LIFE SCIENCE PHD MEETING INNSBRUCK 2018 ABSTRACT BOOK
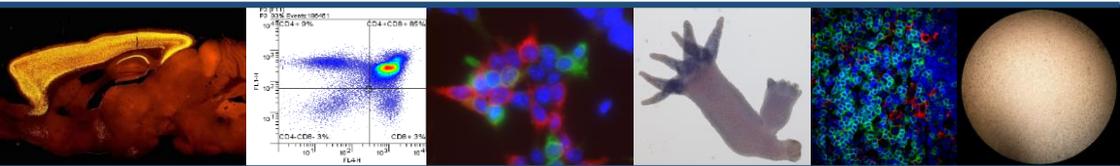
Mission Statement

The Life Science PhD Meeting provides a platform for students from various scientific backgrounds to share their knowledge, experience and critical thinking. We are proud to present excellent scientific work from numerous fields, which is only possible due to the sheer variety of scientific interests of the groups, represented in the Meeting. Therefore the organizing committee would like to take the opportunity to thank all the represented research programs:

Medical University of Innsbruck
- HOROS (Host Response in Opportunistic Infections)
- MCBO (Molecular Cell Biology and Oncology)
- SPIN (Signal Processing in Neurons)
- Regulation of Gene Expression during Growth, Development and Differentiation
- Image-guided Diagnosis and Therapy
- Molecular Cell Biology
- Musculoskeletal Sciences
- Genetics and Genomics
- Infectious diseases
- Molecular Oncology
- Neuroscience
- Clinical PhD: Applied Morphology and Regeneration (AMR), Clinical Cancer Research (CCR), Clinical Neurosciences, Intensive Care and Emergency Medicine, Cardiovascular PhD, Clinical Imaging Science

University of Innsbruck CMBI (Center for Molecular Biosciences Innsbruck)
- Pharmaceutical Sciences
- PhD Biology
- PhD Chemistry

MCI Technology & Life Sciences

GSN - LMU

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Pre-event Wednesday, April 4th 2018
MCBO Concluding Symposium

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Program Thursday, April 5th 2018

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13:40-14:00 M.EG.180	**Flash talks** (2min presentations) Thursday session: Poster #20, 23, 30, 46, 52, 54, 57

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Aula / Foyer	Please be at your poster if your abstract number is:	
	even: 13:00-14:00	odd: 14:00-15:00

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	MCBO best paper award lecture: **Fabian Schuler**
	Checkpoint kinase 1 is essential for normal B cell development and lymphomagenesis
	CMBI PhD project award
	PhD program info – MCBO & doc.funds CaV-X
	SPIN alumnus talk: **Kai Kummer**
	Neuron function in Fabry disease - more than lysosomal storage disturbance?
	MCBO alumna talk: **Marta Campiglio**
	STAC - CaV1 interactions in skeletal muscle EC coupling
	Best microscopy picture award
	Poster/talk awards
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	The mycobiome of the respiratory tract: does it reflect what you breath in?
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Selected short talks

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Selected short talks

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Huang	Anming	14	Effects of CENP-A post-translational modifications on its assembly in Drosophila melanogaster
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Mayrhofer	Johanna	21	Compartmentalization and structure of the macromolecular GPR161:PKA complex
Enzler	Florian	22	Proteomic mapping of cAMP-dependent kinase interactions and substrate dynamics
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Aschenbrenner	Bertram	24	Molecular background to effectively combat breast cancer metastasis using ionizing radiation

Gain-of-function mutation of a voltage-gated Cav1.3 L-type Ca2+-channel associated with a neurodevelopmental disorder of unknown cause

Background: Low voltage-gated Cav1.3 L-type Ca2+-channels are key regulators of neuronal excitability controlling neuronal development and different types of learning and memory. Recently, large-scale genetic analysis revealed de-novo missense mutations in their pore-forming α1-subunit (CACNA1D gene) in 6 patients associated with a neurodevelopmental syndrome including varying degrees of sporadic autism spectrum disorder (ASD; G407R, A749G) with and without intellectual disability, neurological manifestations (including seizures; V401L) and endocrine symptoms (G403D, I750M). Typical hallmarks of these mutations are severe gating changes compatible with a gain-of-channel-function. Here we investigated if similar gating changes are observed in a de-novo CACNA1D mutation (IIS4-S5 linker, S652L) which could explain symptoms in a patient diagnosed with a severe neurodevelopmental disorder of unknown cause.

Methods: Mutant (S652L) and wild-type (WT) Cav1.3 α1 subunits were co-expressed together with β3 and α2δ-1 subunits in tsA-201 cells and calcium or barium currents (15mM) were measured using the whole cell patch-clamp technique.

Results: Very similar to the previously characterized mutation V401L (IS6), A749G and I750M (IIS6), mutation S652L dramatically shifted the voltage-dependence of Cav1.3 steady-state activation and inactivation to more negative potentials (~20 mV) with a faster inactivation especially at low potentials. A complete biophysical analysis revealed that these changes are compatible with a mutational gain-of-function phenotype.

Conclusion: By demonstrating the typical gating changes previously shown by us for CACNA1D de-novo missense mutations we propose that mutation S652L also explains the symptoms in this patient with a severe developmental disorder. Our work confirms CACNA1D as a causal disease gene contributing to the risk for the development of a broad range of neurodevelopmental disorders. Patients carrying such mutations may benefit from treatment with already available L-type Ca2+ channel blockers, such as nimodipine. Such CACNA1D missense mutations are likely underreported in large-scale genetic analyses.

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Cholinergic processing in the medial prefrontal cortex of neuropathic mice

The medial prefrontal cortex (mPFC) plays a role in the modulation of pain processing. Disruption of cholinergic transmission to mPFC has been associated with the impairment of cognitive functions, which is a frequently observed comorbidity of chronic pain. Therefore, we investigated the effect of cholinergic stimulation on neuronal activity in acute mPFC slices from spared nerve injury (SNI) and sham-operated mice.

Seven days following surgery, multielectrode array recordings (MEA, Multi Channel Systems) of neuronal activity in acute mPFC slices in response to superfusion with cholinergic agonists (carbachol, muscarine, nicotine) were performed. By employing optogenetics and whole-cell patch clamp recordings postsynaptic responses to endogenous acetylcholine release in layer V mPFC pyramidal cell were investigated and mRNA expression was analyzed for relevant cholinergic receptors, ion channels and enzymes.

Using MEA recordings no differences in the average mPFC baseline activity were observed between SNI and sham treated animals. Carbachol dose-dependently induced activity of neurons in all recorded mPFC regions (i.e., prelimbic, infralimbic and anterior cingulate cortices). Neurons in slices obtained from SNI mice exhbited significantly lower spiking frequencies when superfused with 50 and 100µM carbachol. Moreover, similar findings were obtained in the hemisphere contralateral to the operated paw and not in the ipsilateral when superfused with 100µM muscarine. In addition, mRNA expression levels of cholinergic receptors, ion channels and enzymes were similar for both treatment groups.

These results suggest that neuropathic pain alters cholinergic transmission in mPFC and address a need for further investigation of underlying mechanisms.

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Sleep studies in MSA mice utilizing EEG biomarkers for disease progression and drug testing

OBJECTIVE: To characterize age related progression of EEG biomarkers and effects of clonazepam in a mouse model for multiple system atrophy (MSA).

BACKGROUND: MSA is a sporadic, rapidly progressive neurodegenerative disease. Besides motor symptoms and autonomic features, sleep disorders are defining characteristics. REM sleep behavior disorder (RBD) as it occurs in most MSA patients, is defined by REM sleep without atonia (REM-A), dream enactment behavior and changed EEG properties ('EEG slowing'). RBD is one of the symptoms of early MSA that develops, before motor symptoms. Clonazepam is the currently recommended symptomatic treatment for RBD. The PLP αSYN MSA mouse model shows RBD like behaviors. Effectiveness of clonazepam in this model could establish predictive validity.

METHODS: Chronic in vivo electroencephalographic and electromyographic recordings of PLP αSYN MSA mice (MSA mice) and C57BL¥6 controls at 24 weeks of age (N=6), 16 weeks (7 MSA, 8 control) and 44 weeks (10 MSA, 6 control). HD videos were recorded time-locked with EEG recordings. Five MSA mice, at 44 weeks of age, received a single dose of clonazepam (0.3mg/kgBW, p.o.).

RESULTS: MSA mice showed an age related increase of motor activity during REM sleep. MSA mice showed altered EEG characteristics similar to human EEG slowing, featuring an age related progression. Clonazepam treated animals showed restored muscle atonia during REM sleep for 3-4 hours after treatment.

CONCLUSIONS: Sleep-related pathologies in the MSA mouse model resemble key characteristics of human RBD. As these become relevant early on and show an age-related progression, EEG biomarkers can be utilized as readouts for drug testing. Clonazepam treatment, as a proof of concept study, theoretically establishes predictive validity for these biomarkers.

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Molecular regulation of the oncogenic miR-17-92 cluster

MicroRNAs are a class of small non-coding RNAs that posttranscriptionally regulate gene expression by sequence-specific repression of mRNA. Since almost every cellular pathway is fine-tuned by miRNAs, they form an essential regulatory layer in multicellular organisms. However, due to their widespread regulatory potential, it is also not surprising that aberrant expression of miRNAs is often correlated with human pathologies such as cancer.

The miR-17-92 cluster is essential for B cell development since the deletion of the cluster results in a block of the pro to pre B cell transition. Further the polycistronic miR-17-92 cluster, which encodes 6 individual miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, miR-92a-1) is one of the first described miRNA genes with a clear oncogenic role in different types of human cancer (e.g. lymphoma). Interestingly, recent data indicate that the cluster members do not equally contribute to the progression of cancer as previously suggested. In contrast, it seems that individual members can confer oncogenic or tumor suppressive functions. This suggests that not the overexpression of the cluster per se, but the imbalanced expression of individual cluster members may be a critical contributor to tumor development. However, little is known about how such an imbalanced expression of miRNAs within a cluster is established on the molecular level, warranting an in-depth investigation.

We established the workflow for a genome-wide CRISPR/Cas9 loss-of-function screen aimed to identify novel regulators of the miR-17-92 cluster. In short, cells expressing fluorescence-based reporters that allow the quantification of miRNAs activity were transduced with a CRISPR library comprising about 120,000 small guide RNAs (sgRNAs) targeting all coding genes. Cells exhibiting altered miRNA activity as measured by flow cytometry were isolated by cell sorting and the corresponding sgRNAs were retrieved by next generation sequencing and thorough bioinformatics data analysis. Several of those candidates we partially reviewed, but these need further confirmation and validation experiments.

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Novel role for the TGF-beta related transcriptional effector FoxH1 in the repression of a key microRNA in early embryonic development of zebrafish

FoxH1 is a conserved transcription factor with well-described essential activities in mesoderm-induction and left-right patterning downstream of the Nodal/TGF-beta signaling pathways. Here a complete new function in the regulation of the conserved microRNA-430 family (miR-430) is described in zebrafish. As miR-430 is required for clearing maternal transcripts during maternal-zygotic transition (MZT) and for balancing Nodal-signaling during gastrulation, these results hint for novel important embryonic FoxH1 activities not only downstream but also upstream and parallel to Nodal signaling.

To define direct targets of FoxH1 during gastrulation we performed ChIP analyses in zebrafish. We noticed a large number of FoxH1 binding sites that were lacking FoxH1 consensus motives including several sites at the miR-430 loci. Up-regulation of miR-430 expression in foxH1 mutants but not in Nodal-deficient embryos emphasizes biological relevance of these non-canonical binding sites in Nodal independent miR-430 repression. FoxH1 mediates Nodal-signals via two protein domains, a Forkhead-domain (Fkh) that binds to DNA and a Smad-interaction domain (SID), which brings Nodal-induced Smad proteins to these loci. We find that up-regulation of miR-430 in foxH1 mutants was rescued by full-length FoxH1 but not by Fkh- or SID-mutant FoxH1-variants, indicating that also miR-430 regulation requires both major FoxH1 protein domains. However, binding of FoxH1 cannot be direct at the miR-430 loci as fusions of the Fkh-domain to transcription activating (VP16), inactivating (EN) or neutral (GFP) elements all cause a similar up-regulation of miR-430.

Our data provide first evidence for a novel Nodal independent mechanism for early embryonic gene regulation by FoxH1 and they identify the miR-430 cluster as a major target for this regulation. We propose a model in which FoxH1 indirectly represses miR-430 by SID-dependent targeting of the miR-430 loci to a distal repressive chromosome environment, which is directly bound by the Foxh1 Fkh-domain. Accordingly, in foxH1 mutants, the lack FoxH1 or expression of truncated forms of FoxH1 both prevents proper chromosome looping and in consequence expression of miR-430 will not be blocked anymore.

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Influence of mitochondrial-derived tRNAs, located in introns of nuclear protein-coding genes, on gene expression of host genes

Computational analysis of next-generation sequencing data revealed the presence of mitochondrial-like tRNAs in introns of 27 nuclear encoded protein-coding genes. For example, intron 84 of the human DYNC2H1 gene showed that an about 7800 bp large region of the mitochondrial genome was integrated into the nuclear genome, including 14 different mtl-tRNAs. Although some of these mitochondrial-like tRNAs (mtl-tRNAs) displayed point mutations, compared to their mitochondrial counterparts, the canonical secondary/tertiary structure of tRNAs was maintained, pointing to an evolutionary conserved function of intron-encoded mtl-tRNAs.

As one of several potential models, we have investigated the possibility whether mtl-tRNAs influence splicing efficiency, thereby acting by a similar mechanism as reported for splicing regulatory elements in nuclear introns. Indeed, introduction of mtl-tRNAs into an intron of an eGFP splicing reporter construct resulted in an increase in mRNA abundance. When introducing different numbers of mtl-tRNAs into the intron of the eGFP splicing reporter construct, a dosage-dependent increase of mRNA abundance was observed, as shown for bona fide splicing enhancer sequences. Introducing mtl-tRNAs into the eGFP intron, containing a less efficient 5'-splice site resulted in an increase of mRNA abundance by about 20-fold, indicating that mtl-tRNAs are indeed involved in promoting splicing efficiency.

The effects of mtl-tRNAs on mRNA abundance were shown to be position-dependent. Introduction of five mtl-tRNAs in close proximity to the 5'-splice site of the eGFP reporter construct resulted in a 3-fold increase in mRNA abundance. Remarkably, introduction of these mtl-tRNAs into the central part of the intron induced only a 1.5-fold increase, while positioning mtl-tRNAs close to the 3'-splice site had no influence on mRNA abundance. Position dependent effect were corroborated by introduction of mtl-tRNAs into the intron of a β-globin splicing construct, exhibiting a significantly shorter intron, which showed little effects on mRNA abundance. Interestingly, despite the significant effects of mtl-tRNAs on splicing of host genes, no fully processed mtl-tRNAs were observed by northern blotting, pointing to a rapid turnover or degradation of mtl-tRNAs. Thus, our data demonstrate that mtl-tRNA genes located in nuclear introns might represent a novel post-transcriptional mechanism to regulate gene expression of nuclear genes.

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Periodontal Ehlers-Danlos syndrome is characterized by aberrant secretion pattern and altered posttranslational processing of complement 1 subunits C1r/s

Periodontal Ehlers–Danlos syndrome (pEDS) is a connective-tissue disorder characterized by early and severe periodontitis and various joint and skin manifestations. Other EDS subtypes are caused by mutations in connective tissue proteins such as collagens or protein-modifying enzymes. In contrast, periodontal subtype is caused by heterozygous missense or in-frame insertion/deletion mutations in C1R or C1S which code for subunits C1r and C1s of complement 1. This finding opens a previously unknown connection between the inflammatory complement pathway and connective tissue homeostasis.

Extensive functional studies of all known pEDS mutations with HEK cells as an in vitro over-expression system show that all pEDS mutations retain enzymatic function in the complement cascade. Interestingly, mutated C1r/s proteins reveal domain-specific abnormalities of intracellular processing and secretion. For instance, mutations within the C1r CCP1 domain lead to retention inside the cell, whereas mutations located in the CUB2 domain are in part secreted as aggregates. Secretion like C1r WT was only observed for one C1R missense mutation that affects a C1q binding site. Immunofluorescence analyses suggest a non-classical secretion pathway for several C1r/s mutant proteins: C1r WT co-localizes with the trans Golgi Network (TGN) but mutant C1r proteins studied are diffusely distributed within TGN and the cytosol. Moreover, we observed vesicle formation exclusively for C1r WT proteins so far.

Patient fibroblasts show mild enlargement of the endoplasmic reticulum compatible with a derangement of intracellular processing. We hypothesize that the unique connective tissue and periodontal inflammatory phenotype reflects abnormal interaction of the mutant complement subunits with collagens or other ECM proteins. Clarifying the effects of ER stress due to misfolded C1r/s as well as the impact of alternative interaction partners of mutated C1r/s will support understanding the disease pathogenesis of both pEDS and other connective tissue pathologies.

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Candida albicans factor H binding molecule Hgt1p – a molecule also executing non-canonical functions.

Background: The opsonisation of fungal surface by C3b represents the key for the killing by immune cells. Factor H (FH) is a negative regulator of the complement alternative pathway for this reason FH is acquired by pathogens, conveying resistance to complement attack.

Objectives: The aim of the study was to highlight whether the blockage of FH binding molecule „high affinity glucose transporter 1" (CaHgt1p), a transmembrane protein in Candida albicans, increases the C3b deposition on the fungal surface due to the impairment of FH acquisition and whether this increases human PMN phagocytosis.

