/acetaminophen (APAP) is a commonly used analgesic whose site and mechanism of action remain controversial. 5–15% of APAP is converted into a reactive intermediate, NAPQI (*N*-acetyl-*p*-benzoquinone imine), by cytochrome P450 enzymes. Neuronal subtypes of voltage-gated potassium channels (K_V7 family) give rise to the characteristic M current, which plays an important role in regulating neuronal excitability and has translational significance in pain management. Similarly, TRP channels, specifically TRPV1 and TRPA1 play critical roles in processing nociceptive input. Therefore, effects of APAP and its metabolites were investigated on these ion channels in dorsal root ganglia (DRG) and spinal dorsal horn (SDH) cultures prepared from rats.

Methods: Electrophysiological recordings were made using the perforated patch-clamp technique.

Results: Currents through K_V7 channels in SDH- and TRPV1-positive DRG neurons showed an irreversible enhancement up to 250% and 120% of control with 3 μM NAPQI, respectively. TRPV1 currents in DRG neurons were irreversibly enhanced up to 200% of control with 3 μM NAPQI. Application of 1 μM NAPQI for 10 minutes resulted in an initial period of depolarization followed by significant hyperpolarization of the membrane potential in 70% of DRG neurons while the others showed a persistent hyperpolarization irrespective of their TRP status. There was a concomitant decrease in the excitability of DRG neurons. DRG neurons showed a depolarization of the membrane potential on application of 30 μM linopirdine, an antagonist

of K_V7 channels, which was adjusted for; on further co-application with 1 µM NAPQI for 10 minutes, the membrane potential steadily depolarized with no significant change in excitability. Another APAP metabolite, AM404 (10 µM) did not affect the magnitude of the K_V7 current but caused a significant depolarization of the membrane potential in DRG neurons without affecting excitability. Persistent hyperpolarization of the membrane potential and a significant decrease in excitability in response to 1 µM NAPQI for 10 minutes was seen in SDH neurons. 100 µM APAP applied alone did not affect any of these parameters.

Discussion: These results suggest that the analgesic action of APAP may involve an enhancement of K_V7 and TRPV1 currents by NAPQI as an active metabolite.

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A4.4

Role of monoamines in the mouse basolateral amygdala in the regulation of fear and anxiety

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Background: The amygdala has been identified as a key player in aversive learning and memory processes. Although our understanding of intra-amygdala connectivity and synaptic plasticity has considerably grown in recent years, the modulatory influence of monoaminergic systems in the amygdala still remains to be fully elucidated. The main catecholaminergic systems, dopamine (DA) and noradrenaline (NA), provide a highly discrete innervation to the functionally distinct subnuclei of the amygdala. While NAergic projections are quite homogenously distributed, DAergic fibres densely innervate GABAergic cell populations (*i.e.* intercalated cells and central amygdala), suggesting a substantial influence of DAergic signaling on amygdaloid information processing and output.

Methods: We stereotactically delivered the neurotoxin 6-hydroxydopamine (6-OHDA) into the basal amygdala (BA) of C57BL6/J mice to selectively lesion midbrain DA neurons innervating the amygdala and associated limbic structures.

Results: Stereological analysis of the two main mesencephalic DA nuclei, the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA), revealed that intra-BA 6-OHDA injections resulted in a significant cell loss in the SNc, questioning the widely accepted fact that the DAergic innervation of the amygdala is mainly derived from the VTA. The DAergic denervation compromised most amygdala substructures (except the central nucleus) as well as the ventral hippocampus, but not the prefrontal cortex. Intra-BA 6-OHDA injections also concomitantly produced a nearly complete loss of noradrenergic terminals in the BA. The loss of monoaminergic signaling in these structures produced an anxiogenic phenotype, but did not affect motor performance and fear learning and memory.

Discussion: Our results bring new light on the role of monoamines in the amygdala on emotional behaviors. Our findings also suggest a substantial participation of mesolimbic SNc neurons in the DAergic innervation and control of amygdala circuits. Further studies will have to clarify the exact origin of DAergic innervation to functionally distinct amygdala subnuclei in order to understand their potential role in anxiety-related behavior.