Methods: FACS analyses were performed to study the blockage of CaHgt1p by an antibody anti-Hgt1p to decrease FH acquisition and thus increase C3b deposition on C. albicans surface. A knock-out strain (hgt1Δ/Δ) was used as a control. Candida strains were opsonized with human serum (HS) and probed by anti-FH and anti-C3b antibodies. In parallel they were stained with FITC and co-cultured with fresh human PMNs. Positive PMNs, with internalized C. albicans, were detected by FACS. Western analyses detected Hgt1p on the cell wall. An in-vivo experiment with mice was performed to show the effect of the blocking of CaHgt1p on mice survival. The mice were injected with SN-152 wild type strain or wild type blocked by anti-Hgt1p before injection.

Results: FACS analyses showed a significantly ($p<0.05$) lower C3b deposition on the SN-152 wild type strain compared to hgt1Δ/Δ knock-out strain or SN-152 blocked by anti-Hgt1p. The significantly higher ($p<0.05$) FH acquisition on wild type SN-152 led to an increase in C3b deposition. The lower FH acquisition on hgt1Δ/Δ knock-out or the blocked SN-152 strains increased significantly phagocytosis by PMNs. The western blotting of extracted cell wall proteins from SN-152 showed the presence of CaHgt1 in this compartment.

Summary: CaHgt1p on C. albicans is significantly increasing FH acquisition on wild type strain, inducing a decrease of C3b opsonisation and also of phagocytosis by PMNs. We speculate that CaHgt1p also exhibits non-canonical functions as FH binding molecule, potentially beneficial factor in mice and also as "moonlighting" protein in the cell wall.

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Identification of the immunodominant T cell epitopes of AQP4 & MOG in patients with NMOSD

Objective: CD4+ T-cells, are key players in the pathogenesis of autoimmune diseases, which initiate central nervous system (CNS) inflammation and blood brain barrier disruption. Mutually exclusive autoantibodies targeting the aquaporin-4 (AQP4)-water-channel-protein or the myelin-oligodendrocyte-glycoprotein (MOG) are associated with a broad spectrum of human CNS demyelinating diseases including neuromyelitis optica spectrum disorders (NMOSD). In this regard, a significant proportion of the AQP4-immunoglobulin(Ig)G seronegative NMOSD patients harbour antibodies to MOG indicating a possible role of MOG to NMOSD pathogenesis or vice versa an involvement of AQP4-specific T-cells in MOG-IgG seropositive (MOG-IgG+) NMOSD patients.

Methods: Peripheral blood T-cells from nine AQP4-IgG+, eight MOG-IgG+, one seronegative NMOSD patients and eight healthy controls (HC) were examined for recognition of AQP4 and MOG peptides using the carboxyfluorescein succinimidyl ester (CFSE)-proliferation assay. The functional phenotype of proliferated T-cells was determined by evaluating the cytokine secretion in cell culture supernatants (granulocyte-macrophage-colony-stimulating factor (GM-CSF), interferon(IFN)-ɣ, interleukin(IL)-4, IL-6, IL-17A) using commercial ELISA kits and cytokine production (IFN-ɣ, IL-17A) using a flow-cytometry-based intracellular staining. In addition, a human leucocyte antigen (HLA)-DR und -DQB1 genotype determination of all participants was performed using the PCR with sequence-specific primer technique (PCR-SSP).

Results: Significant T-cell proliferation in response to AQP4, but not to MOG peptides, was found in AQP4-IgG+ and MOG-IgG+ NMOSD patients when compared to HC. The cytokine secretion was either not detectable (IL-4, IL-17A) or not specific (GM-CSF, IFN-ɣ, IL-6). However, the intracellular staining revealed a strong IFN-ɣ production indicative of a pro-inflammatory Th1 phenotype.

Interpretation: These results not only highlight AQP4 as the main target of autoreactive T cells and antibodies in AQP4-IgG+ NMO but even more strikingly show that AQP4-specific T-cells are also present in MOG-IgG+ NMOSD, which could explain the clinical features of these patients and may justify the inclusion of MOG-IgG+ patients under the umbrella of NMOSD. We hope that our results yield the basis for the development of new individualised immune-tolerance therapies.

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How to improve diphtheria vaccination for the elderly

Immunization is one of the most successful health intervention against infectious diseases. However, the efficacy of vaccination is reduced in old age. Previously, we demonstrated that booster vaccinations with multivalent tetanus/diphtheria vaccines provided long-term protection to tetanus, while inducing an insufficient long-lasting immunity against diphtheria in humans, and particularly in the elderly. To investigate the reason for that, we set up a long-term study with a mouse model, where we applied different vaccination regimes with varying primary and booster vaccinations and measured diphtheria-specific and tetanus-specific antibodies. Furthermore, we tried to target dendritic cells (DCs) by applying GM-CSF to the same injection site as the vaccine, and measured humoral immune responses by ELISA and also cellular immune responses by flow cytometry.

For tetanus we can show, that animals who received a primary immunization with three shots of Infanrix® and additionally three booster shots with Boosterix® had the same tetanus-specific antibody titers than animals which received only the three booster shots with Boosterix®. However, the diphtheria-specific antibody titers were much lower of the mice that received only the booster shots, compared to the mice with primary and booster shots. By applying GM-CSF next to the vaccine, we can show a significantly better diphtheria-specific antibody response in young as well as in old mice. GM-CSF treated mice had also an increased T-cell response, with more diphtheria-specific CD4+ T-cells producing IL-2, IL-6 and TNF-a. Interestingly, GM-CSF was leading to a higher number of DCs at the injection-site 24 hours after vaccination and also to a higher number of splenic DCs with upregulated MHC-II expression.

In conclusion, our findings demonstrate that the imbalanced level of protection against tetanus and diphtheria provided by multivalent tetanus/diphtheria vaccines is most likely due to the vaccine composition and not because of the vaccination regimes. Moreover, targeting DCs with GM-CSF improves the diphtheria-specific immune response following vaccination and this might be a useful strategy to improve the vaccination situation for the elderly.

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Molecular mechanism of ESCRT-III assembly during reverse membrane budding

The endosomal sorting complexes required for transport (ESCRT) drive the formation of multivesicular bodies (MVB), membrane abscission at the end of cytokinesis, HIV budding, nuclear envelope closure and repair of holes in the plasma membrane. Common denominators of this complex molecular machinery, in those processes are the AAA ATPase Vps4 and the ESCRT-III complex.

ESCRT-III is a hetero-oligomer consisting of the four proteins Vps20, Snf7, Vps24 and Vps2 and its assembly is essential for the recruitment of the Vps4 complex.

By quantitative fluorescence lattice light-sheet microscopy, we have shown that ESCRT-III subunits polymerize rapidly on yeast endosomes, together with the recruitment of at least two Vps4 hexamers. During their 3-45 second lifetimes, the ESCRT-III assemblies accumulated 75-200 Snf7 and 15-50 Vps24 molecules. Productive budding events required at least two additional Vps4 hexamers. Membrane budding was associated with continuous, stochastic exchange of Vps4 and ESCRT-III components, rather than steady growth of fixed assemblies, and depended on Vps4 ATPase activity. An all-or-none step led to final release of ESCRT-III and Vps4.

We propose a model in which multiple Vps4 hexamers (four or more) draw together several ESCRT-III filaments. This process induces cargo crowding and inward membrane buckling, followed by constriction of the nascent bud neck and ultimately ILV generation by vesicle fission. Tomographic electron microscopy demonstrated that acute disruption of Vps4 recruitment stalled membrane budding.

How individual ESCRT-III filaments are formed and how Vps24 and Vps2 bind to each other and to the Snf7 filament is poorly understood. Our goal is to understand how Vps24, Vps2 and Snf7 bind to each other and therefore, how the of the ESCRT-III filament is organized.

We will use purified ESCRT complex to reconstitute the ESCRT machinery on artificial liposomes for analyzing budding reactions in vitro.

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BORC Regulates Endosomal Biogenesis and Size Maintenance Through Phosphatidylinositol- Phosphate Species

Fundamental cellular functions such as proliferation, differentiation, migration and apoptosis require the precisely coordinated action of a number of proteins in macromolecular assemblies. These provide spatial and temporal specificity to a signal, which needs to trigger a specific defined response at a designated location. The Late endosomal/lysosomal Adaptor and MAPK and mTOR activator (LAMTOR) complex facilitates MAP kinase signal transduction and is also essential for mTORC1 kinase signaling and amino acid sensing on this specific subcellular location (Teis et al, J Cell Biol. 2006 Dec 18;175(6):861-8.; Rebsamen et al, Nature. 2015 Mar 26;519(7544):477-81.).

The recently described BORC complex (Pu J et al, Dev Cell. 2015 Apr 20;33(2):176-88.) interacts with the LAMTOR complex and facilitates endosomal positioning via its interaction with the small GPTase Arl8b. Interestingly, upon generating CRISPR/Cas9 knock outs (KOs) of different components of the BORC complex, we noticed some minor, but yet significant differences between the phenotypes that resulted. Whereas all KOs manifested a redistribution of Arl8b from endosomes to the cytoplasmic space, only C10orf32KO and LOH12CR1KO had significantly smaller late endosomes/lysosomes, compared to wild type (WT) or C17orf59KO cells. We have shown in this work that this phenotype is tightly linked to the Phosphatidylinositol (PI) phosphate levels, which are significantly increased in C10orf32KOs. Furthermore, as recently demonstrated in Bissig et. Al (2017), the PI phosphates are a driving force for reformation of terminal storage lysosomes from endolysosomes. Therefore, we could attribute an unexpected and new function to the BORC components of C10orf32 and LOH12CR in the process of lysosomal biogenesis and size maintenance of this important organelle.

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The LAMTOR-complex in regulation of fat metabolism

The LAMTOR complex is anchored to late endosomal/lysosomal membranes. It is known to regulate mTORC1 signaling in an amino acid and cholesterol dependent manner and MAPK signaling. Both signaling pathways play a crucial role in cellular homeostasis.

Deletions of LAMTOR components are early embryonic-lethal in mice, but conditional knockouts allow the study of the complex. Microarray and proteomic studies in LAMTOR2 -/- mouse embryonic fibroblasts (MEF) and macrophages point to a regulation of lipid metabolism including lipid synthesis, uptake, transport and degradation. Furthermore, the LAMTOR complex seems to play an important role in adipocyte differentiation since LAMTOR2 -/- MEFs are deficient in adipogenesis.

The LAMTOR complex was deleted specifically in adipose tissue using an AdipoqCRE-trangenic mouse line. Metabolic and biochemical studies of the brown and white adipose tissue were performed at 30°C, room temperature and 5 °C as well as in fasting and refeeding condition.

The AdipoqLAMTOR2 -/- mice show an accumulation of lipids e.g. triglycerides in the blood, brown adipose tissue (BAT) and liver in chow diet. Under fasting and refeeding conditions a defect in activating mTORC1 signaling in BAT of these mice was observed. The reason for the lipid accumulation is on one hand an increased lipid and glucose uptake. On the other hand, the LAMTOR complex is involved in the regulation of lipolysis.

Although thermogenesis genes are downregulated in BAT of AdipoqLAMTOR2 -/- mice, cold treatment reverses the phenotype observed in BAT and blood. The defect in mTORC1 activation is still present after cold exposure.

In summary, an adipose tissue specific knock out of LAMTOR2 disrupts BAT homeostasis and has effects on the whole body lipid metabolism.

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Effects of CENP-A post-translational modifications on its assembly in Drosophila melanogaster

CENP-A is a histone H3 variant that is in normal cells exclusively deposited at the centromeric region of a chromosome. The special chromatin architecture of the centromere enables the assembly of the kinetochore for faithful segregation of sister chromatids during cell divisions. CENP-A is incorporated into chromatin in a replication-independent fashion, however, the question of precisely how CENP-A is targeted to centromeres and loaded in a cell cycle-specific manner is not completely understood. Multiple studies in different organisms have revealed that a complex network of factors is required to ensure timely and spatially constrained incorporation. However, there are still large gaps to our understanding of CENP-A/Cid loading pathways in Drosophila melanogaster.

Previous work from our group revealed the presence of at least three different CENP-A preloading complexes in Drosophila. Furthermore, CENP-A in these complexes shows differential patterns of posttranslational modifications. To study the functional significance of these modifications, we generated stable cell lines expressing various mutant versions of CENP-A. Analyses of these cells showed that phosphorylation, but not acetylation, at distinct sites considerably affected CENP-A loading speed and turn-over rates. So far, the data suggest that phosphorylation regulates the amount of free CENP-A protein, thereby preventing the deleterious ectopic incorporation of CENP-A that occurs when CENP-A levels become too high.

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Analysis of the Concepts Race, Ethnicity and Population in Biomedical Literature

There is ample evidence that race cannot be explained by genomic sequence or biomarkers. Nevertheless, an increasing number of studies look for correlations of race or similar categories such as ethnicity or population with e.g. disease risk or drug response. This may be explained by current trends of personalizing medicine and an effort to acknowledge human diversity but also reiterates the old and biased theme of so-called race-based medicine. Given the fact that particularly race is a highly ambiguous sociocultural/socioeconomic concept rather than a biological fact, and that ethnicity or population are as well poorly characterized categories, we were interested to see for which purpose biomedical papers make use of these concepts. To this end, we focussed on the following questions: How is race, ethnicity or population defined in these studies? Is there a statistically significant link of the categories race, ethnicity or population to outcome that would justify their use in the context of the study? How are the results contextualized by the authors?

We therefore analysed open-access papers published in PubMed between 2009 and 2014 by systematic review. All the included papers (930) dealt with clinical trials in any of the three categories, as this was the most abundant study type in this context. Consistent with previous findings, in most of the studies identification of race, ethnicity, population was self-reported ($\approx$ 95 %) or not reported at all ($\approx$ 5 %). Nevertheless, these concepts were widely used without any further consideration regarding their definition or usefulness. In the vast majority of studies the rationale behind including these categories was not revealed and no obvious benefit, i.e. statistically relevant correlation of the categories to certain outcomes became evident.

We therefore suggest that race and related concepts such as ethnicity or population should be used with more caution and awareness about potentially misleading and hence harmful connotations. These concepts are not helpful to develop more personalized approaches in medicine as they are ill-defined, highly complex and not suitable for better understanding human diversity and its possible impact on health and disease.

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The effect of cell culture media lipid content on mitochondrial lipid composition and functions

Cell culture media contain components necessary for cellular proliferation. One important class of additives are lipids, either supplemented as part of serum, or in serum-free medium as a separate lipid extract. Although many lipids are synthesised by the cells own anabolism, cells take up and incorporate nutritional lipids as can be seen by the example of essential polyunsaturated fatty acids. Lipids serve as energy source and building blocks for membranes, and they are also involved in multiple other functions including signalling, protein stabilization, or scavenging of oxidative damage. Beside the integration of nutritional lipids into the different lipids, especially the length and degree of saturation distribution of the fatty acyl pool defines the specific molecular lipid species composition.

For cardiolipins, a mitochondria-specific phospholipid class substituted with four fatty acyls, we have shown that these profiles are not arbitrary, but follow an organism-, tissue-, and condition-dependent rationale that changes in response to fatty acyl availability, the selectivity of enzymes/transporters, or their remodeling activity. Cardiolipins are important components of mitochondrial membranes, where they stabilize transmembrane protein complexes to increase the efficiency of the electron transport chain, while simultaneously capturing ROS and control apoptotic signalling.

To elucidate the impact of lipid supplementation on cardiolipin compositions, we used HeLa cells and cultivated them in serum- and lipid-free medium, allowing the addition defined lipid pools. Comparing the cardiolipin profiles in cells grown under lipid-free conditions and in medium supplemented with heart lipid extracts using LC-MS/MS, we found a strong increase of linoleic acid rich cardiolipins. To assess the functional impact of these treatments we performed high-resolution respirometry analyses and biochemical assays. Interestingly, we observed a reduction of basal oxygen consumption in heart lipid treated cells, which was not associated with changes in their proliferation rates. Additional experiments revealed an increased Complex I activity that could be responsible for the more efficient oxidative phosphorylation in heart lipid treated cells. In the future this impact of lipid supplementation on mitochondria membrane composition and function could be used to design more realistic cell culture experiments, for example by establishing lipid environments that mimic a specific tissue of interest.

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Effect of cell-permeable succinate and malonate prodrugs on mitochondrial respiration in prostate cancer cells

Succinate is a substrate mainly metabolized to fumarate in mitochondria by succinate dehydrogenase (SDH) or Complex II. SDH is located at the inner mitochondrial membrane, coupling the oxidation of succinate to fumarate in the tricarboxylic acid cycle (TCA) with electron transfer to ubiquinone. Inhibition or downregulation of SDH leads to an impairment of TCA cycle and respiratory activity, and consequently to accumulation of succinate. This, in turn, transmits an oncogenic signal from mitochondria to the cytosol. Cytosolic succinate inhibits the hypoxia inducible factor 1α (HIF1α) prolyl hydroxylase (PHD) leading to HIF1α stabilization. In this "pseudohypoxic" state angiogenesis and anaerobic metabolism are enhanced, ultimately leading to tumour progression.

While succinate has essential implications on prostate cancer development, it is difficult to control intracellular succinate concentrations in intact cells due to the low permeability of plasma membranes to the compound. To overcome this limitation, we applied novel plasma membrane-permeable succinate (NV118) and malonate (inhibitor of SDH, NV161) prodrugs in high-resolution respirometry (Oroboros O2k-FluoRespirometer). Mitochondrial respiration was assessed in three cell lines: RWPE-1 (prostate; noncancerous), LNCaP (prostate; cancer), and HEK293T (embryonic kidney; control).

NV118 (250 µM) stimulated ROUTINE respiration in LNCaP cancer cells by 18% as compared to vehicle (DMSO), while respiration remained unchanged in RWPE-1 (4% increase) and HEK 293T cells, even at higher concentrations of the prodrug. NV161 (66 µM) had no effect on ROUTINE respiration of HEK 293T cells.

Our results indicate enhanced utilization of external, plasma membrane-permeable succinate in mitochondrial respiration in LNCaP prostate cancer cells but not in control cell lines. The cell-permeable prodrugs offer promising research tools to elucidate the roles of succinate and inhibition of SDH in metabolic reprograming towards a malignant phenotype.

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Fast-track development of an in vitro 3D lung/immune cell model to study Aspergillus infections

To study interactions of airborne pathogens, e.g. Aspergillus (A.) fumigatus with upper and lower respiratory tract epithelial and immune cells, we set up a perfused 3D human bronchial and small airway epithelial cell system. Culturing of normal human bronchial or small airway epithelial (NHBE, SAE) cells under air liquid interphase (ALI) and perfusion resulted in a significantly accelerated development of the lung epithelia associated with higher ciliogenesis, cilia movement, mucus-production and improved barrier function compared to growth under static conditions. Following the accelerated differentiation under perfusion, epithelial cells were transferred into static conditions and antigen-presenting cells (APCs) added to study their functionality upon infection with A. fumigatus. Fungi were efficiently sensed by apically applied macrophages or basolaterally adhered dendritic cells (DCs), as illustrated by phagocytosis, maturation and migration characteristics. We illustrate here that perfusion greatly improves differentiation of primary epithelial cells in vitro, which enables fast-track addition of primary immune cells and significant shortening of experimental procedures. Additionally, co-cultured primary DCs and macrophages were fully functional and fulfilled their tasks of sensing and sampling fungal pathogens present at the apical surface of epithelial cells, thereby promoting novel possibilities to study airborne infections under conditions mimicking the in vivo situation.

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Quantitative surgery

We present an open source concept, in which by using the navigated surgical stereo microscope with two grayscale cameras, surface reconstruction of the patient is performed. The obtained model is bound with registration algorithm to a model, segmented from the preoperative images (DICOM), that are registered with the patient, with the use of surgical navigation. The resulting model is projected, to the microscope oculars, as an overlay of the patient that defines the certainty of resection borders.

The Leica M500N microscope is calibrated with the optically tracked checkerboard pattern for different zoom and focus levels, that are controlled by can-bus communication. The calibration gives the parameters of cameras (distortion, field of view, transformation) and form an epipolar geometry. Tracking part provides a relation between microscope and checkerboard. With the use of stereo algorithms, characteristic features, that are scale, illumination and occlusion invariant are found in the left and right images, and then matched to define the correct epipolar lines. Homogeneous structures, such is skin, or inhomogeneous ones, that are highly illuminated and provide speculiarities, did not provide enough features to determine a valid disparity map. Therefore, we exclude the visible light with the bandpass filter and use an NIR source with DLP technology, in order to project an irregular set of points to the surgical scene. This irregularities give enough features, since they form an inhomogeneous structure on the skin.

These features are triangulated with the camera parameters, in order to form a point cloud of the patient surface, with correct scaling, that consist of valid inliers, while outliers are excluded by K-D tree.

We use NDI Optotrak Certus navigation, with which preoperative images are registered with the patient fiducials, and we form a segmented object from the same DICOM series. The microscope surface is bound to a model segmented object, by using the ICP algorithm, that results in the correct alignment of the two point clouds. By forming the transformation chain and tracking the position of microscope, patient, checkerboard and the DRF, the back projection of the resulting model to the microscope oculars is done.

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Catalytic mechanism of human oxaloacetate decarboxylase FAHD1.

Fumarylacetoacetate hydrolase domain containing protein 1 (FAHD1) was identified in eukaryotes as oxaloacetate decarboxylase. We gathered evidence that FAHD1 regulates mitochondrial function and proliferation in human cells by acting in an antagonistic pathway to pyruvate carboxylase. FAHD1 is a key metabolic enzyme at the crossroads of lipid, carbohydrate and amino acid catabolism, representing a potential pharmacological target in oncology. We were able to synthesize protein crystals of high resolution that enabled the formulation of a presumed catalytic mechanism of FAHD1, that is comprised of a series of induced-fit type changes in the tertiary structure of the protein. In this talk we present the novel data on FAHD1 where we talk about its catalytic mechanism, and new data on the protein's regulatory role in mitochondrial function and cell proliferation.

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## Compartmentalization and structure of the macromolecular GPR161:PKA complex

Scaffolding proteins organize the information flow from activated G protein-coupled receptors (GPCRs) to intracellular effector cascades both spatially and temporally. By this means, signaling scaffolds, such as A-kinase anchoring proteins (AKAPs), compartmentalize kinase activity and ensure substrate selectivity. In previous studies we have identified dynamic protein:protein interactions (PPI) of the compartmentalized protein kinase A (PKA) with distinct molecular switches downstream of receptor cascades. Recently we showed that the orphan GPCR GPR161 contains the structural features to function as selective high-affinity AKAP for PKA regulatory subunits type I. We demonstrated that GPR161 is involved in recruiting the PKA holoenzyme complex into the primary cilium. In the cilium the cAMP-sensing GPR161:PKA complex is a compartmentalized signalosome which integrates cAMP signals and receptor activities. To determine the dynamic structure of the PKA holoenzyme in complex with the GPR161 we generated a chimeric protein consisting of the transmembrane regions of the beta-adrenergic receptor and the carboxy terminus of GPR161. We aim to purify a functional GPCR:PKA complex which will be stabilized with ligands of the beta-adrenergic receptor. This approach should form the basis to determine the macromolecular structure of the GPR161:PKA complex using Cryo-EM studies. Our ultimate goal is to visualize the architecture of this macromolecular GPR161:PKA complex in the context of GPCR/kinase activation and G protein coupling.

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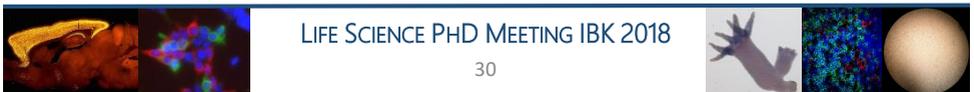
Proteomic mapping of cAMP-dependent kinase interactions and substrate dynamics

Cellular membrane receptors sense and convert the vast array of extracellular input signals and transmit information through intracellular signaling circuits [1]. To spatiotemporally control the information flow, it is required to channel the signal through central signaling nodes. Hereby, diverse scaffolding proteins take the center stage by interlinking receptors and intracellular effectors [1]. A collection of scaffolds such as A-kinase anchoring proteins (AKAPs) or the kinase suppressor of RAS (KSR) play key roles in redirecting the information flow. Deregulation of kinase pathways contribute to the etiology and progression of cancer. Activating mutations in the G-protein lead to persistent adenylate cyclase activity and therefore deregulated protein kinase A (PKA) activities [2]. We pursue our target to determine the composition of macromolecular PKA complexes in colon cancer cells along with glioblastoma [3]. Due to the fact that kinases work together in complex signaling networks, we hypothesize that elucidation of whole kinase interaction networks help to explain the molecular basis for deregulated kinase activities. First, we performed affinity isolations along with a subtractive phosphoproteomic approach to establish a PKA protein interaction network [4]. Therefore we affinity-isolated PKA complexes from different colon cancer cell lines (KM12, SW620, SW480, SKCO-1 and SNU-175) and human Glioblastoma tissue biopsies by the use of a cyclic AMP antagonist. As a second step we applied bioinformatics in order to select proliferation relevant substrates from our network. We plan to subject them to biochemical and cell-based interaction studies and to functional proliferation assays. In our preliminary processed network we already identified a prospective crosstalk between PKA and major components of the RAS-RAF-ERK pathway. Moreover we are able to substantiate the strength of our protein hits by citing recently published data [5]. By identifying and mapping these proliferation relevant PKA substrates combined with analysing distinctive signalling alterations in different cell types we hope to identify feasible drug targets to interfere with cancer cell proliferation.

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The Role of the Nuclear Orphan Receptor NR2F6 in the Germinal Center Reaction

Objective: The germinal center reaction (GC) is the site of B cell affinity maturation, plasma cell and memory formation. As such the GC is critical for humoral immunity and vaccine responses. Follicular CD4+ helper T (Tfh) cells are important for the formation and maintenance of the GC reaction through several mechanisms including, cytokine production and co-receptor stimulation, and the subsequent recruitment of follicular B cells to the GC. Because of their importance in GC regulation Tfh cells must be tightly regulated as altered Tfh activity can lead to pathogenic immune responses and auto-immunity. We have previously demonstrated the role for NR2F6 as a check on CD4+ Th17 driven auto-immunity (1,2) and CD4+ Th1 and CD8+ T cell cancer immune surveillance (3). In humans Nr2f6 expression in CD19+ B cells isolated from systemic lupus erythematosus patients is significantly reduced (4). Therefore we wanted to investigate the role of NR2F6 in GC derived auto-immunity.

Results: Loss of Nr2f6 leads to a dramatic increase in the GC reaction as both Tfh cell and GC B cell numbers are enhanced during the immune response to sheep red blood cells (SRBC) in vivo. Adoptive cell transfer of low numbers of CD4+ OT-II Nr2f6 deficient T cells into wild-type hosts followed by OVA-alum immunization was sufficient to replicate the GC phenotype of globally Nr2f6 deficient mice, indicating a primary role for NR2F6 in the CD4+ population during the GC reaction. In vitro NR2F6 acts as a suppressor of the critical Tfh cytokines interleukin (IL)-21 and IL-4.

Outlook: Investigate the effect of Nr2f6 deficiency in affinity maturation and class switch recombination. Define what if any role NR2F6 has in the B cell compartment. Characterize long-lived plasma cell formation in the absence of Nr2f6, in both B and T cells. Determine which cytokines are responsible for the increased GC response in Nr2f6 deficient mice.

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Molecular background to effectively combat breast cancer metastasis using ionizing radiation

Metastatic breast cancer (BCa) is an incurable disease with limited therapeutic response. Radiation therapy (RT) is widely used in the management of metastatic BCa, however, efficacy of conventional RT is often not satisfying due to occurrence of resistances. It is suspected that BCa cells with increased invasive potential exhibit intrinsic radiation resistance; however, there is a lack of models that allow investigating the underlying mechanisms. Therefore, the aim of this project is to establish BCa cell lines with increased invasive potential (INV), and to elucidate differences in responsiveness to irradiation between parental and INV cell lines.

MDA-MB-231, T47D and Au565 were subjected to repetitive migration through an uncoated 8µm-pore membrane to obtain INV BCa cells. Invasive potential was validated by CytoSelect™ Invasion Assay (Collagen I or Laminin). To assess metastatic abilities of INV versus parental cells, in vivo experiments using nude mice were performed. Moreover, we employed Deep Profiling Mass Spectrometry assays (MyOmix Dx, USA). Radiation response was evaluated by clonogenic assay.

All INV cell lines exhibit increased ability to invade collagen-I and laminin-coated membranes. While MDA-MB-231-INV cells invaded both, collagen I and laminin with a comparable efficiency, T47D-INV and Au565-INV cells showed pronounced invasiveness through Laminin. In vivo experiments showed that tumors formed by INV cells grew slower at the injection site but, importantly, lead to increased lymph node size, thus confirming increased metastatic potential. MDA-MB-231 parental and INV cells exhibited comparable clonogenic survival after exposure to photon-based ionizing radiation. Interestingly, however, while T47D-INV cells display increased clonogenicity, Au565-INV cells revealed lower clonogenic capacities after irradiation, compared to their parental counterparts. Moreover, we identified a number of proteins that were differentially regulated in INV compared to parental BCa cells, demonstrating a close relationship between invasive and migratory capacities and cell death, cell cycle regulation and DNA damage response.

We successfully established 3 BCa cell models with increased invasive capacities. Our results demonstrate that enhancement of invasiveness is accompanied by modulation of cell migration, resulting in increased metastatic potential of BCa cells in vivo. Interestingly, we detect differences in the sensitivity to irradiation in the 3 INV cell lines, pointing to a heterogeneous regulation of radiation response in different cell lines.

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Tyrolean Cancer Research Institute

Poster abstracts

- Posters should stay up for the whole duration of the meeting and must be taken down immediately after the poster session on friday.
- Poster presentations should last approximately 3min.
- even posternumbers at the first hour of your assigned postersession
- odd posternumbers at the second hour of your assigned postersession

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    #54-58: Genetics and Genomics
    #59-69: Developmental Biology and Ageing
    #70-86: Immunity, ID, Oncology and Clinical Medicine
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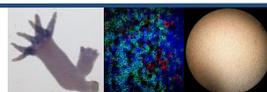
Do iron salophene complexes exert their anti-leukemic effect via ferroptosis?

Iron complexes bearing Schiff base ligands are described as promising candidates for antitumor therapy. The complex [Fe(III)salophenCl] is a representative that displayed promising anti-tumor properties against various cancer cell lines. Moreover, [Fe(III)salophenCl] was found to be competent to overcome multiple drug resistance in vincristine and daunorubicine resistant leukemia cells.

Comprehensive biological testing was conducted with derivatives of [Fe(III)salophenCl] in order to evaluate their potential to serve as compounds with anti-leukemic effects.

The metabolic activity of the complexes was investigated on the chronic myelogenous (K-562), the acute lymphatic (SD-1) and the acute myelogenous (HL-60) leukemia cell lines, and - as negative control - fibroblasts, by an assay based on the reduction of a tetrazolium salt to colored formazan. The anti-proliferative activity of the complexes was assessed by 3H-thymidine incorporation assay. The formation of reactive oxygen species (ROS) was determined by staining with mitotracker red and subsequent analysis by confocal microscopy and induction of cell death was measured by annexin /propidium iodide staining.

[Fe(III)salophenCl] and its derivates dose-dependently reduced the proliferation and the metabolic activity of all cell lines. Furthermore, the compounds induce necrosis and ROS, whereby [Fe(III)salophenCl] is more active than its derivatives. These modes of actions implicate that the compounds' effects might be considered as contribution of ferroptosis.

The influence of the complexes on induction of necrosis and ferroptosis was additionally analyzed by means of administering Necrostatin-1 and Ferrostatin-1, concomitantly with the compounds during the metabolic activity assay. Both substances were capable to suppress the inhibitory capacity of the compounds. Hence, [Fe(III)salophenCl] and its derivatives are supposed to exert their anti-leukemic potential via ferroptosis.

Ferroptosis is caused by iron that was internalized by endocytosis of the transferrin receptor 1 after iron loaded transferrin bound to it. The extent of [Fe(III)salophenCl] to bind to apo-transferrin was indirectly quantified employing atomic absorption spectrometry and revealed a high degree of binding. Besides, stability of [Fe(III)salophenCl] in the presence of Ferrostatin-1 and the iron chelator deferoxamine was proven.

In conclusion, [Fe(III)salophenCl] was characterized as promising lead structure with strong anti-leukemic property and ferroptosis can be suggested as potential mode of action.

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pH dependence of mitochondrial respiration and H2O2 production in oral cancer cells – a pilot study

Introduction: Cancers are characterized by a high metabolic plasticity resulting from mutations and the selection of metabolic phenotypes. Metabolic transformations involve mitochondrial adaptation or mitochondrial dysfunction. Metabolism and mitochondria have become focal targets for anticancer therapy. In the progression of tumor, cancer cells upregulate glucose uptake and glycolysis. The different environmental and intracellular pH due to the metabolic reprogramming can change the behavior of the cells. Therefore, we compared the effects of pH on mitochondrial respiration and H2O2 production in different cell lines.

Methods: We measured respiration and H2O2 production in permeabilized human embryonic kidney cells (HEK239T), human gingival fibroblasts (HGF) and human oral squamous carcinoma cancer cells (SCC25) in the pH range from 6.6 – 7.5 using High-Resolution FluoRespirometry including the Amplex UltraRed assay. We used substrate-uncoupler-inhibitor titration protocols to access mitochondrial respiration in different coupling and pathway control states, activating the succinate (S)-, NADH- (N) and fatty acid (F)- pathways separately or in combination.

Results: With increasing pH, H2O2 flux decreased in HEK239T cells in the S- and N-pathways, while O2 flux was significantly increased. A similar result was observed in HGF. Conversely, the SCC25 cells showed an opposite trend. In these cancer cells, electron transfer pathways and Complex IV activity were depressed at high pH. HGF and SCC25 showed a higher sensitivity to intracellular pH compared to HEK293T cells.

Conclusion: SCC25 cancer cells showed a response of respiration to changes in pH that was opposite compared to normal HGF and HEK293T cells. The sensitivity of mitochondrial respiration to modulations of intracellular pH may have implications on chemotherapy sensitivity. Therefore, the experimental design evaluated in the present study will be applied in radioresistant SCC25 cells and radiochemotherapy sensitive SCC-090 cells.

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Functional Analysis of Rbm26

Human Rbm26 and its homolog Rbm27 are two poorly characterized nuclear proteins that harbor three protein domains: a PWI, a ZF of the C3H1-type and RRM domains, known to be present in RNA processing proteins. Rbm26 has been identified in mRNA processing multiprotein complexes, but the precise function of these proteins is still very poorly understood. The Drosophila homolog, Swm, is an essential gene and has been suggested to be a cell cycle regulator, an inhibitor of Hedgehog signaling and a facilitator of Notch signaling, by regulating the expression of the fucosyl transferase (FucTA) mRNA, required for Notch glycosylation. Previous studies in our lab showed that Rbm26 is required for zebrafish development and cell cycle progression. Rbm26-depleted Zebrafish embryos showed hallmarks of ciliopathies, including situs inversus, kidney cysts and malformation of the retina. Depletion of RBM26 in human RPE1 cells also resulted in impaired proliferation and abnormal ciliogenesis. These data suggest that RBM26 might be a novel ciliopathy gene.