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A4.5

The selective κ-opioid receptor partial agonist HS666 produces antinociceptive, antiseizure and anticonvulsant effects without causing sedation or motor dysfunction after systemic administration in mice

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Background: Differential modulation of the κ-opioid (KOP) receptor is nowadays regarded as a promising strategy for developing pharmacotherapies for human disorders including pain, drug addiction, mood disorders (***e.g.*** depression and anxiety), neurological conditions (***e.g.*** epilepsy), and itching skin and inflammatory diseases. Accumulating evidence indicates that KOP receptor-mediated beneficial effects (antinociception, anticonvulsive, anti-pruritus) result from G protein-mediated signaling events, while alternative signaling pathways (*i.e.* β-arrestin-2) may promote adverse effects (dysphoria, sedation, motor dysfunction). The concept of biased agonism at the KOP receptor has gained significance to drug discovery, with G protein-biased KOP agonists emerging as prospective drugs with an improved benefit/risk profile. Encouraged by the recent *in vivo* findings on a new ligand from the class of diphenethylamines, HS666, a selective KOP partial agonist, with reduced liability for sedative/motor impairment and aversive effects after central (intracereboventricular) administration to mice, correlating to its low efficacy in the β-arrestin-2 signalling pathway *in vitro*, we further investigated the pharmacology of HS666 after systemic administration in mice.

Methods: Antinociceptive activity was assessed in the mouse acetic acid-induced writhing test. Pentylenetetrazole (PTZ)-induced acute seizures were induced in pDyn-knockout mice, and the kainic acid-induced model of temporal lobe epilepsy was performed in wild-type mice. The rotarod test was used for assessing sedation and the potential loss of coordinated locomotion.

Results: Dose-dependent inhibition of the writhing response was produced by HS666 after subcutaneous administration with an antinociceptive potency (ED_{50} = 3.23 mg/kg) comparable to the standard KOP agonist U50,488. Intraperitoneal administration of HS666 increased the threshold for PTZ-induced seizures and reduced paroxysmal activity in the mouse model of intra-hippocampal injection of kainic acid in a dose-dependent manner. The antinociceptive and antiseizure/anticonvulsant effects of HS666 after systemic administration were completely blocked by pre-treatment with the selective KOP antagonist nor-binaltorphimine, demonstrating a KOP receptor-mediated mechanism of action. HS666 did not impact motor performance of mice at therapeutic doses.

Discussion: The current results establish the specific KOP receptor-mediated beneficial effects (antinociceptive and antiseizure/anticonvulsant) of HS666 following systemic administration in mice and its reduced liability for adverse effects. In summary, these findings offer valuable insights into the discovery of drugs targeting the KOP receptor with improved pharmacological profiles and enhanced therapeutic efficacies for the treatment of human disorders.

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A4.6

Partial releasers rescue misfolded serotonin transporters by conformational trapping

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Background: Transporters for serotonin (SERT) and dopamine (DAT) display promiscuous binding to a vast array of exogenous ligands. These include napthylpropane-2-amine analogues (PAL series): some of these have been characterised as 'partial releasers' because their efficacy in inducing neurotransmitter efflux through SERT and DAT is lower than that of amphetamines. We hypothesize that this occurs due to the ability of partial releasers to trap SERT/DAT in certain conformational states during the transport cycle. This conformational trapping may also favour protein folding, conferring them the ability to rescue function of misfolded transporters. We explored this conjecture by analysing the effect of PAL binding on (i) SERT transport cycle using electrophysiological recordings, and (ii) rescuing surface expression and transport activity of the folding-deficient mutant SERT-^{601}PG^{602}-AA.

Methods: SERT undergoes a series of conformational changes during the transport cycle, which can be inferred from analysing transporter currents. Substrate-induced SERT currents consist of a peak component, which reflects substrate-induced charge movement, and a steady-state component, which represents a conducting state of the transporter associated with completion of the transport cycle. These currents were measured in HEK 293 cells stably expressing human SERT in whole-cell patch clamp recordings. The currents induced by PAL1045 (a partial releaser for SERT/DAT) were compared to those induced by the full releasers PAL287, PAL1046 and *para*-chloroamphetamine (pCA) with serotonin (5-HT)-induced currents as a reference. Rescue of transporter function by PAL compounds on SERT-^{601}PG^{602}-AA was checked by combining radioactive transport assays with immunoblotting and confocal microscopy.