From the results obtained so far, both Rbm26 and Rbm27 show a nuclear pattern by immunofluorescence and Rbm26 expression seems to be cell cycle phase-dependent. We found that RBM26 co-localises with SC-35 to nuclear speckles, suggesting that it might be involved in the regulation of mRNA metabolism. To determine its cellular function, Rbm26/27 shRNA knockdown and CRISPR knockout cell lines were generated. Rbm26-depleted cell lines were analysed for cell cycle progression and ciliogenesis and these results suggest a potential role of Rbm26 in regulating these two cellular processes. In order to promote our understanding of RBM26 function we want to identify the RNA targets of RBM26 as well as its protein interactors. We have generated cell lines for inducible expression of HS-tagged-Rbm26 to perform tandem affinity purification experiments to define its protein and RNA interactome. Preliminary data showed the presence of Rbm26 in RNA complexes. We plan to perform a detailed study on the protein characterization and its function in the regulation of RNA processing mechanisms. In parallel we will perform RNAseq experiments of RPE wildtype and RBM26 knockout cells to determine changes in mRNA abundance or alternative splicing.

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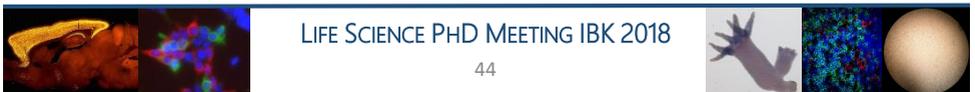
Biosynthesis of histidine, arginine, riboflavin and pantothenic acid, but not siroheme, is crucial for virulence of Aspergillus fumigatus

Aspergillus fumigatus is the most prevalent airborne fungal pathogen causing invasive fungal infections in immunosuppressed individuals. Limitations in antifungal therapy arise from non-specific symptoms of infection, poor diagnostics and comparatively few options for treatment. The aim of this study is to explore the metabolism of A. fumigatus on a comprehensive scale as essential virulence determinant to generate a collection of A. fumigatus strains with a focus on primary metabolism to target fungal pathways that are absent in mammals. Based on the annotated genome of A. fumigatus, metabolic network reconstruction served to identify fungal-specific pathways and key reactions. Predictions for unique enzymes resulted in a candidate list of genes, the inactivation of which is likely to result in an auxotrophic phenotype. The virulence potential of the generated auxotrophic mutant strains was then analyzed in various host niches. We identified five A. fumigatus pathways that are essential for growth in minimal medium: biosynthesis of the amino acids histidine and arginine, the vitamins riboflavin and pantothenic acid, and the heme-like prosthetic group siroheme, which is essential for sulfate and nitrate assimilation as well as nitric oxide detoxification. Inactivation of biosynthesis of histidine, arginine, riboflavin and pantothenic acid, but not siroheme, resulted in attenuated virulence of A. fumigatus in murine models for invasive aspergillosis with intranasal and systemic infection. The results characterize the availability of nutrients in the host niche and reveal targets for development of novel antifungal therapeutic approaches.

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Activation of Metallothionein in MTF-1 -/- cells

Anthropogenic activities in industry and agriculture lead to environmental pollution in various types, especially soil pollution increased dramatically in the past decades. To cope with heavy-metal stress in general, detoxification mechanisms like the expression of Metallothioneins (MTs) evolved throughout the animal kingdom.

Cadmium (Cd), a highly toxic soil pollutant, is one of the main inducers of MT transcription, activating MT genes in vertebrates and insects in a highly conserved mechanism via the Metal Transcription Factor-1 (MTF-1). Interestingly, MTF-1 or an orthologue is absent in invertebrates like molluscs and annelids. Instead, we recently confirmed the involvement of the Activating Transcription Factor-1 (ATF-1), a member of the cAMP responsive binding protein (CREB) family, as transcriptional regulator of MT in earthworms.

However, the exact mechanisms of MT activation in invertebrates are still unknown.

Here we identify the exact promotor region responsible for MT regulation in earthworms.

Mouse embryonic fibroblasts, more specific a double-knockout cell line lacking the expression of MTF-1 (Dko7), enabled the implementation of reporter gene assays to study earthworm MT promotor activity. Truncated earthworm-MT2 promotors allowed us to identify the exact regions responsible for basal and Cd-induced MT expression.

Our results contribute to a better understanding of the transcriptional regulation of MT in invertebrates other than insects and the evolution of signal mechanisms reacting to environmental stress.

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An apple a day keeps the doctor away - Insights into apple allergen Mal d 1 isoforms

With about 10 million tons of apples being produced in Europe annually, it is the most cultivated fruit in central Europe. Nevertheless, its consumption provokes allergic reaction to a significant proportion of patients who suffer from birch pollen allergy, an estimated 20 % of all central Europeans. Apple allergy is the result of initial sensitization to the major birch pollen allergen, Bet v 1, and subsequent immunologic cross reactivity of the Bet v 1 specific IgE antibodies with the structurally homologous apple allergen Mal d 1. Vice versa, it is possible to develop immunotherapies against birch pollen allergy by desensitizing patients towards Bet v 1 using low allergic apples.

The 17.5 kDa protein Mal d 1 is encoded by a multigene family, and 4 isoform clusters of Mal d 1 have been identified on the basis of their DNA sequence similarities. Isoform expression levels are depending on the cultivar. Yet, immunological data is based on most abundant Mal d 1.0101, but is not taking other isoforms into account. To characterize an apples allergic potential, it is crucial to gain insights into the molecular structure of this proteins.

Recently, we developed 13C-15N labeled protein expression and purification protocols for 5 isoforms, Mal d 1.0101, and Mal d 1.0105, Mal d 1.0106A1, Mal d 1.0201, and Mal d 1.0302 to perform NMR triple-resonance experiments for structure modelling. Assigned NMR protein-backbone shifts were used as input for CS-Rosetta, a program that correlates experimental shifts in structural terms with those in the BMRB data bank and known 3D structures.

We could extract structural models for each isoform, revealing that the Mal d 1.0101 fold is highly conserved among isoform clusters. This suggests that differences in IgE binding are not related to the fold topologies of the isoforms, but result from discrepancies of the amino acid sequence. Our experiments open up the possibility to perform NMR 3D structure determination with structural restraints along with studies of dynamic properties of residues of interest, and enables us to perform IgE antibody binding studies as well as immunological tests with Mal d 1 isoforms on patients.

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Voltage-gating of Cav 1.1 based on Molecular Dynamics Simultions

Voltage-gated calcium channels control various functions in nerve and muscle cells. The Cav 1.1 subtype is one of the ten subtypes of calcium channels in mammals and plays a central role in the excitation-contraction coupling in skeletal muscle cells. Exhaustive structure function studies and in silico structure models based on the recent cryo electron-microscopy structure of the active-state Cav 1.1 suggest a molecular mechanism for voltage sensing according to the transitions of positively charged amino acids in sodium and potassium channels. The knowledge of the active, intermediate and resting conformations are essential in terms of understanding the gating mechanism.

Molecular Dynamics simulations and enhanced sampling techniques are used to provide this missing information. By simulating the movement of the gating charges in the S4 (I) a sequential formation of ion pairs and the forming of a 3-10 helix is observed in agreement with experimental results.

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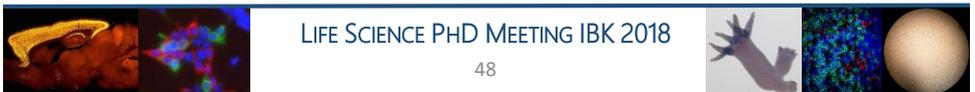
A respirometric cell viability test for peripheral-blood mononuclear cells and platelets

Mitochondrial (mt) respiration of blood cells represents a powerful diagnostic model for evaluation of mt-function in health and disease. However, standard operating procedures have to be established. We developed a substrate-uncoupler-inhibitor titration protocol to measure respiratory control in intact cells and cell viability in an integrated assay: the coupling control and cell viability protocol (CCVP) using mitochondrial respiration medium MiR06 and cell culture media RPMI and M199.

Respiration was determined in isolated peripheral blood mononuclear cells (PBMC) and platelets from healthy volunteers by high-resolution respirometry. ROUTINE respiration of intact cells (ce) was measured before and after addition of pyruvate. Oligomycin (0.05 – 2.5 µM, inhibitor of F-ATPase) was added to obtain LEAK respiration. Electron transfer-capacity (E) was stimulated by titration of CCCP (uncoupler), followed by glucose addition (Crabtree effect). Malate was added to stimulate respiration of cells without intact plasma membrane (dead cells, dce). Rotenone inhibits intact cells to the level of residual oxygen consumption (Rox), after which succinate stimulates respiration of dce. The entire cell population was permeabilized by titration of digitonin, providing a reference level of succinate respiration (permeabilized cells, pce). Cytochrome c was added to test the intactness of the mt-outer membrane (mtOM). CIII was inhibited by antimycin A, to measure CIV activity by addition of ascorbate and TMPD and later inhibition by azide.

ROUTINE respiration in MiR06 was higher in PBMCs but lower in platelets compared to cell culture media. Pyruvate stimulated respiration (p<0.02) in both cell types and respiration was higher in MiR06 than in cell culture media containing glucose after uncoupling. 0.1 µM oligomycin inhibited LEAK respiration as much as 2.5 µM, without or only slight inhibitory effect on E in PBMCs and platelets, respectively. In PBMCs, the CCVP viability test, 1-(dce-Rox)/(pce-Rox), resulted in 94% viability which correlated with 96% obtained with acridine orange/propidium iodide staining.

The respirometric CCVP viability test was equally applied to platelets. Additionally, the CCVP protocol yields a measure of the integrity of the mtOM and CIV activity. Taken together, our results suggest that the CCVP offers a standard operating procedure for mitochondrial respiratory studies in blood cells.

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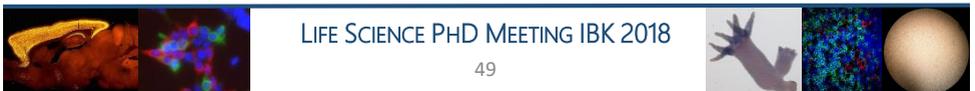
Dissecting the molecular basis of sterile inflammation upon centrosome aberrations

Tight control of cell ploidy is fundamental for organism health. Failures in cell division (cytokinesis) create polyploid cells accompanied by an accumulation of extra centrosomes, the main microtubule organizing centers in animal cells. Extra centrosomes promote aneuploidy in proliferating cells by causing errors in chromosome segregation, underlying a series of human pathologies, most notably cancer and premature ageing. Our group has reported that these supernumerary centrosomes trigger the activation of the PIDDosome multi-protein complex, leading to Caspase-2-mediated MDM2 cleavage, p53 stabilization, and p21-dependent cell cycle arrest. However, the PIDDosome also triggers NF-kB activation in response to DNA-damage, forming a complex that involves PIDD1, RIP1 and NEMO. The absence of PIDD1 causes a NF-kB activation deficit, similar to loss of RIP1, that does not affect cell death but impairs cytokine release after acute radiation which can also induce centrosome amplification. Hence, we aim to elucidate the role of PIDDosome in NF-kB signaling upon centrosome accumulation. To approach this study, we evaluated the effects of PIDD1 loss in pathway competent and cell cycle arrest proficient CRISPR/Cas9 edited cells and assessed NF-kB activation under cytokinesis failure (centrosome accumulation) or PLK4 OE (centrosome amplification) conditions. This approach shall help to identify the molecular basis of sterile inflammation in response to cytokinesis failure and centrosome aberrations.

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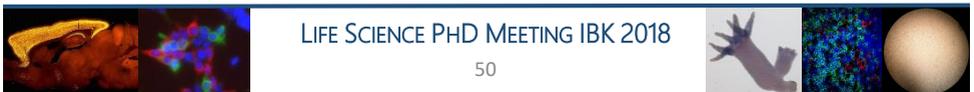
The dynamics of the apoptosis regulating BCL2 family during extended mitotic arrest

Cell death during extended mitotic arrest is considered arguably most critical for the efficacy of microtubule-targeting agents (MTAs) in anticancer therapy. While the molecular machinery controlling mitotic arrest on MTA treatment, the spindle assembly checkpoint (SAC), appears well defined, the molecular components executing cell death, as well as factors connecting both networks remain poorly understood. We conducted a mini screen exploring systematically the contribution of individual BCL2 family proteins at single cell resolution to death on extended mitotic arrest, and demonstrated that NOXA leads to the degradation of MCL1, enabling BIM-dependent cell death. We could also show that NOXA accumulates in G2 phase and is – similar to MCL1 – degraded during mitotic arrest which lead us to hypothesize that MCL1 and NOXA could be codegraded. To better understand the cell cycle specific regulation of NOXA stability we set out to define the E3 Ubiquitin Ligase that controls NOXA – and by extension MCL1 – turnover. We found that knockdown or knockout of the mitochondria-resident E3 Ligase MARCH5 stabilized both NOXA and MCL1 during mitotic arrest. This was best observed when cell death execution was blocked since otherwise the depletion of MARCH5 strongly sensitized cells to mitotic death, leading to the degradation of MCL1 and to an unexpected accumulation of NOXA. Furthermore we could show that the cell death sensitization by MARCH5 depletion was mostly NOXA dependent. These results argue for a strong involvement of MARCH5 in the stability of both MCL1 and NOXA, therefore influencing the progression of extended mitotic arrest

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The role of CD24 in adipose stem/progenitor cells

Adipose derived stem/progenitor cells (ASCs) are essential for adipose tissue regeneration und homeostasis. These cells can be isolated from the stromal vascular fraction (SVF) of adipose tissue. The SVF is a heterogeneous cell population that consists of pericytes, endothelial cells, and several other cell types besides ASCs. Several cell surface proteins are used as markers to define ASCs. However, the functions of these proteins in ASCs remain to be elucidated. The glycophasphatidylinositol-linked cell surface receptor CD24, that is expressed by various stem cell populations and thought to play a role in cell proliferation and differentiation, is one interesting candidate for a functional cell surface marker of ASCs. We isolated a cell surface DLK1-/CD34+/CD24+/CD45-/CD31- ASC subpopulation from the SVF of human subcutaneous white adipose tissue and present data on the functional characterization of these cells.

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Atomic mutagenesis of stop codon nucleotides reveals the chemical prerequisites for release factor-mediated peptide release.

Termination of protein synthesis is triggered by the recognition of a stop codon at the ribosomal A site and is mediated by class I release factors (RFs). Whereas in bacteria, RF1 and RF2 promote termination at UAA/UAG and UAA/UGA stop codons, respectively, eukaryotes only depend on one RF (eRF1) to initiate peptide release at all three stop codons. Based on several structural as well as biochemical studies, interactions between mRNA, tRNA, and rRNA have been proposed to be required for stop codon recognition. In this study, the influence of these interactions was investigated by using chemically modified stop codons. Single functional groups within stop codon nucleotides were substituted to weaken or completely eliminate specific interactions between the respective mRNA and RFs. Our findings provide detailed insight into the recognition mode of bacterial and eukaryotic RFs, thereby revealing the chemical groups of nucleotides that define the identity of stop codons and provide the means to discriminate against noncognate stop codons or UGG sense codons.

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Metabolic Regulation of Nutrient Transporter Endocytosis

Cells precisely control the repertoire of nutrient transporters and growth factor receptors at the plasma membrane in response to changes in their environment. This involves the addition of new transporters and receptors to the cell surface, but also their selective ubiquitination and endocytic removal from the cell surface. These processes are therefore essential to control cell growth and homeostasis. Defects are associated with diseases, including nutrient malabsorption, cancer and neurodegeneration. The goal of this project is to characterize the molecular mechanism leading to the selective endocytosis of nutrient transporters. Our preliminary results using budding yeast as model system, indicate that nutrient transporter endocytosis is directly coupled to the cellular metabolism (Mueller et al., eLife 2015) and differs under conditions of nutrient excess or limitation. Depending on the nutrient status, different ART-Rsp5-ubiquitin ligase complexes become active. They selectively ubiquitinate different nutrient transporters, leading to their removal from the plasma membrane in response to changes in nutrient availability. To understand how nutrient limitation is coupled to nutrient transporter endocytosis, we performed a genome-wide genetic screen in yeast to identifiy all nonessential genes that are required for this process. The function of the most interesting genes will be characterized. We predict that similar genetic networks operate and regulate starvation-induced endocytosis in mammalian cells. We are currently testing different cell lines (hTERT RPE1, HT1080 cells) and growth conditions to identify model cargos, such as Glut1 (SLC2A1), CAT1 (SLC7A1) and LAT1 (SLC7A5), suited to follow nutrient transporter endocytosis in humans. The goal of this project is to help to understand how cells regulates their nutrient transporter distribution on the cell surface in response to nutrient availability and thus have important implications for the regulation of cellular homeostasis.

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Understanding the role of the BCL-2 protein family in the maintenance of genomic stability.

Microtubule-targeting agents (MTAs) are standard of care for a number of human cancers. Through their inhibition of the mitotic spindle, the so-called "spindle assembly checkpoint" (SAC) becomes activated and prevents the cells to progress through mitosis. SAC execution involves the assembly of the mitotic checkpoint complex (MCC) containing - besides other proteins - MAD2. MAD2 sequesters the co-activator of the Anaphase-promoting complex (APC), i.e., CDC20, thereby preventing mitotic exit. Cells kept in mitosis by MCC activation can either die, mainly via apoptosis which involves the BCL-2 protein family or adapts to the MCC, which causes exit from mitosis in a process termed slippage. Such cells frequently miss-segregate their chromosomes and often fail cytokinesis, both leading to genetically instable cells that are prone to die by so far poorly defined mechanisms. Overexpression of MAD2 was shown to lead to extended mitotic arrest, chromosome miss-segregation and aneuploidy as well as increased cell death, thought to be induced to avoid chromosomal instability (CIN) that is otherwise linked to tumorigenesis.

In our study, we make use of a mouse model with Tetracycline-inducible overexpression of HA-tagged MAD2 in combination with BCL-2 protein overexpression to explore the effects on chromosomal stability in vitro and cancer progression in vivo. We hypothesize that overexpression of BCL-2 will increase tolerance of MAD2-induced aneuploidy and accelerated tumour development.

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Metabolic characterization of human T-cells during differentiation

CD8+ T-lymphocytes play a crucial role in immunity against pathogenic agents and cancer. In response to antigen presentation and in the presence of co-stimulatory signals, naïve CD8+ T-cells undergo a highly orchestrated developmental program characterized by a distinct phases of expansion, contraction and persistence of longed-lived memory T-cells. To meet the bioenergetics and biosynthetic demands, T-cells face major metabolic challenges upon differentiation.

The link between metabolic reprogramming and lymphocyte differentiation is a rising theme in immunology. In our work we aim at clarifying the metabolic programs adopted by the T-cells during differentiation and the impact that these programs have on T-cell function. We isolated human PBMCs from blood of healthy donors and naïve CD8+ T-cells have been FACS sorted to high purity using population-specific antibodies (CD8a, CD45RA, CD45RO, CD197, CD95). Naïve CD8+ T-cells (TN) have been differentiated in vitro to obtain stem cell memory (TSCM), central memory (TCM), and effector memory (TEM) cells. Using a Seahorse Extracellular Flux Analyzer we have dissected the metabolism of human CD8+ T-cells during in vitro differentiation.

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Characterizing biological phospholipid compositions with HPLC-MS/MS

The biological membranes of every living cell are composed of well-defined mixture of lipids, which are critical for their stability and fluidity. Such membranes are formed as bilayers with two hydrophilic surfaces and a hydrophobic core. To establish robust membranes, in which active proteins can be integrated, a balanced composition of different lipid species such as phospholipids, other glycolipids, or cholesterol is crucial. Apart from these 'static' membrane forming functions, phospholipids are also involved in diverse signalling pathways, as for example in the mediation of inflammatory reaction via the Lands cycle.

We have recently established an analytical setup for the qualitative and quantitative analysis of phospholipids, allowing us to characterize the phospholipid composition in cellular lipid extracts. In this high performance liquid chromatography – tandem mass spectrometry (LC-MS/MS) approach the great variety of phospholipid species is first separated on a reversed phase C8 column and then analysed with an online coupled mass spectrometer in negative ESI full scan mode. This allows us to detect more than 1100 features, corresponding to about 400 molecular species within seven different phospholipid classes. Furthermore, data-dependent acquisition of fragment spectra enables us to structurally characterize the individual lipid species using a mathematical modelling strategy we developed in earlier work. At the moment this approach is limited to abundant, typically membrane forming lipid species, but if transferred to a high resolution mass spectrometer, would for example allow the detection of low abundant signalling lipids as well. In summary, setting up this LC-MS/MS based method represents the first step towards a broader lipidomics platform that allows comprehensively characterizing lipid compositions in different biological samples.

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Production of high-value metabolites from soil-algae

Over the last decades of research, micro-algae have been proven to have a great potential as a source of highly valuable bioactive compounds such as proteins, lipids, carbohydrates, vitamins or carotenoids for medical, pharmaceutical, food and cosmetic production. Microalgae have special fundamental attributes that can be converted into technical and commercial advantages. They are a genetically highly diverse group of organisms with a wide range of biochemical and physiological characteristics like producing naturally unusual lipids, bioactive compounds, pigments or sugars and they comprise a large, unexplored group of organisms, which provide a virtually unused source of products.

Micro-algal species are potential resource of high-value metabolites and their production is growth dependent. Microalgae live in complex habitats and are subjected to stress and/or extreme conditions to survive and thus produce a variety of bioactive secondary metabolites that are not found in other organisms. This means that production of high-value metabolites is growth depended. Growth parameters can be screened for the selection of novel micro-algal species that produce molecules of interest. In this study high-value metabolites are produced under influence of various physico-chemical stresses such as nutrient starvation or nutrient deficiency, high salinity or under high light intensities.

A unique collection of algae named as ASIB 505 from the LFU Innsbruck comprised >500 soil-, air- and lichen-algae from the Alpine area of Central Europe witch will be screened for a specific compounds. This is possible through cooperation between the MUI, MCI, ADSI and LFU. The purpose of this project is to cultivate and extract soil algae from the ASIB 505 collection in order to test for anti-inflammatory, anti-oxidative and antimicrobial activity in a cell/biological-based screening.

After cultivation the algae biomass is collected and lyophilized for further specific extraction.

Because of a wide variety of possible bioactive compounds, the extraction method is based on the polarity of the substances. In further for understanding soil-algae metabolic network, which is crucial for production of wanted/specific bioactive compounds cultivation optimization and analytic profiling, should be investigated.

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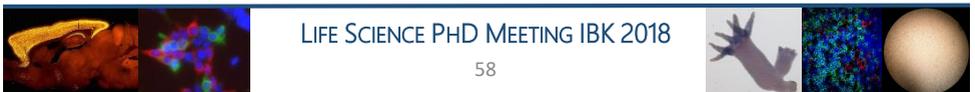
Development of an in vitro Potency Assay for Human Skeletal Muscle Derived Cells

Background: Potency is a quantitative measure of the desired biological function of an advanced therapy medicinal product (ATIVIP) and is a prerequisite for market approval application (IVIAA). To assess the potency of human skeletal muscle-derived cells (SMDCs), which are currently investigated in clinical trials for the regeneration of skeletal muscle defects, we evaluated acetylcholinesterase (AChE), which is expressed in skeletal muscle and nervous tissue of all mammals.

Methods: CD56+ SMDCs were separated from CD56- SMDCs by magnetic activated cell sorting (MACS) and both differentiated in skeletal muscle differentiation medium. AChE activity of in vitro differentiated SMDCs was correlated with CD56 expression, fusion index, cell number, cell doubling numbers, differentiation markers and compared to the clinical efficacy in patients treated with SMDCs against fecal incontinence.

Results: CD56- SMDCs did not form multinucleated myotubes and remained low in AChE activity during differentiation. CD56+ SMDCs generated myotubes and increased in AChE activity during differentiation. AChE activity was found to accurately reflect the number of CD56+ SMDCs in culture, their fusion competence, and cell doubling number. In patients with fecal incontinence responding to SMDCs treatment, the improvement of clinical Symptoms was positively linked with the AChE activity of the SMDCs injected.

Discussion: AChE activity was found to truly reflect the in vitro differentiation Status of SMDCs and to be superior to the mere use of surface markers as it reflects not only the number of myogenic SMDCs in culture but also their fusion competence and population doubling number, thus combining cell quality and quantification of the expected mode of action (MoA) of SMDCs. Moreover, the successful in vitro Validation of the assay proves its suitability for routine use. Most convincingly, our results demonstrate a link between clinical efficacy and the AChE activity of the SMDCs preparations used for the treatment of fecal incontinence. Thus, we recommend using AChE activity of in vitro differentiated SMDCs as a potency measure in end stage (phase III) clinical trials using SMDCs for skeletal muscle regeneration and subsequent market approval application (MAA)

Keywords: Acetylcholinesterase (AChE), Cell- and Tissue-Based Therapy, Fecal Incontinence, Skeletal Muscie Fibers, Myoblasts, Regeneration, Potency Assay

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Structure-based mutational analysis of the twister-sister ribozyme and implications on the cleavage mechanism

The twister-sister RNA motif is one out of four novel ribozyme classes that have been recently identified by comparative genomics analysis. It belongs to the group of self-cleaving ribozymes, catalyzing cleavage of the phosphodiester backbone in site-specific manner. Because of the new discoveries, there has been considerable interest towards an in-depth understanding of the cleavage mechanism of these catalytic RNAs. Here, we present structure-guided mutational analyses based on our observed crystal structure of the twister-sister ribozyme. Eleven conserved spatially separated loop nucleotides are brought into close proximity at the C-A cleavage site. Cleavage assays on key base substitutions and different ribose mutations, as well as Mn^{2+} for Mg^{2+} replacements in this twister-sister construct, have revealed that the interactions between a guanine and a hydrated Mg^{2+} with the non-bridging phosphate oxygens at the cleavage site are important for the cleavage activity.

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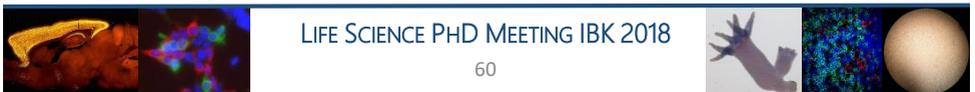
Iron sensing is governed by mitochondrial, but not by cytosolic iron-sulfur cluster biogenesis in Aspergillus fumigatus

For optimal growth, microorganisms have to adapt their iron metabolism to the requirements of their ecological niche to avoid iron shortage as well as iron toxicity. Therefore, mechanisms have been evolved to tightly regulate iron uptake, consumption and detoxification, respectively, which depend on sensing the cellular iron status. In the facultative anaerobic yeast *Saccharomyces cerevisiae,* iron sensing has been shown to depend on mitochondrial (MIA) but not cytosolic iron-sulfur cluster assembly (CIA), while in mammals the cellular iron state is sensed via cytosolically synthesized iron-sulfur clusters. To address the question how the obligatory aerobic mold *Aspergillus fumigatus* senses the cellular iron state, mutant strains allowing down-regulation of MIA and CIA were generated. These studies revealed that: (i) Af-Nfs1 (Afu3g14240) and Af-Nbp35 (Afu2g15960), which are required for MIA and CIA, respectively, are essential for growth; (ii) inactivation of the Frataxin homolog Af-FxnA (Afu4g10510), which is involved in MIA, is not lethal, but results in a severe growth defect; (iii) a decrease in MIA (Af-Nfs1 depletion, Af-FxnA-deficiency) but not CIA (Af-Nbp35 depletion) results in an iron starvation response accompanied by increased iron toxicity; and, likewise, (iv) a decrease in mitochondrial iron import results in an iron starvation response. Taken together, these data underline that iron sensing in *A. fumigatus* depends on the mitochondrial, but not the cytosolic iron-sulfur cluster machinery. Moreover, depletion of the glutathione pool caused an iron starvation response underlining a crucial role of glutathione in iron sensing in *A. fumigatus*.

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The Tmk1 MAP kinase governs mycoparasitism and negative chemotropism in Trichoderma atroviride

Trichoderma atroviride is a mycoparasitic fungus used as biological control agent against plant-pathogenic fungi. To study sensing and recognition of prey-derived signals we applied an assay that quantifies the chemotropic response of the mycoparasite towards selected chemical compounds and culture supernatants of different fungal preys such as Rhizoctonia solani, Botrytis cinerea, and Sclerotinia sclerotiorum. T. atroviride wild type and the Δtmk1 deletion mutant lacking the Fus3/Kss1-type MAP kinase were included in our analysis. In chemotropism assays comparing the responses of conidial germlings to chemicals promoting negative chemotropism, the Δtmk1 mutant showed a higher chemotropic index than the wild type, whereas similar positive chemotropic responses were elicited in both strains by culture supernatants of prey fungi. However, analyses of the mycoparasitic behavior in macro-colony confrontation assays revealed a reduced ability of the Δtmk1 mutant to overgrow and lyse any of the fungal preys tested indicating a role of the Tmk1 MAP kinase in the later stages of the mycoparasitic interaction.

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Identification and characterization of anti-inflammatory compounds from soil algae extracts

A novel cell-based reporter assay was developed for primary screening of soil algae extracts with anti-inflammatory potential. The spontaneously immortalized human keratinocyte cell line HaCaT has been used to generate a stable NF-κB reporter cell line by retroviral transduction. Activity of the transcription factor NF-κB has been utilized as a representative marker of the inflammatory state of cells due to its key role in various inflammatory processes within essentially all human cells. The assay concept is based on induction of an inflammatory response in the NF-κB reporter cell line and treating them with selected soil algae extracts. NF-κB reporter activity was induced either "receptor independent" with UV irradiation or "receptor mediated" with TNFα. In the course of assay development the optimal time point for quantification of reporter activity, impact of different doses of UVA and UVB radiation, interaction of FBS and extract solvent concentration on assay performance and establishment of functional assay controls were investigated. The biological relevance of the reporter cell line was verified by correlating increased reporter activity in response to UVB radiation with the increased secretion of relevant pro-inflammatory cytokines. Cytotoxic concentrations of soil algae extracts on the NF-κB reporter cell line were analyzed with a resazurin based cell viability assay prior to screening. To screen for anti-inflammatory activities, cells were treated with non-cytotoxic concentrations of algae extracts either prior to UVB irradiation ("pre-treatment") or afterwards ("post-treatment"). We tested 106 extracts and found that several of them had a pronounced inhibitory effect on the reporter activity. Treatment with several algae extracts have led to reduced reporter activity in this primary screening, suggesting their potential inhibitory effect on NF-kB. Those hits will be tested more thoroughly in secondary screenings to verify their anti-inflammatory potential in physiologically highly relevant 3D human skin models. For characterization of anti-inflammatory metabolites extracts will be fractionated to simplify identification of active compounds by analytical analysis with HPLC/GC-MS. In conclusion the established inflammatory-response assay is a cost-effective and reliable screening tool to identify algae metabolites capable to produce potent agents which will be used for further investigations and drug development.

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Effect of Nuclear Magnetic Resonance on the Circadian Clock and the Hypoxic Signaling Pathway

The circadian clock and the hypoxia signaling pathway interact bidirectionally, and both are important targets for several diseases, such as osteoarthritis or cancer. Nuclear magnetic resonance (NMR) has been used as therapy for the treatment of human osteoarthritis and osteoporosis, but the biological effects on living systems, such as cells or organisms are still not clearly understood. We therefore analyzed the impact of NMR treatment on the zebrafish cell line Z3, which is well characterized in terms of circadian rhythmicity and hypoxic signaling. Both pathways are highly conserved across the animal kingdom and play major roles in the development and progression of osteoarthritis. Our results revealed that circadian rhythm of Hif1 protein expression, the major regulator of hypoxia signaling pathway, and Peroxiredoxin-SO3, an antioxidant for tuning the cellular redox status, was significantly altered after NMR exposure. Also reactive oxygen species levels were significantly altered correlating to Peroxiredoxin-SO3 results, both significant markers as well as effectors of the cellular redox environment, which might serve as the actual signal transduction system, similar to the light induced reset of the circadian clock. In summary, we found that NMR therapy selectively affects pathways which play major roles in several diseases such as the development and progression of osteoarthritis or cancer. This study gives further indications of the molecular mechanism of NMR exposure in a cell system.

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High-throughput screening to identify regulators of PIDDosome activation

The PIDDosome is a multi-protein complex composed of the death domain proteins PIDD1 and RAIDD. This complex facilitates dimerization and activation of procaspase-2, a protease implicated in cell death effector. Along with its poorly understood role in apoptosis, the PIDDosome/caspase-2 axis has also been implicated in other cellular processes. One such process is cell cycle arrest in response to centrosome amplification. Centrosome amplification can occur as a result of cytokinesis failure. It is considered a major driver of chromosome mis-segregation and aneuploidy. Upon cytokinesis failure, the PIDDosome drives a p53-mediated and p21 dependent cell cycle arrest. Although PIDD1 can localize to centrosomes, it is still unknown how the presence of extra centrosomes drive PIDDosome activation. Here, we report the results of a screening effort to identify novel regulators of PIDDosome assembly. Towards this end, we have adapted a bifluorescence complementation reporter of caspase-2 dimerization to identify compounds which extinguish or enhance PIDDosome activation upon cytokinesis failure. This work will enhance our understanding of PIDDosome formation and provides lead compounds for efforts to target tumor growth through forced PIDDosome activation.

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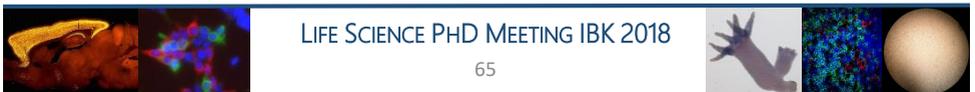
The erythropoietin / p27Kip1 axis in erythroid cell proliferation and differentiation

Erythropoiesis is a complex process during which hematopoietic stem cells (HSC) differentiate into mature erythrocytes. The earliest committed progenitors of the erythroid lineage are the burst forming unit-eryhoid cells which differentiate into colony-forming unit-erythroid (CFU-E) progenitors. CFU-E progenitors divide rapidly and they are highly dependent on the presence of Erythropoietin (Epo), a hormone which binds the type I erythropoietin receptor (EpoR). Binding of Epo to EpoR triggers multiple signal transduction pathways, through the activation of JAK2, which in turn regulate the proliferation, survival and differentiation of the erythroid progenitors.

Activation of EpoR is essential for erythropoiesis and the receptor is not only highly expressed in the CFU-E progenitor cells but remains expressed during differentiation in the late stages of erythropoiesis. It was previously observed that erythroid differentiation is associated with accumulation of the cyclin-dependent kinase inhibitor p27Kip1, but little is known about its role and regulation during erythroid proliferation. We observed previously that phosphorylation of p27Kip1 on tyrosine residue 88 (Y88) by a number of tyrosine kinases including JAK2 and Lyn, impairs its CDK-inhibitory capacity and can initiate its ubiquitin-dependent degradation. Since Epo/EpoR signaling depends on JAK2 and Lyn, we were wondering if p27Kip1 can be regulated upon Epo stimulation.

In this project, we investigate the role of p27Kip1 in response to Epo stimulation in vivo and in vitro in proliferation and differentiation. We found that p27Kip1 is phosphorylated on tyrosine 88 (pY88p27) upon Epo stimulation and inhibition of JAK2 prevents this phosphorylation. Furthermore, endogenous p27Kip1 is in a complex with EpoR in the Epo-dependent erythroleukemic UT-7/Epo cells in vivo and pull down assays using bacterial produced recombinant proteins suggest a direct binding of the N-terminus of p27Kip1 to EpoR in vitro. Since EpoR is also expressed in the late stages of erythropoiesis, we aim to determine the Epo-mediated regulation of p27Kip1 in erythroid differentiation. To gain further insight into the physiological role of the pY88p27 by Epo, we are planning to investigate the generation of erythroid cells in p27Y88F-knock-in and wild type mice in steady state and stress-induced erythropoiesis.