Results: At physiological pH, all PALs stimulated steady-state currents with a bell-shaped concentration response curve over the range of 0.3–30 µM as opposed to a saturation hyperbola for 5-HT and pCA. This is indicative of internal PAL accumulation and rebinding due to substrate diffusion. This interpretation was confirmed by lowering the extracellular pH to 5.5 during electrophysiological recordings, a manipulation which eliminated the descending limb at high concentrations of PALs. We hypothesized that PAL compounds were ligands, which bound to both outward-open and inward-open states of SERT with high affinity. SERT binding-affinity estimates calculated separately from binding assays and kinetic characterization of substrate-induced peak currents revealed PAL1045 having low nM affinity followed by PAL1046, PAL287 and pCA with increasing IC_{50}s. This high affinity is governed by poor dissociation rates and thus consistent with conformational trapping. Owing to high-affinity binding to multiple conformational SERT states, all 3 PALs restored surface expression and transporter activity of the non-functional folding-deficient mutant SERT-^{601}PG^{602}-AA, with PAL1045 being the most efficacious.

Discussion: PAL1045 acts as a partial releaser and a pharmacochaperone by virtue of its high-affinity binding to SERT. Slow dissociation rates result in longer dwell time at the binding site. This not only precludes intracellular 5-HT binding and subsequent efflux but also facilitates proper folding of the otherwise ER-retained ^{601}PG^{602}-AA mutant form of SERT, leading to restoration of surface expression and transporter function.

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A4.7

Nuclear effectors of oncogenic BRAF signaling and their pharmacological inhibition

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Background: Most key proteins in mitogenic signal transmission and gene regulation are encoded by proto-oncogenes from which many had been originally identified in transforming retroviruses. The BRAF and MYC oncoproteins are master switches in cell proliferation and represent major drivers in human tumorigenesis. Signals transmitted by the BRAF protein kinase are relayed into the nucleus via MAP kinases leading to activation of the transcription factors MYC and AP-1. Similar to MYC, AP-1, consisting mainly of JUN and FOS proteins, has a pivotal role in oncogenic processes leading to malignant cell growth and invasion.

Methods: Using small molecules, we aim to pharmacologically inhibit AP-1, MYC, or upstream-acting regulatory MAP kinases, which could represent a suitable strategy to modulate or even to revert signaling processes leading to the initiation or maintenance of neoplastic cell transformation. For efficient analysis of small organic molecules or peptides for their potentials to interfere with oncogenesis initiated by human BRAF, MYC or AP-1, a cell transformation system was established based on quail embryo fibroblasts (QEF). In these cells, either single or combinatorial oncogene expression leads to efficient cell transformation and tumorigenesis within days, and therefore this system represents a major experimental advantage.

Results: Co-expression of *BRAF* and *MYC* orthologues in avian fibroblasts leads to activation of AP-1 at transcriptional and post-transcriptional levels, suggesting that this transcription factor complex has a crucial role in cell transformation initiated by oncogenic BRAF signaling. Novel promising compounds have emerged in the last years, which specifically interfere with dimer formation between MYC and MAX thereby inhibiting MYC-specific transcriptional regulation and oncogenic transformation [1,2], or selectively inhibiting AP-1. These compounds will be employed either individually or in combination to interfere with growth and viability of therapy-resistant human cancer cells. In addition, specific inhibition of critical AP-1 target gene products could provide an additive value.

Discussion: The identification of compounds selectively interfering with initiation of oncogenic BRAF signaling in cell culture should yield novel targets to attack BRAF-dependent tumorigenesis in cancer types, which have so far remained untreatable by known specific inhibitors.

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A4.8

Different neuronal activation patterns in different amygdala nuclei after fasting and fear extinction

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Background: Anxiety disorders are the most frequent brain disorders imposing a significant burden to affected individuals, their families and the whole society. Dysregulation of fear, anxiety and related behavioral disturbances are hallmarks of anxiety disorders. On the other hand, eating disorders are emotional disorders linked to anxiety and depression. However, how feeding affects cognitive skills and anxiety- or fear-related processes is not known. To investigate this interaction and the underlying neuronal circuitries is the focus of this project.

Methods: Fear was investigated by Pavlovian fear conditioning, in which an initially neutral stimulus, such as a tone (CS), is repetitively paired with an unconditioned stimulus (foot shock, US). The resulting fear memory is characterized by increased freezing behavior to the CS. Importantly, repetitive exposure to the CS in the absence of a US, gradually reduces the acquired fear response, a phenomenon called fear extinction. To identify the involved neuronal ensembles, we accomplished immunohistochemistry against the immediate early gene c-Fos, a marker of neuronal activity. We analyzed changes in neuronal activation patterns in key brain areas of the fear circuitry between fasted and non-fasted animals, exposed to fear extinction or without conditioning.

Results: Interestingly, mice fasted during extinction learning displayed faster fear extinction than non-fasted controls, suggesting a direct relation between feeding and fear circuits in the brain. Fasting during the extinction process increased neuronal activation in the basolateral nucleus of the amygdala, a key structure of the fear response. In addition, we also detected changes in the central nucleus of the amygdala, a pivotal brain area for fear expression and in the paraventricular nucleus of the thalamus, a relay structure of sensory inputs.

Discussion: These experiments suggest several brain structures as possible interaction sites between feeding and fear circuits. We are now planning to manipulate neuronal ensembles in these brain areas to elucidate their role during feeding and fear-dependent challenges.

Acknowledgements: This study was supported by the Austrian Science Fund FWF (grants P 25851 and P 29952).

A4.9

Neuropeptide Y and Y_2 receptors in hippocampus-dependent fear and spatial learning

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Background: Neuropeptide Y (NPY) is abundantly expressed throughout the central nervous system and especially enriched in limbic areas, such as the hippocampus and amygdala. NPY is well known to mediate anxiolytic and fear-suppressing effects, mainly through activation of Y_1 receptors. However, in particular in the hippocampus, presynaptic Y_2 receptors (Y_2R) are also highly expressed, but their role in fear learning is not clear yet. Our aim was to investigate the effect of NPY acting on Y_2Rs in the hippocampus in fear conditioning as well as spatial learning.

Methods: Y_2R knockout (KO) and wild-type mice were tested in hippocampus-dependent context fear conditioning. The time spent freezing was used as a measure of the fear response. To test whether Y_2Rs play a role also in non-emotional learning, animals were tested in the Barnes maze. Furthermore, we performed rescue experiments by re-expressing the Y_2Rs specifically in the hippocampus of Y_2R KO mice via microinjection of recombinant adeno-associated viral vectors (rAAVs), and repeated the same battery of behavioural tests. To determine the appropriate concentration of viral vectors and to confirm whether the vector-mediated Y_2Rs are functional, we employed classical receptor binding assays and functional GTPγS receptor binding, respectively.

Results: In context fear conditioning, Y_2R KO mice displayed increased freezing during fear recall and delayed fear extinction. Locally restricted re-expression of Y_2Rs specifically in the dorsal hippocampus did, however, reverse these behavioural deficits and restore extinction learning. On the other hand, Y_2R KO mice displayed improved spatial memory performance in the Barnes maze, which was reduced after re-expression of hippocampal Y_2Rs. Receptor binding suggested that 10^9 viral particles of rAAV6-Y_2R were sufficient to yield expression levels reminiscent of the wild-type hippocampus. In addition, GTPγS binding assays confirmed functional coupling of exogenously introduced Y_2Rs.

Discussion: Here, we demonstrated that Y_2Rs play a crucial role in contextual fear learning, since germline Y_2R deletion led to elevated fear expression and delayed fear extinction. Viral-vector-mediated re-expression of Y_2R in the hippocampus restored these deficits. In contrast, Y_2R re-expression impaired long-term spatial memory. Thus, Y_2R may inhibit fear conditioning by suppressing memory processes in the hippocampus.

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A4.10

Light-induced ganglion cell responses in $Ca_V1.4$-mutant mouse retinas

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Background: Mutations in the *CACNA1F* gene encoding for the α1 subunit of $Ca_V1.4$ channels are known to cause congenital stationary night blindness type 2 (CSNB2). Typical symptoms in CSNB2 are moderately low visual acuity, myopia, nystagmus and variable levels of night blindness or progressive photophobia. The $Ca_V1.4$ I745T (IT) mutation is associated with this disease, and in a heterologous expression system has been shown to result in gain of $Ca_V1.4$ channel function. How such abnormal calcium influx can affect the retinal circuits is hardly known. Using multielectrode array recordings upon visual stimulation in mesopic conditions, we have demonstrated previously that the IT mutation caused disturbances in the signal transmission of mouse retinas.