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Membrane recruitment of neurofibromin to regulatory interaction of Ras protein

Neurofibromatosis type 1 (NF1) is a common autosomal dominant genetic disease that affects 1 in 3500 newborns. Patients have an increased risk to develop the disease typical neurofibroma, benign and malignant tumors of the nervous system and display additional hallmarks including pigmentation anomalies, bone deformations and learning disabilities. The tumor suppressor gene NF1 encodes the giant signal regulator neurofibromin (320 kDa) which is a Ras specific GTPase activating protein (RasGAP). Neurofibromin uses its central GAP related domain (GRD) to down regulate the biological activity of the small G-protein Ras that is catalytically mutated in 30% of human malignancies and rendering Ras insensitive to the presence of RasGAPs. A regulatory protein of Ras/MAPK pathway is Sprouty related protein with an EVH1 domain 1 (Spred1) which involve to interact with neurofibromin. This observation links NF1 to the only recently described Legius Syndrome (LS) that shares mild features with NF1 and is characterized by genetic alteration of the SPRED1 gene. Indeed, binding Spred1-EVH1 to GRD induces the plasma membrane localization of neurofibromin close to Ras, which is subsequently down-regulating activated RAS level. The follow up analysis shed important light on mechanisms of membrane recruitment of neurofibromin that is required for its regulatory interaction with membrane associated Ras. Despite a rather detailed dissection of the interaction on the Spred1 side, the site of interaction on the 2800 residues of the coding sequence of neurofibromin remained elusive. Our aim is the depth characterization of the neurofibromin Spred1 interaction expecting novel insights considerable amount of membrane translocation of currently most important signal regulator of Ras.

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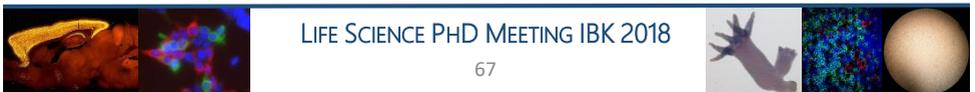
Synthesis of Leucosceptroid Natural Product Derivatives for Target Identification

The leucosceptroid natural products were isolated from Leucosceptrum canum Smith, a native plant in Nepal and China, by Li and coworkers. These sesterterpenoids display nanomolar antifeedant activity against both the cotton bollworm and the beet armyworm, some of the most destructive agricultural pests in nature.

The recent total synthesis of sixteen members of the leucosceptroid family of natural products by our laboratory provided enough material for further investigations of the biological activity of these terpenoids. In addition to the significant antifeedant activity, the group of Adibekian (University of Geneva, Scripps Research Institute - Florida) found that some leucosceptroid natural products interact highly selectively with proteins that are involved in the development of cancer. In this respect, leucosceptroids K, L and M revealed the most favourable effects. It is hypothesized, that the remarkable structure-activity relationship of these natural products arises from the butenolide moiety, which they have in common. Suitably modified leucosceptroids for target identification via affinity proteomics could help to understand the mode of action with which small molecules can influence cancer development.

Herein, we describe the synthesis of three derivatives, which correspond to leucosceptroids K, L and M. The synthesis intercepts our general entry to the antifeedant leucosceptroids and is based on a late stage intermediate of the synthetic route.

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Regulators of cardiomyocyte proliferation and regeneration in mouse and human

Rationale: The adult mammalian heart has little regenerative capacity after myocardial infarction (MI) while neonatal mouse hearts regenerate without scarring or dysfunction. However, the underlying pathways and responsible coding and non-coding transcripts are poorly defined.

Objective: We sought to derive insights into the pathways regulating neonatal development of the mouse heart and cardiac regeneration post-MI.

Methods and Results: Performing RNA-seq on neonatal mouse hearts through the first 10 days of postnatal life revealed changes in the coding and non-coding transcriptome after neonatal MI, with evidence of essentially complete healing by P10. Over two thirds of each of the mRNAs and microRNAs that were differentially expressed in the post-MI heart were also differentially expressed during normal postnatal development, suggesting a common regulatory pathway for normal cardiac development and post-MI cardiac regeneration. We selected exemplars of miRNAs that were implicated in our data set as regulators of cardiomyocyte proliferation. Several of these showed evidence of a functional influence on mouse cardiomyocyte cell division. In addition, a subset of these microRNAs, miR-144-3p, miR-195a-5p, miR-451a and miR-6240 showed evidence of functional conservation in human cardiomyocytes. Promising targets of the coding transcriptome were validated in neonatal mice in-vivo using a rAAV9 mediated knock-down system. Candidates like Igf1r, Myl9, Lamc2 and Spp1 were confirmed as potentially important players in the process of neonatal cardiac regeneration.

Conclusions: The sets of mRNAs and miRNAs that we report here merit further investigation as gatekeepers of cell division in the postnatal heart and as targets for extension of the period of cardiac regeneration beyond the neonatal period. Results of rAAV9 mediated knock-down experiments furthermore strengthen the validity and relevance of our screening results in the process of neonatal cardiac regeneration.

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The lipoxygenase gene lox1 affects growth and the injury response in Trichoderma atroviride

Trichoderma atroviride is a necrotrophic mycoparasite antagonizing various fungal plant pathogens and finds application as biological control agent. Secondary metabolites are among the main agents determining the strength and progress of the mycoparasitic attack. The main secondary metabolite produced by T. atroviride is 6-pentyl-α-pyrone (6-PP) which exhibits anti-fungal and plant growth-promoting activity. 6-PP is a volatile, unsaturated lactone derived from fatty acid metabolism, synthesized in a hitherto unknown biosynthetic pathway.

Isotopic labelling experiments proposed the oxidation of linoleic acid by a lipoxygenase (lox) as the first step in 6-PP biosynthesis (Serrano-Carreon et al 1993, Applied and Environmental Microbiology 59). Accordingly, 6-PP producing species such as T. atroviride encode a lox gene (lox1) in their genomes while non-producing relatives do not. In T. atroviride lox1 expression as well as 6-PP levels furthermore are upregulated during the mycoparasitic interaction with fungal preys.

Based on these evidences indicating a role of lox1 in 6-PP biosynthesis, we generated T. atroviride Δlox1 gene deletion mutants. Δlox1 mutants exhibited unaltered mycoparasitic and antifungal activities, but showed a slightly increased growth rate on solid media compared to the wild type (WT). In addition, mechanical injury did not result in the formation of asexual reproduction in the mutants, while T. atroviride typically shows this injury response. GC-MS analysis of 6-PP levels revealed similar quantities produced by the mutants and the WT. We hence conclude that lox1 affects radial growth and the response of T. atroviride to injury, but is not involved in the biosynthesis of 6-PP.

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Protein kinase A regulates RNA:protein interactions of TAF15

Many diseases can be thought as pathological alterations of molecular interactions. Besides protein:protein and protein:DNA interactions, in particular RNA-binding proteins are involved in the regulation of physiological but also pathological cell functions. Examples are the RNA-binding proteins of the FET family including the TATA binding-protein-associated factor 2N (TAF15) [1]. TAF15 is predominantly localized to the nucleus participating in transcription and splicing and has been found to form cytoplasmic inclusions in the neurodegenerative disease amyotrophic lateral sclerosis (ALS) [2-4]. It is still an open question how the cytoplasmic interaction between TAF15 and specific RNA species contribute to disease etiology or progression.

In a phospho-proteomics screen for protein kinase A (PKA) substrates and interaction partners, we have identified a possible functional connection between the kinase pathway and TAF15 [5, 6]. Following PKA affinity isolation and mass spectrometry analyses we identified and confirmed the phosphorylation of one of the TAF15 RNA-binding modules, the RanBP2 zinc-finger domain (ZnF). We assume that PKA-mediated TAF15 phosphorylation might affect RNA binding and/or protein:protein interactions (PPIs) and it may exert an effect on deregulated TAF15 functions.

Currently we are performing cross-linking and inmunoprecipitation experiments (iCLIP) to identify TAF15 and ZnF-bound RNA species which are regulated by PKA phosphorylation. In the first experiments we could see that aminoacid substitution in the phosphorylation site changes global TAF15 RNA-binding properties indicating that it might regulate molecular interactions between TAF15 and its RNA targets. We plan to identify these RNAs to unveil the impact of PKA phosphorylation on TAF15 physiological and pathological functions.

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Efficient Expression and Purification of Toxic, Instable and Intrinsically Disordered Proteins

Structural studies of proteins require large amounts of homogeneous material, typically produced recombinantly. Unfortunately, toxicity and proteolytic instability often render heterologous expression methods ineffective. A novel approach is presented here that circumvents these issues by redirecting proteins to bacterial inclusion bodies via a small, well-expressed and highly insoluble fusion partner. The crude insoluble protein fraction is denatured and incubated at low pH, a comparatively mild treatment that selectively disrupts an acid-sensitive linker between protein of interest and fusion partner. The target is subsequently purified to homogeneity via metal-affinity chromatography in the denatured state. Effectively transforming the expression problem into a renaturation challenge, the method is ideally suited for the production of inherently disordered proteins (IDPs), as well as peptides and small proteins that readily refold in vitro. Using lysozyme as an example, the chemical cleavage procedure is shown to be fully compatible with high-resolution structural studies.

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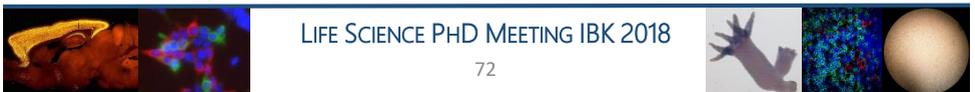
The Dsc-E3 ligase complex targets the membrane protein Orm2 for proteasome dependent degradation at the Golgi to restore sphingolipid homeostasis.

Ubiquitin-dependent protein degradation pathways and their associated stress responses are essential for cellular integrity. Ubiquitinated membrane proteins in post-ER compartments (at the plasma membrane, the Golgi, on lysosomes and on endosomes) are typically sorted by the endosomal sorting complexes required for transported (ESCRT) along the MVB pathway into lysosomes for degradation.

We have now identified an additional post-ER membrane protein degradation pathway. At the Golgi, the membrane embedded Dsc-E3 ubiquitin ligase complex functions in a proteasome-dependent membrane protein degradation pathway to restore sphingolipid (SL) homeostasis in response to TORC2-Ypk1 signaling in yeast. Activation of TORC2-Ypk1 signaling (by membrane stress and SL depletion) phosphorylates Orm2, a negative regulator of serine palmitoyltransferase (SPT) at the ER. Phosphorylated Orm2 exits the ER and moves to a post-ER compartment, most likely the Golgi, and there interacts with the Dsc complex. The RING E3-ligase of the Dsc complex, Tul1, ubiquitinates phosphorylated Orm2 on N-terminal lysine residues, which results in its proteasome-dependent degradation. As a result Orm2 levels decrease, the serine palmitoyltransferase becomes more active, SL biosynthesis is stimulated and membrane homeostasis restored.

Our results show for the first time that membrane proteins at the Golgi are selectively degraded in a proteasome-dependent manner, and we implicate this process in membrane stress signaling.

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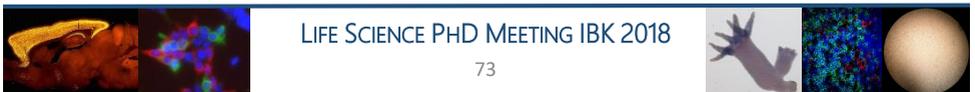
Metabolic regulation of nutrient transport in eukaryotic cells

Nutrient uptake fuels cellular metabolism and thereby promotes cell growth and survival. Little is known about the molecular mechanisms that control nutrient transport across the plasma membrane in response to nutrient availability. We have discovered that changes in amino acid and nitrogen availability selectively triggers endocytosis and lysosomal degradation of over 30 different nutrient transporters in Saccharomyces cerevisiae (Müller et al. eLife 2015). We refer to this process as starvation induced endocytosis which promotes cellular adaptation and ensures entry into quiescence and cell survival during nutrient limitation.

To characterize the regulatory networks that couple nutrient availability to nutrient transporter endocytosis we use the methionine transporter Mup1 as a model because it is removed from the cell surface, both in response to nutrient excess and starvation. Mup1 fused pHluorin was introduced into the yeast nonessential knockout collection (5133 genes). The capability of 4962 mutants to sort MUP1-pHluorin to the vacuole where the fluorescence of pHluorin is quenched was analysed by 96-well fluorescence-activated cell sorting (FACS). Potential hits were re-assayed by live cell epifluorescence microscopy.

We identified 231 nonessential genes that regulate the starvation induced endocytosis of Mup1 including expected hits known to be involved in endocytosis and response to starvation as well as unexpected hits like genes involved in mitochondrial organisation and oxidative phosphorylation. Today I present the initial characterization of the most promising hits from the screen.

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Application of Real-Time Breath Monitoring to Generate Metabolomic Data

Breath gas analysis is a promising method for the non-invasive monitoring of metabolomic processes. With modern mass-spectrometric methods it is possible to take samples rapidly and in real-time, and hence follow individual breath cycles. The standard procedure to gather breath data is usually one sample per minute or to deliver one breath sample into a bag every 5 - 10 minutes. This yields only a little amount of samples, and in addition the volatiles in the bags are unstable for periods of longer than an hour. Here we demonstrate the advantage of using high time resolution measurements for breath analysis, but this comes at the expense of resulting in a higher error in volatile concentrations. Often studies work around this by claiming to work under stationary conditions, but this is postulated and not proven. We propose that the application of high time resolution measurements can provide the necessary information to determine if a stationary condition has been reached or not, whilst providing an accurate measure of volatile of concentrations, which can be further improved by down-sampling. However, there are particular computational challenges to overcome, namely:

i) the breath sample can be affected by environmental matrix effects,

ii) the division of a continuous signal into breath and ambient air samples,

iii) modelling for estimation of latent parameterand

iv) achieving reproducibility.

While individually these challenges have been addressed, applying these in a complex setting is non-trivial. As part of this thesis we have developed a pipeline for rocessing such data, in order to provide a framework for reproducibility. We will present a study on three volunteers inhaling diluted acetone vapours and the relevant extracted data. These data are compared with the results of other established measurements.

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Bio-assay guided isolation of terpene ester derivatives of the roots of Ferula hezarlalehzarica and their cytotoxic activity on breast cancer cells

The genus Ferula (Apiaceae) comprises about 180 species spreading throughout the world and some species are used in folk medicine of different countries for treatment of stomachache, hysteria and others. In Iranian flora, genus Ferula is represented by 32 species including 16 endemic representatives. In Iranian traditional medicine Ferula species are highly appreciated e.g. the well-known representative F. assa-foetida of which the gum has been used as a remedy for abdominal pain, constipation, diarrhea, spasms and others. Ferula hezarlalehzarica Ajani, identified in 2008, is an endemic species growing in Kerman province, Iran. This plant is used by the local people for the treatment of gastrointestinal disorders.

Bio-assay guided isolation of the dichloromethane extract of the roots of F. hezarlalehzarica resulted in the isolation of twelve compounds. One of the ten isolated daucane sesquiterpene derivatives were identified as new compounds. The two remaining compounds were identified as known borneol derivatives. Determination of absolute configuration of the new compound was done by means of electronic circular dichroism and quantum chemical calculation methods

An initial pharmacological investigation of the isolated compounds in a colorimetric assay (MTT) in MDA-MB-231 cells revealed two active compounds, the p-hydroxy benzoic acid as well as the 3-methoxy-4-hydroxy benzoic acid analog of ferutinol, which showed identical IC50 values of 42 µM after 24h incubation.

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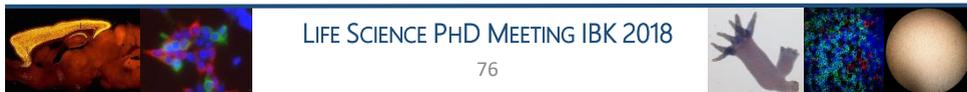
Characterizing O-Glycosylated (N-Terminal) Pro-B-Type natriuretic peptide forms in blood plasma of patients with severe heart failure

Background: N-terminal pro–B-type natriuretic peptide (NT-proBNP) and its C-terminal physiologically active counterpart, B-type natriuretic peptide (BNP), have become well-accepted heart failure (HF) biomarkers and were implemented into the guidelines for HF-management more than 10 years ago. Both forms, as well as their unprocessed precursor proBNP circulate as various N- and C-terminally truncated and O-glycosylated forms in severely ill HF patients.

As commercial antibody-derived immunoassays show different affinities to the varying molecular forms and thereby do not reflect their entirety and impede comparisons between platforms, understanding the structural attributes of the molecular heterogeneity in the diverse settings of HF displays a critical prerequisite for improved design of assays as well as their clinical application.

In this study we set out to define the specific O-glycosylation sites of human endogenous (NT-) proBNP in HF patients. We also demonstrate a variable distribution of glycoforms in different patients.

Materials and Methods: Heparinized plasma samples of HF patients were purified by Immunopurification using either a biotin-conjugated polyclonal antibody directed against amino acids 1–21 or 42–46 of (NT-) proBNP bound to streptavidin-coated magnetic microparticles. Eluates were partially deglycosylated with an exoglycosidase cocktail and subsequently proteolytically digested with trypsin or endoproteinase Glu-C or a combination of both enzymes. To verify results, additional samples were also treated with BEMAD chemistry.

Samples were analyzed by nanoflow liquid chromatography electrospray ionization multistage mass spectrometry (nano-LC ESI-MSn) on an LTQ Orbitrap XL (Thermo Fisher Scientific).