Methods: We used multielectrode array recordings to further examine the ganglion cell (GC) activity of IT mouse retinas under both dim light (scotopic) and bright light (photopic) conditions, and by means of multiple light stimuli aimed to detect specific GC response patterns.
Results: We confirmed a higher spontaneous firing rate in the absence of stimuli and a delayed response in both light conditions in IT whole-mount retinal preparations. In addition, compared to controls, IT retinas showed a diminished firing frequency within the stimulus (wild type, WT: 16 Hz, IT: 6.7 Hz, mean of 5 and 4 experiments respectively). The higher spontaneous firing rate and the decreased light-driven firing response likely account for the inability of GC to efficiently transduce visual signals. Of note, many GC previously did not respond to full-field stimulation under mesopic light conditions. In this study, the analysis of the same cell in two different light conditions showed that ON and OFF responses of IT GC are largely lost during bright light: while 275 GCs responded to dim light, only 80 GC ($n = 4$) responded using bright light stimuli. Gaussian white noise stimulus analysis instead showed a loss of GCs response also at scotopic level.
Discussion: These preliminary data indicate that, although scotopic and photopic pathways show similarly impaired responses, in the IT CSNB2 model the cone pathway might be more severely affected. Together, our findings reflect what is seen in electroretinographic analyses of CSNB2 patients.
Acknowledgements: This study was supported by the European Marie Skłodowska-Curie Innovative Training Network ITN (grant 674901 to A.K.) and the Austrian Science Fund FWF (grant P26881 to A.K.).

A4.11

Functional neuroanatomy of prodynorphin

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Background: Dynorphins (Dyn) and κ opioid receptors (KOR) are abundantly expressed throughout limbic brain areas and were shown to be involved in the regulation of anxiety and stress control. Moreover, the Dyn/KOR system is implicated in the pathophysiology of depression, anxiety and addiction. However, the organization of the Dyn/KOR system is highly complex. Understanding and potentially interfering with this complex system to target specific functions depends on a detailed understanding of specific functional roles of individual dynorphinergic neurons as well as neuronal population.
Methods: In order to investigate the specific functional implication of distinct dynorphinergic projections in emotional control, we implemented independent, yet complementary strategies. based on restricted prodynorphin (pDyn) knock-out—achieved so far in the bed nucleus of the stria terminalis (BNST), the central nucleus of the amygdala (CeA) or neurokinin B (NKB)-expressing cells—or re-expression (BNST and CeA). These mice underwent behavioural testing related to anxiety (open-field, elevated plus maze, light–dark choice test) and stress-coping behaviour (tail suspension test). We also employed the cocaine-induced conditioned place preference paradigm to investigate the extinction and stress-induced reinstatement of the place-conditioned response.
Results: Behavioural tests related to anxiety and stress-coping did not show any significant differences between the investigated groups. By contrast, behaviour in the cocaine-induced conditioned place preference paradigm was altered. Mice with deletion of pDyn in NKB-positive cells displayed no stress-induced reinstatement, whereas control mice did.
Discussion: Our data so far revealed first indications for regional differences in Dyn functions. These are in line with the known role of Dyn in fear and anxiety and stress control. Further studies on the role of Dyn in specific brain areas in addictive process and fear extinction are in progress.
Acknowledgements: The study was supported by the Austrian Science Fund FWF (SPIN W1206-B18).

Pharmacoepidemiology and Pharmacovigilance

A5.1

Prescription of antibiotics for systemic use in Austria, 2006–2014

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Background: Antibiotics for systemic use represent one of the most widely prescribed groups of pharmaceuticals in outpatient care (rank 14 in Austria). This study analyses data (expenses, packages, daily defined doses) from all prescriptions of antibiotics for systemic use filled at the expense of the Austrian national health system at substance (ATC-5) level.
Methods: Data from all prescriptions filled in Austrian pharmacies on public expense by outpatients (2006–2014) were obtained from the Main Association of Austrian Social Security Organisations (Hauptverband der Sozialversicherungsträger). Prescriptions and costs of antibiotics for systemic use were analysed in detail using WHO drug statistic methologies.
Results: Public expenses on antibiotics for systemic use totalled €66,07m in 2014, (−0,85% compared to 2013, −12,86% compared to 2006). Defined daily dose figures rose from €41m (2006) to €47m in 2009 and dropped to €43.5m in 2014 (this equals to 2009). Prescription and defined daily dose figures are displayed in detail in tables at substance level, a relevant savings potential can be calculated.
Discussion: In contrast to prescription numbers of most pharmaceutical groups, dose equivalents of antibiotics for systemic use prescribed on public expense have stabilized and even declined over the last years. However, these figures still impose as higher than expected if prevalence numbers of bacterial infectious diseases and population data are taken into account, bearing in mind that careless prescription of antibiotics accounts not only for a waste of public funding but also for rising numbers of bacterial resistance. A close look entertains suspicion of antibiotics being prescribed without adequate indication nor precaution.

A5.2

Adverse drug reactions reported to the regional pharmacovigilance center Zurich in 2014–2016: a retrospective, monocentre cohort study

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Background: Serious adverse drug reactions (ADRs) resulting in death, hospitalization, disability or life-threatening effects must be reported to regional pharmacovigilance centers (RPVC) in Switzerland. All ADRs reported to the RPVC Zurich, as the biggest RPVZ in Switzerland, over the last 3 years were analyzed. The aim of the retrospective study was to identify reporting trends within and between the respective years, with a focus on drug classes, severity of the ADRs as well as causality assessment between drug exposure and ADRs.
Methods: All ADR reports from the RPVC Zurich collected between January 2014 and December 2016 were included. Relevant data on the ADRs were extracted from the official reports, Swissmedic forms and internal documents. Information included labelledness of the reaction, its severity, outcome and causality of the ADRs besides other reporting factors. Further aspects such source of the reports and reporting methods of the notifications were also integrated. Statistical analyses were performed with SPSS Statistics (v. 23; 2015).
Results: Over 3 years, 2,060 ADRs were collected at the RPVC Zurich, with a mean of 687 annual reports, and with a slight increment over the 3 years. A dominant trend within the severity of the ADRs was observed with 684 (33.2%) hospitalizations overall. Other severe outcomes were decisively less frequent, displaying 64 (3.1%) cases of death, 76 (3.7%) permanent disabilities, and 330 (16.0%) reactions leading to outpatient treatment categorized as medically important. The remaining outcomes were either "medically non-important" or "not reported". Causality assessment between the suspected drug and the respective ADR was categorized as "possible" in 1,323 (64.2%) cases, in 564 (26.5%) as "likely", and in 95 (4.6%) as "certain". In most of the cases, patients "completely recovered" at the time of the report (48.5%), whereas only 18.9% were still affected. In 1,531 (74.3%) notifications, the ADR was specified in the professional Swiss product information of the suspected drug ("ADR labelled"), while in 419 reports the information on the ADR was not mentioned in the product information. The most common drugs implicated in death cases were antineoplastic agents (25.0%), antithrombotic agents (18.8%), immunosuppressants (9.4%) and analgesics (7.8%). The prevailing conditions leading to death were hemorrhage (18.8%)—mostly affecting the central nervous system (58.3%) and the gastrointestinal tract (33.3%)—, sepsis (9.4%), anaphylaxis (9.4%) and sudden cardiac death (7.8%).
Discussion: In 2014–2016, ADRs leading to hospitalizations were reported in approximately one-third of the reported patients, while 3% reported that ADRs caused death. The prevailing drug classes implicated in death were antineoplastics, immunosuppressants and anticoagulants. The majority of the patients completely recovered of the respective ADR at the time of the notification.