Results: A new glycosylation site could be pinpointed to Ser5. An additional newly described site, either Thr14 or Ser15, was found to be glycosylated in substoichiometrical amounts. Data evaluation of differently proteolytically digested samples proved that Thr36, Ser37, Ser44, Thr48, Ser53, Thr58 and Thr71 are modified by glycans. Interestingly, relative amounts of glycoforms varied between patients.

Conclusion: In this study we characterized nine specific O-glycosylation sites on human (NT-) proBNP circulating in plasma of patients with severe HF. Most strikingly, all peptides characterized coexist also in an unmodified form and relative amounts of glycoforms varied among patients.

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Imaging of Biological Tissue Using Desorption Electrospray Ionization (DESI)

Mass spectrometry imaging (MSI) has emerged as a powerful technique in biological sciences. It provides direct information of the spatial distribution and the (structural) characterization of compounds in biological tissues. Desorption electrospray ionization mass spectrometry (DESI-MS) is an ambient mass spectrometry method that allows direct sampling of surfaces with no or little sample pre-treatment in the open air. We investigated the use of DESI mass spectrometry imaging (DESI-MSI) as a direct analytical technique for lipid profiling and imaging of mouse brain as well as mouse heart tissues. DESI imaging experiments were performed on a Thermo LTQ ion trap and a LCQ DecaXP ion trap mass spectrometer equipped with an in-house built prototype 2D DESI ion source. Data were processed using the open-source R environment. Capillary size, angles and distances between sprayer, sample and mass spectrometer inlet, as well as solvent composition and solvent flow rate were optimized to improve signal intensity for the detection and spatial localization of lipids of biological tissues. Results will be discussed.

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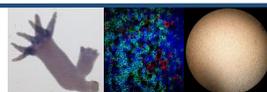
Enhancing proteomic throughput in capillary electrophoresis-mass spectrometry by sequential sample injection

Introduction: In this study we demonstrate the potential of sequential injection of samples in capillary electrophoresis-mass spectrometry (CE-MS) for rapid and sensitive proteome characterization of human lymphoblastic T-cells (line CCRF-CEM). Proteins were extracted, enzymatically digested, and the resulting peptides fractionated by RP-HPLC. Twenty fractions were thereafter analyzed by CE-MS within a single MS analysis. The CE-MS method was designed that every 10 minutes a new fraction was injected into the CE system. Without any rinsing or equilibration steps we were able to generate a continuous stream of peptides feeding the mass analyzer.

Methods: Proteins were extracted from $1x10^6$ CCRF-CEM cells, reduced, alkylated and digested using trypsin. Peptides were thereafter separated by reversed-phase chromatography. A total of 240 fractions were collected, lyophilized and dissolved in ammonium acetate. Peptide analysis was performed using a CESI 8000 Plus (Sciex, Brea, CA) which was coupled to a Q Exactive HF. Sample injection and application of separation voltage was carried out alternately. Samples were injected by applying a pressure of 3 psi for 50 sec (25 nL). In between sample injections a separation voltage of +30kV was applied for 10min with a simultaneous pressure of 2 psi applied at the capillary inlet. A total of 20 peptide fractions were analyzed successively.

Results: In 250 min, the total analysis time of a single sequential injection experiment, we were able to identify roughly 28.000 peptide sequences counting for 4.800 proteins. High total ion current was observed between 15 and 240 min, which indicates peptides continuously entering the MS for 3.75 h. A stable electrospray is generated as soon as the liquid in the capillary moves forward, during sample injection as well as during separation. No electrospray is generated during the switchover from sample to BGE vial and back again. Importantly, during those few seconds no flow is generated, meaning that no peptides emerge at the capillary tip and thus cannot get lost.

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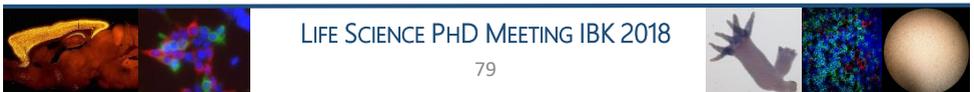
Development and validation of a rapid ultra-high performance liquid chromatography diode array detector method for Verbena officinalis

This study presents a fast and validated ultra-high performance liquid chromatography diode array detector (UHPLC-DAD) method for the simultaneous determination of the major compounds in Verbena officinalis (Verbenaceae) herb, a medicinal plant listed in the European-, British-, and, Chinese- Pharmacopeia. In order to get reference substances, nine compounds, belonging to iridoids, flavonoids, and phenylpropanoids, were isolated from the herb extract. Two of them, namely cistanoside D and leucoseptoside A, were found in this plant species for the first time. Chromatographic separation was achieved in less than 7 min on a Kinetex 1.7u XB-C18 (50 x 2.10 mm) column by using a solvent gradient of water-acetonitrile modified with 0.1% formic acid. Method validation confirmed the assays sensitivity, linearity ($R^2 \geq 0.997$), precision (intraday precision $\leq 1.71\%$; interday precision $\leq 1.46\%$) and accuracy (recovery rates between 93.9% and 108.8%) for the quantitative analysis of the eight selected marker compounds. Identity and peak purity of the analytes was confirmed by coupling the UHPLC instrument to a quadrupole time-of-flight mass spectrometer via an electrospray ionization interface (ESI-QTOF-MS) operating in the negative ionization mode.

Finally, the applicability of the developed UHPLC-DAD method was successfully proven for the sensitive quantitation of the major compounds in Verbena officinalis herb extracts, thereby providing a reliable tool for its rapid quality control.

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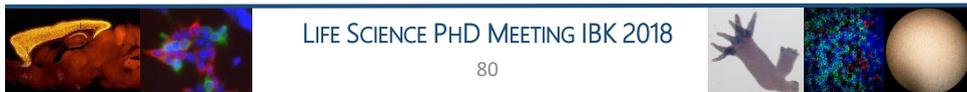
pH Dependent Local Fold Stability

Proteolytic digestion at distinct pH values is a key aspect of allergen processing. Plant pollen allergens have been shown to exhibit a strongly pH dependent proteolytic susceptibility. However, the atomistic details of this pH dependence and those of the resulting structural and dynamical consequences remain unclear.

Our goal is to unravel the structural determinants of the pH-dependency of foldstability. We profile proteolytic stabilities and molecular flexibilities of plant pollen allergens on a broad range of timescales using molecular dynamics simulations and proteolytic degradation experiments. The use of enhanced sampling techniques allows us access to protein dynamics on the millisecond timescale. Time resolved degradation assays enable us to identify key structural features of the proteins which we link with the dynamics captured in our simulations.

We investigated two plant allergens, the major birch pollen allergen Bet v 1a and the major timothy grass allergen Phl p 6. We found a striking correlation between the molecular flexibilities captured with our MD simulations and the thermal and proteolytic stabilities. The strong influence of the pH value on the proteolytic susceptibility of the systems is also compelling: small changes in the pH value are clearly enough to completely change the kinetics of local unfolding and consequently proteolytic degradation. We employ constant pH molecular dynamics simulations and NMR techniques to determine the pKa values for the systems' titratable residues to unravel the molecular mechanisms responsible for this reversal in stability.

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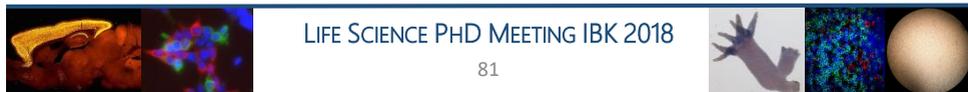
Describing the CDR-H3 Loop as a Conformational Ensemble

The H3 loop of the complementary determining region (CDR) substantially influences the binding properties of antibodies. In contrast to the five remaining loops, no canonical structure models have been identified for the CDR-H3. As most modeling tools rely on these canonical structure models[1,2], prediction of CDR-H3 loop conformations remains a major challenge[3].

Here we introduce a molecular dynamics (MD) based approach to characterize the ensemble of the CDR-H3 loop. MD simulations allow to generate diverse structural ensembles and simultaneously capture information on the thermodynamic and kinetic properties of the CDR loops. We use different crystal structures of the Anti-HBV e-antigen monoclonal Fab fragment (e6) with strongly varying CDR-H3 loop conformations as starting points[4]. In our simulations, we observe a diverse ensemble of conformations for the CDR-H3 loop, while the remaining five loops tend to sample only distinct conformational states. The usage of two different starting conformations allows us to trace the transitions from one crystal state to the other.

Our results clearly indicate that the CDR-H3 loop does not fit into the description of canonical structure models. As the CDR-H3 loop is intrinsically more flexible than the remaining five loops we propose that it has to be characterized by a conformational ensemble for each individual antibody.

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Novel insights into the specificity of mushroom-tyrosinase towards ubiquitous polyphenols found in plant extracts

Purpose: Tyrosinase is a key enzyme in skin pigmentation, catalyzing the first two rate-limiting steps in human melanogenesis. Melanin, or in general skin pigmentation, is a valuable protection against UV-radiation and ROS. Hence, deregulation of melanogenesis can represent a cosmetic issue or – more dramatically – hamper melanoma treatment, turning tyrosinase inhibitors into significant pharmacological agents.

Methods: The Mh-Tyr inhibition assay is typically performed using the cheaper mushroom-tyrosinase (Mh-Tyr), a fungal orthologue derived from Agaricus bisporus. Mh-Tyr is incubated with the potential inhibitors in competition with L-DOPA, the physiological substrate of both Mh-Tyr and human tyrosinase. Furthermore, LC-MS was used to verify the reaction and the respective molecular weights.

Results: In the course of our ongoing search for Mh-Tyr inhibitors, we discovered that in contrast to literature, ubiquitous polyphenols such as p-coumaric acid and caffeic acid are rather substrates of Mh-Tyr than inhibitors. The enzymatic reaction results in the formation of the corresponding di-quinones and leads to a time-dependent increase of UV absorption of the test solution, especially at wavelengths at or close to the absorption maximum of reaction products of L-DOPA.

Conclusion: According to our recent observations, it is highly likely that many published plant-derived Mh-Tyr inhibitors are actually substrates of Mh-Tyr and their utilization in cosmetic and pharmaceutical products has to be considered carefully. Furthermore, it seems likely that many Mh-Tyr inhibitors were falsely classified as inactive (false negative) in the past, since in extracts their inhibitory potential could be masked by the absorption created by such converted polyphenols.

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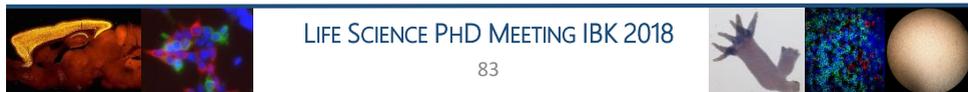
2D MALDI Imaging Mass Spectrometry for the Localization of Lipids in Human Aortic Tissue

Lipids play an essential role in all kind of tissues as constituents of cell membranes, act as regulators for various biological processes and are associated with many diseases in which lipids show a modified distribution in tissue. Previous metabolome-based studies provided evidence that lipids might also be potential biomarkers for the occurrence of thoracic aortic aneurysms.

In the present study, MALDI imaging mass spectrometry (MSI) in the positive and negative ion mode was used to localize lipids in human aortic tissue slices. Since a homogenous matrix coating of the tissue section is essential for high quality mass spectra and MSI ion images, we optimized the deposition of 1,5-Diaminonaphthalene (DAN) matrix by sublimation and recrystallization using different organic solvents. The sensitivity was further improved by performing a tissue washing-step prior to matrix sublimation. Lipids were identified with respect to high-resolution data and chemometric analyses using the open-source R environment were utilized to characterize and monitor the spatial distribution of multiple different lipids found in aortic tissue. Results will be discussed.

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The effects of dietary supplementation on the human gut metabolome

Human state of health has been shown to be strongly influenced by nutrition. Lately, the effects of gut microbiota and their metabolism moved into the focus of research whereby different methods are applied to elucidate bacterial metabolism and its influence on human state of health. An evolving technique in this field is metabolomics in which metabolites are measured based on different principles of detection. In a recent study we applied NMR spectroscopy to shed light on the human gut metabolome. We conducted a study with 19 healthy participants over a period of 9 weeks while in weeks 4-6 their diet was supplemented with soluble fiber from guar beans. Clinical data on individuals' state of health as well as a fecal sample were collected every week. The fecal samples were analyzed by NMR, and the metabolite profiles were evaluated by means of single and multivariate statistics. We were able to identify numerous metabolites from the profiles and we compared the signal intensities of these metabolites between individuals as well as cohort sub-groups. Additionally, we traced for miscellaneous metabolites by monitoring the developing of signal intensity values over time. Here we present some preliminary results of our study and illustrate the tremendously high variability of human fecal metabolome not only between different individuals but also for a particular individual over time. Despite the high variability of the gut metabolome we are able to detect weak influences of dietary supplementation with fiber and we show the clinical relevance of this approach.

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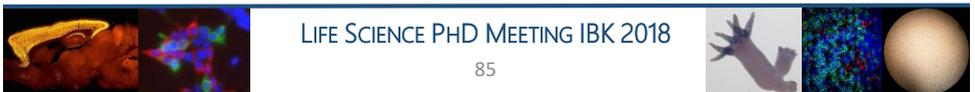
Determination of Kava-Lactones in Piper methysticum by Supercritical Fluid Chromatography

On many Pacific islands the roots and rhizomes of Piper methysticum are traditionally used to prepare a ceremonial drink known as kava-kava, whereas in the Western world respective products are a remedy to treat mild to moderate forms of anxiety. Recent studies also point to other interesting bioactivities (e.g. anticancer, antihyperglycemic); however, these positive effects are usually associated with lactones present in the plant. Their analysis is commonly achieved by reversed or normal phase HPLC; the use of Supercritical Fluid Chromatography (SFC) has only been described once in a nearly 20 year old publication.

In SFC a compressed gas (mainly CO_2) serves as mobile phase, combining liquid like density with gas-like viscosity and diffusivity; together with specific stationary phases of a particle size below 2 µm extremely fast and efficient separations are possible. As a consequence we were able to baseline resolve six kava kavalactones, i.e. dihydrokavain, demethoxyyangonin, kavain, yangonin, dihydromethysticin and methysticin, in only 4 min using an Aquity UPC2 BEH 1.7 µm column. The chosen mobile phase comprised CO_2 and methanol with diethylamine, column temperature, which had a great impact on separation, was set to 70°C. The procedure was fully validated and revealed excellent correlation coefficients above 0.998 and recovery rates between 95.9 and 104.1 %. Practical use of the procedure was confirmed by analysing commercial kava-kava products, which contained dihydrokavain (2.06 – 7.28 %), yangonin (1.98 – 4.64 %) and kavain (1.87 – 7.09 %) as major lactones. Regarding speed, selectivity and environmental friendly "green" operation this SFC approach surpasses all previously described methods.

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Siderophore-mediated diagnosis of invasive aspergillosis in urine samples

Current diagnostic tools for aspergillosis lack sensitivity and/or specificity. In Aspergillus fumigatus, iron starvation induces secretion of siderophores (low molecular mass iron chelators) for iron acquisition. Previously, three lines of evidence indicated induction of the siderophore system in mouse models for pulmonary A. fumigatus infection: (i) transcriptional upregulation of the siderophore system in vivo, (ii) avirulence of siderophore-deficient A. fumigatus mutant strains, and (iii) recently shown imaging of aspegillosis via radioactively-labelled siderophores. Moreover, the major siderophore of A. fumigatus, triacetylfusarinine C (TAFC), showed rapid renal excretion in intact form. From 2012 to 2015, 44 urine samples were prospectively collected from 24 hematological malignancy patients twice weekly. TAFC, GM and creatinine urine levels were retrospectively determined in patients with probable (7 patients/21 samples), possible (1 patient/2 samples) or no IA (16 patients/21 samples) according to EORTC/MSG criteria 2008. TAFC was determined by mass spectrometry and normalized to the creatinine content. The optimal cut-off for TAFC/creatinine index was calculated by receiver operating characteristics curve (ROC) analysis by using the Youdens index. TAFC/creatinine index values ≥ 3 and GM/creatinine index values ≥ 0.25 were considered as positive. Per patient ROC analyses revealed an area under the curve (AUC) for TAFC/creatinine index for probable versus no IA of 0.835 [95% confidence interval (95% CI) 0.585 – 1.000; p=0.012]. GM/creatinine index analysis per patient for probable versus no IA revealed an AUC of 0.830 (95% CI 0.579 – 1.000; p=0.013). TAFC/creatinine sensitivity, specificity, positive and negative likelihood ratio for probable versus no IA for per patient analysis were 0.86 (95% CI 0.49 – 0.97), 0.88 (95% CI0.64 – 0.97), 6.86 (95% CI 1.81 – 25.96) and 0.16 (95% CI 0.03 – 1.01); and for per sample analysis 0.81 (95% CI 0.60 – 0.92), 0.90 (95% CI 0.71 – 0.97), 8.5 (95% CI 2.2 – 32.3) and 0.21 (95% CI 0.09 – 0.51). Reliable biomarkers for IA in urine would offer several advantages in clinical routine such as easy repetition and non-invasive sampling. The TAFC/creatinine index showed promising results in this study for diagnosis of IA. Sensitivity and specificity were similar as reported for galactomannan determination in bronchoalveolar lavage fluid samples, the current gold standard for IA diagnosis.

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Sex-/Gender and Diversity – What does this mean in scientific research?

Sex/Gender and Diversity – What is all about?

Talking about gender normally is connected to the topics of equality of women and men, gender balance in team building or leadership. The aspects of gender and diversity in research are not at all self-explaining. They concern the research content itself and awareness is necessary to consider professionally these aspects during all phases of a scientific project.

Sex/Gender and Diversity – Why does it matter?