A5.3

Statin-associated immune-mediated necrotising myopathy: a retrospective study of WHO pharmacovigilance safety reports

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Background: Statins represent an effective treatment for hyperlipidemia. Recently, immune-mediated necrotising myopathy (IMNM), a new form of a non-self-limited statin myopathy, became evident. The incidence of IMNM is approximately 2 to 3 patients per 100,000 patients treated with statins, mostly older than 50 years. Clinically, IMNM mostly manifests as symmetrical proximal muscle weakness of the arms and legs. Laboratory values can show significantly raised creatine kinase (CK) levels up to 13.000 U/l. The onset of the disease may be acute (days to weeks) or subacute (weeks)—with even ongoing symptoms after discontinuation of statin therapy. Treatment options of autoimmune-related myopathy consists of immunosuppressive regimens. All reported cases of IMNM in the WHO pharmacovigilance database were analysed to characterise this rare phenomenon.
Methods: International individual case safety reports (ICSR) concerning IMNM until October 1st, 2016, were extracted from VigiBase™, the WHO database of pharmacovigilance. The responsible authorities were contacted to maximise ICSR information and improve quality of the reports by implementing information of the corresponding case narratives. Duplicates of similar pairs of ICSR were identified. Demographic data, drug administration information, *e.g.* dose and duration, seriousness of the adverse drug reactions (ADR), latency time and patients' outcomes were analysed.
Results: Overall, 159 ICSR related to IMNM were identified. 101 ICSR with IMNM under statin administration from 17 countries (52 from Norther America, 45 Europe, 3 Australia, 1 Japan) were used for analysis after identification of duplicates. The majority of the European cases ($n = 51\%$) were reported by physicians, in contrast to only 9.6% from North American reports. Males and females were equally affected (59 males *vs.* 41 females). The average age was 67 years (range 16–87). 100 reports were classified as serious, 6 of them were subgrouped as life-threatening and 7 as disabling/incapacitating. The mean latency time between diagnosis of IMNM and start of treatment was over 4 years (range 1–300 months). The median CK values were 6460 U/l (range 24–35000). For 33 patients HMGCoA antibody testing was documented: 24 (73%) of those patients were tested HMGCoA-positive. In total, 8 patients recovered from IMNM, 6 only recovered with sequelae and 28 were recovering during the reported ICSR. A 63-year-old American female patient died due to IMNM. In 76 patients one statin was reported, in 22 ICSR 2 statins, and in 3 patients 3 statins were documented. Atorvastatin was the most frequently coded statin in 80% of the reports (simvastatin 27%, rosuvastin 18%, pravastatin 3%). The median daily statin dose was 40 mg (range 5–80 mg) for a median time of 36 weeks (range 1–232 weeks).
Discussion: IMNM associated with statin treatment was reported in different countries worldwide with the main focus in Europe and Northern America. IMNM was described with serious consequences even with fatal outcomes. Atorvastatin was the most frequently reported statin.

Training and Education

A6.1

Recognition and training of medical specialists in (Clinical) Pharmacology in Europe: a pilot survey of the UEMS Section of Pharmacology

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Background: UEMS, the European Union of Medical Specialists was founded in 1958 in order to represent all medical specialties on a

European level. Currently UEMS has 31 full members, *i.e.* the national medical associations (NMAs) of all member states of the European Union plus Switzerland, Norway and Iceland. One of the main tasks of UEMS is to aim for harmonising the training and examination requirements for all medical specialties across Europe. Pharmacology, although already recognised as a medical specialty in some European countries for various numbers of years, had not been represented in UEMS until 2016 when the UEMS Section of Pharmacology [1] was founded.

Methods: As a first major undertaking, the UEMS Section of Pharmacology has initiated a pilot survey on the status of Pharmacology as a medical specialty in Europe. In January 2017, a survey form with 16 open questions, regarding representation of the specialty in the NMA, legal framework of the specialty, training requirements, examination procedures, typical occupational environment, interaction of NMAs with national scientific societies of (clinical) pharmacology, current challenges and opportunities, and needs for support of the specialty in the respective country, was sent to all NMAs in Europe. In addition, all delegates to the section and some scientific societies were contacted when NMAs failed to respond.

Results: As of June 2017, preliminary data from 27 UEMS member countries could be collected. Pharmacology is recognised as a medical specialty by the respective national legislation in 23 out of these 27 countries. In 17 European countries the specialty is recognised as a full specialty in its own right, in 7 countries it is (also) a subspecialty of, or additive specialty to, other medical specialties (some countries have established both options). In the majority of European countries, the specialty is named 'Clinical Pharmacology', only Austria and Germany use 'Pharmacology and Toxicology' and 'Clinical Pharmacology' for their two specialties. Length of training for the stand-alone specialty varies between 4 and 8 years, with highly different lengths of time to be spent in other (clinical) specialties; for additive or sub-specialties, training time varies between 1 and 3 years. National legislations define training requirements to highly different degrees with respect to training contents, reflecting also the manifold professional roles that medical pharmacologists are supposed to perform.

Discussion: In light of the obvious considerable differences in the legal framework and in the definition of (Clinical) Pharmacology as a medical specialty, a more detailed follow-up survey, which shall collect more detailed information about the specific regulations governing training contents for medical specialists in Europe, is in preparation. Given the large variety of possible professional roles for medical pharmacologists, defining harmonised training requirements on a European level will be a major challenge for the near future. Rather than defining specific common training contents, developing a common competency profile for professional medical pharmacologists—a strategic approach already successfully pursued by a growing number of other, similarly diverse, professions in the fields of biomedical sciences [2]—is an option that is currently explored by the UEMS Section of Pharmacology.

Links

1. http://www.uems-pharmacology.eu/ [last accessed: 07/07/2017]
2. http://www.lifetrain.eu/competencies/competency-profiles/ [last accessed: 07/07/2017]

Drug Research

A7.1

Pharmacological and molecular modeling studies on 6-desoxo-*N*-methylmorphinans as potent agonists interacting with the μ-opioid receptor

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Background: Pain remains one of the main challenges in human medicine at the beginning of the third millennium, with ca. 20–30% of people worldwide suffering from chronic pain. Opioid analgesics are the cornerstone drugs for the treatment of moderate to severe pain, but their clinical use is hampered by numerous adverse effects. Pharmacological actions of opioids are primarily mediated through activation of the μ-opioid (MOP) receptor. One long-standing focus in drug discovery has been the search for opioids exhibiting a favorable dissociation between analgesia and side effects. Morphinans are the most common and highly effective analgesics. Oxymorphone, a clinically relevant analgesic, represents a valuable scaffold for the design of MOP ligands, with examples including the 14-*O*-methyl (14-OMO)- and 14-*O*-benzyl (14-OBO)-substituted derivatives, and the 5-methyl-substituted analogue, 14-methoxymetopon (14-MM). Position 6 on the morphinan skeleton was shown to play a key role on opioid activity *in vitro* and *in vivo*. In this study, the consequence of 6-carbonyl (6-CO) group deletion in targeted *N*-methylmorphinans on ligand–MOP receptor interaction, signaling and antinociceptive activity was evaluated.

Methods: *In vitro* radioligand binding and [^{35}S]GTPγS functional assays were performed with membranes from Chinese hamster ovary (CHO) cells stably expressing the human opioid receptors. Antinociceptive activities were determined using the hot-plate test in mice after subcutaneous administration. For molecular docking to the murine MOP receptor crystal structure, ligands were prepared using the LigandScout (version 3.1), and docking was performed using the GOLD (version 5.1) software.

Results: Binding studies indicated that the 6-desoxy-*N*-methylmorphinans display affinities in the subnanomolar range at the human MOP receptor, and are MOP receptor-selective. The loss of the 6-CO group was not favorable when comparing oxymorphone and 14-MM to their 6-desoxy counterparts, while a significant increase in MOP binding was observed for 6-desoxo-14-OBO. The 6-desoxo derivatives activate G proteins with high potency as full MOP agonists, with 6-desoxo-14-OMO, 6-desoxo-14-OBO and 6-desoxo-14-MM retaining or displaying an improved agonism than the parent compound, exception being 6-desoxooxymorphone. *In vivo*, they were effective against acute thermal nociception in mice, with comparable potency to lead molecules. Docking of the 6-desoxo-*N*-methylmorphinans to the crystal structure of activated MOP receptor revealed important molecular interactions, which these MOP agonists share and distinguish them.

Discussion: The absence of a 6-CO group in targeted *N*-methylmorphinans strongly impacts binding to the MOP receptor and post-receptor signaling, with all presented derivatives evolving as potent MOP agonists. The current results expand the understanding of the impact of the 6-CO-to-6-CH_2 alteration in *N*-methylmorphinans on ligand–MOP receptor interaction and molecular mode of action, and may aid in identification of new opioid analgesics with improved pharmacological profiles.

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