The evidence that sex and gender differences matter in health is constantly increasing. Not only biological differences as hormones, genes, immunological differences play an important part, but also sociocultural differences like nutrition, profession, financial background or role understanding. The interaction of the two dimensions sex and gender and additional influences like age make it even more complex. If the aim of health research is to find adequate answers and therapies for patients, it is necessary to say goodbye to the model of a standardized patient and the dogma that "one size fits for all" and to consider sex-/gender- and diversity aspects in the design of research projects from the beginning. At the level of life science research sex factors might be in the foreground as research mostly is done with cells and mice, later on in the research chain, gender factors gain considerable more importance.

Sex/Gender and Diversity – Legal background

Meanwhile the requirement to consider the sex and gender dimension in science and research is introduced in national and European legislation. It is obligatory to consider both sexes in research and innovation content in grant application. The differentiation of the sexes not only is a matter of legislation, it is also a sign of scientific quality and reliability of studies.

Sex/Gender and Diversity –Information and tools

The introduction of sex/gender and diversity during all phases of projects requires methodological competence. The poster presents methods and tools and gives further information.

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Measurement of Respiratory Movements of Patients with Magnetic and Optical Technology

The aim of this project (respiTrack) is the evaluation of two methods for measuring the respiratory-induced spatial change of the patient and anatomy during surgical therapies. These are, for example, irradiations or radiofrequency ablation of certain tumors. Currently, the positions of internal organs vary as induced by respiration and "oscillates" during certain surgical interventions. It currently can hardly be taken into consideration. Thereby, extended safety seams must be planned and maintained, so that healthy tissue, often with vital functions, is not damaged by the therapy or intervention. Thus, there is a chance of under- and over-treatment, which is disadvantageous for patients in either both case.

respiTrack uses electromagnetic   and optical tracking systems separately for surgical navigation, predicting the tumor location and Finite Element Method (FEM) to determine the deformation of tumors or inner organs during respiratory changes of patient with tracked sensors (fiducials) in real-time. The sensors are placed on the patient's surface before treatment and a 4D-CT image dataset is acquired. The exact locations of the sensors and tumor/s are determined by a morphology based algorithm in CT image coordinate system preoperatively and stored to use in prediction step. In order to learn and determine the model of the patient's respiratory circle using Principal Component Analysis (PCA), the patient is let breathe freely for a short time (10-15 sec) , while placed sensors are tracked intraoperatively. The real-time prediction of current tumor position during intervention is performed with a machine learning Gaussian Process Regression (GP) algorithm. In first step GP algorithm is trained using preoperatively determined fiducial positions from 4D-CT image dataset and in second step; it takes every measured position of the sensors from tracker and predict current position of tumor based on its trained data. Based on the GP prediction, the deformation of tumor or organ is modeled and visualized using FEM.

Magnetic and optical 3D measurement technology is evaluated in an experimental operating room at suitable realistic objects and tested for suitability for use in the medical environment.

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Triacetylfusarinine C Modification for PET Imaging of Fungal Infection

Iron is an essential nutrient for growth and virulence of pathogenic microorganisms. Aspergillus fumigatus (AFU) produces and secrets the siderophore triacetylfusarinine C (TAFC) for iron assimilation via the MirB transporter, which is highly upregulated during infection. We have shown that TAFC can be labeled with 68Ga, a short-lived positron emitting radionuclide, thereby replacing Fe and [68Ga]TAFC revealed excellent targeting characteristics in an AFU infection model by means of Positron-Emission Tomography (PET). Here we aimed to modify TAFC with different substitutions for 68Ga-labeling and investigate the effect of introduced substituents on preservation of AFU targeting properties in vitro and in vivo by µPET/CT imaging to establish structure activity relationship.

[Fe]fusarinine C, the deacetylated form of TAFC, was extracted from AFU mutant strain ΔsidG. TAFC derivatives were synthesized by acylation(s) at NH2 group(s) of [Fe]FSC with various substituents (different carbon chain length acyl compounds as well as charge substituents). Iron containing TAFC derivatives were demetalated, followed by radiolabeling with 68Ga. Stability, lipophilicity and protein binding were determined. Uptake and growth assays in Aspergillus species expressing the MirB transporter were performed. In vitro uptake value of each [68Ga]compound was expressed as uptake ratio to [68Ga]TAFC from the same experiment. Biodistribution study and µPET/CT imaging of selected compound in healthy and AFU-infected rats were accomplished.

TAFC derivatives were prepared in moderate yields and could be labeled with 68Ga at high specific activities. Lipophilicities as expressed in logP were -0.38 to -3.80 ([68Ga]TAFC -2.1). In vitro uptake assays using AFU wildtype strain showed the recognition of the MirB transporter to monosubstitued-TAFC derivatives whereas uptake assays in MirB-possesing- and wildtype-A. terreus confirmed the specificity for the MirB transporter. The selected compound [68Ga]DABuFC showed low protein binding and was stable in PBS and serum and revealed comparable biodistribution behavior as compared to [68Ga]TAFC. This was confirmed by µPET/CT images in normal and AFU-infected Lewis rats.

Modification of TAFC without losing targeting properties for molecular imaging of fungal infections is possible. Displacement of one acetyl group by non-charge and appropriate chain length results in derivatives that can be recognized by the MirB transporter and release high contrast PET/CT image of infected lung. Introducing functionalities such as antifungal moieties opens new ways for theranostics of infectious diseases.

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Audio and 3D-Visual guidance for optimal placement of an Auditory Brainstem Implant with magnetic navigation and maximum clinical application accuracy

This work presents a dynamic 4D (audio and 3D-visual) feedback system for positional guidance in real-time during surgical placement of an Auditory Brainstem Implant.

At present, dummy and simultaneous preoperative measurements, usually including the CT and/or MRI scans of the patient's head, are used to determine the optimal position for an Auditory Brainstem Implant (ABI) on the nucleus cochlear. When found, the optimal position is marked by the surgeon and in the next phase the surgeon tries to locate the optimal position in the patient´s head and place the implant.

With current technology there are no quantifiable methods for storing the optimal implant position. Among other things, brainstem implants do not always provide satisfying and predictable results in hearing perception. Therefore, the aim is to equip the surgeon with a new navigation system based on magnetic navigation and maximum clinical application accuracy; to provide intuitive audio and 3D-visual guidance for positioning the implant; to store the optimal position of the implant in a database and to reevaluate its optimal position.

The navigation software will be platform-independent and developed in the C++ programming language. The basis for this software is Rhinospider Technology developed by the University Clinic for ENT at the Medical University of Innsbruck. One of the goals is to get the certification of this software as a medical device. This project would be paving the path for intraoperative support during ABI procedures.

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High-resolution Desorption Electrospray Ionization Mass Spectrometry Imaging (DESI-MSI)

Mass Spectrometry Imaging (MSI) utilizing an ambient ionization method such as Desorption Electrospray Ionization (DESI) allows the examination of samples in situ and outside the vacuum system of the mass spectrometer. We developed a DESI method for imaging of biological tissue sections at atmospheric pressure without sample pre-treatment at a lateral resolution of 50µm. Under optimized DESI conditions (solvent composition, flow rate, gas flow rate capillary size, angles and distances between sprayer, sample and mass spectrometer inlet) we investigated the spatial distribution of lipids in mouse brain tissue. High-resolution MS and MSn data were used to identify lipids and adduct formations in the positive as well as negative ion mode. Results will be discussed.

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Novel in-vitro-models for drug screening based on additive 3D-bioprinting and magnetic bioprinting of cancer cells

Bioprinting, a technology to print biological material into a 3D-construct, is a new possibility to optimize medical research and medical applications. Because of cellular complexity, interactions and drug effects are better mimicked in 3D-cell cultures than in conventional 2D-cell cultures. 3D-cell culture techniques are also an optimal method to reduce, refine and replace animal experiments.

A microextrusion bioprinter was used to print 3D-constructs composed of hydrogels and neuroblastoma cells to create novel in-vitro models. Additionally, neuroblastoma spheroids were printed in hydrogels after they were formed by magnetic bioprinting. Neuroblastoma is a pediatric, embyonal tumor, which arises from the sympathetic nervous system. Various biomaterials were developed, which could stabilize and improve the viability of cells. Additives in hydrogels, such as collagen, fibrinogen, FCS or platelet lysate were tested and included to improve and facilitate cellular adhesion, viability and proliferation of the printed cells. Supporting gelatine microbeads were used as a thermoreversible matrix during printing the constructs composed of alginate, cells and other additives to obtain higher resolutions. After printing cells, the viability was measured by qualitative analyzis (microscopical) and quantitative analyzis (alamar blue assay etc.). A biomaterial with ideal additives like collagen and FCS, which supported cell viability, could be established. There is an additional need in varying the components of additives in hydrogels and in modifying the concentrations. Cell-cell and cell-matrix contacts may then be established and thus proliferation, differentiation and migration of cells could occur in hydrogels.

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Optimizing animal-free culture of primary human respiratory cells for live cell analyses

To study respiratory challenges with upper and lower respiratory tract cells in an animal-free in vitro environment over time and for repeated screening, we set up an innovative up-side-down three-dimensional (3D) epithelial cell culture system. This approach of cell seeding offered advantages regarding the additional handling of the cultured epithelium. For further analysis it is not necessary anym

Culturing of normal human bronchial or small airway epithelial (NHBE, SAE) cells under air liquid interphase (ALI) usually is done using rat tail collagen to coat micro porous membranes cells are seeded on. In our approaches a recently developed cellulose nanofibril hydrogel called GrowDex was used instead of collagen to function as extra cellular matrix (ECM) and so supporting cellular growth. Live cell imaging analysis have shown that the animal free hydrogel seems to have a positive impact on the growth and differentiation of human lung epithelium. Not only a faster proliferation of the normal human bronchial epithelial cells could be observed but also the fully differentiation of the epithelium occurred earlier. It seems like the GrowDex animal free hydrogel offers optimal conditions for a 3D cell culture model to study further approaches.

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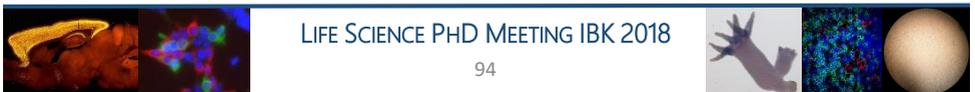
New Antimicrobial Potential of PAFB: A Cationic, Cysteine-Rich Protein from Penicillium chrysogenum Q176

The number of severe microbial infections is steadily increasing, partly due to resistance development against common antibiotics. This requires the invention of novel antimicrobial treatment strategies. Filamentous Ascomycetes secrete small, cationic and cysteine-rich antimicrobial proteins (AMPs), which could be used as templates for the development of new antimicrobial drugs in medicine and agriculture. AMPs are highly stable against harsh environmental conditions and some were tested to be non-toxic to mammalian cells in vitro and in vivo [1, 2, 3].

In the genome of Penicillium chrysogenum Q176 three genes were identified that code for AMPs. The structure and mode of action of the P. chrysogenum Anti Fungal (PAF) protein, has already been extensively examined [4, 5]. In this study, we analysed the antimicrobial activity of the second protein, PAFB, which is a prepro-protein with high similarity to the recently described protein PgAFP from P. chrysogenum R42C [6]. Although we detected timely regulated pafB gene transcripts in Northern blot experiments, we could not identify PAFB in the supernatant of P. chrysogenum, cultured under standard conditions. We therefore used our recently described P. chrysogenum-based expression system to produce recombinant PAFB in high quality and quantity for functional characterization [7, 3].

The purified, mature PAFB inhibited effectively the growth of human opportunistic pathogenic fungi like Aspergillus fumigatus and dermatophytes like Trichophyton rubrum in a low (µM) concentration range. A potent killing activity was also identified against the yeast Candida albicans. Fluorescence microscopy using BODIPY-labelled PAFB revealed that the fungicidal activity is closely linked with protein uptake and subsequent cytoplasmic localization in fungal cells. A surprising and novel observation for small, cysteine-rich, cationic AMPs from filamentous Ascomycetes was the anti-viral activity of PAFB and PAF on the human coronavirus 229E.

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Proteome-wide Profiling of Arginine Methylation in Aspergillus nidulans

Only recently arginine methylation has gained traction as an important post translational modification (PTM). In addition to its role in contributing to the "histone code" this PTM has been identified in numerous non-histone proteins functioning throughout cell biology.

Our main scope of interest is related to epigenetics in filamentous fungi aiming to unravel a presumptive function of protein arginine methylation in fungal pathogenicity and secondary metabolism. To this end, a proteomic analysis was performed in order to (1) study the expression of proteins in pathways that are controlled by arginine methylation and to (2) isolate and identify novel targets of Aspergillus protein arginine methyltransferases (PRMTs).

In Aspergillus nidulans four PRMTs have been identified. RmtA and RmtC reveal homology to mammalian PRMT1 and PRMT5, respectively, while RmtB and RmtD are presumed being highly fungi specific. Whereas, the latter are characterized by distinct substrate specificities, an oxidative stress phenotype was shown for rmtA and rmtC mutants. Our study aims to discover numerous protein targets of PRMTs on a proteome-wide scale. Therefore, quantitative proteomics combined with immuoaffinity enrichment of peptides containing distinct forms of methylarginine and mass spectrometry in a bottom-up approach was envisaged. By introducing 13C-labeled arginine to fungal anabolism, the SILAC method (stable isotope labelling with amino acid in cell culture) enables the quantification of arginine methylation as a response to oxidative stress treatment. Results of our current screen revealed arginine monomethylated proteins of both already described PRMT substrates as well as novel targets. Currently we have started the validation of candidates as well as an experimental redesign for enabling the profiling of low abundance PRMT substrates.Ultimately we seek after links between arginine methylation and the regulation of fungal secondary metabolism as a base of applications in biotech and medicine.

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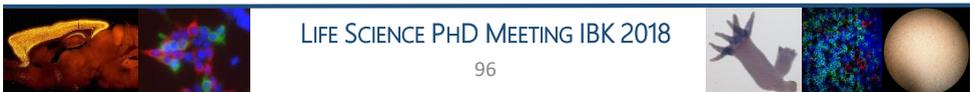
Regulation of B cell development by the miR-26 family

MicroRNAs (miRNAs) are short, non-coding RNAs of about 21-24 nucleotides (nt) length that regulate gene expression and thereby affect physiological as well as pathological processes such as cancer. Several miRNAs have been described to mediate pro-tumorigenic as well as tumor-suppressive functions. One miRNA family involved in cancerogenesis is the miR-26 family, who is aberrantly expressed in different types of cancer. However, little is known about the effect of altered miR-26 expression in the context of immune cell malignancies. Furthermore, despite its prominent expression in early lymphocytes, the role of miR-26 in hematopoiesis is still unknown.

Here we combine in vitro and in vivo approaches to demonstrate an important role for the miR-26 family in early B cell development, as pre-B cells overexpressing miR-26a or b showed reduced differentiation, increased proliferation and a partial inhibition of apoptosis upon IL-7 withdrawal, eventually resulting in their oncogenic transformation. Conversely, a functional knockdown of the miR-26 family by a competitive sponge construct enhanced B cell differentiation. These observations were supported by findings in our miR-26 sponge mouse model. In analogy to our in vitro data, these mice displayed an increased number of immature B cells and a decrease in pre-B cells, indicating enhanced B cell differentiation. Furthermore, the miR-26 family not only plays a role in early B cell development but also in mature B cells, as splenocytes derived from miR-26 sponge mice showed reduced proliferation upon LPS stimulation.

Together, these results clearly indicate the importance of the miR-26 family in early B cell development and suggest a pivotal role in regulating pre-B proliferation versus differentiation. Furthermore, we suggest a potential oncogenic role of miR-26 in B cell leukemia by conferring a hyper-proliferative and anti-apoptotic pre-B cell phenotype.

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Role of the chromatin remodeling factor CHD1 in long-term spatial memory

The condensation of DNA by wrapping the double helix around histone octamers resulting in so-called nucleosomes and the further organization of the nucleosomal fiber into higher order structures is a highly regulated equilibrium, regulating the storage and usage of genetic information. The dynamics of a chromatin fiber relies on proper packaging and opening mechanisms to ensure proper accessibility of the DNA for transcription, replication, recombination or DNA repair. Among the evolved regulator mechanisms are also chromatin remodeling factors (ChRFs), ATPases that are able to change histone-DNA contacts within nucleosomes thereby affecting chromatin structure.

In the recent past, it has become increasingly noted that epigenetic mechanisms, in particular DNA methylation and the posttranslational modification of histones in chromatin, play important roles in the regulation of cognitive processes. Moreover, several ChRFs have been found to be mutated in various disorders affecting the brain and brain functions. However, little is known about implications of ChRFs in cognitive and behavioral processes.

The chromodomain-helicase-DNA binding protein 1 (CHD1) belongs to the Snf2 superfamily of ATP-utilizing motor proteins involved in the regulation of transcription and chromatin functions. Hence, to gain more insights into the connection between chromatin dynamics and cognition, we decided to take a closer look at the role of the chromatin remodeling and assembly factor CHD1 in learning and memory. Previous studies from our lab revealed that a mutation in the Chd1 gene resulted in skewed differentiation of homozygous embryonic stem cells towards neural development. Moreover, we found CHD1 to be dysregulated in a fear conditioning and extinction test in the S1 mouse strain that exhibits impaired fear extinction learning. Based on these findings and making use of a mouse model carrying an N-terminal truncation of the Chd1 gene, we studied the learning and memory abilities of Chd1 mutant mice. The results of these analyses will be presented.

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Role of RNA cytosine methyltransferase Nsun3 in mouse embryonic stem cell differentiation

First epigenetic modifications of both DNA and RNA were already described more than 60 years ago with the DNA modification, 5-methyl-2'-deoxycytidine (m5dC), as the best understood one to date. In contrast to DNA methylation, the study of RNA methylation was more challenging due to the lack of proper techniques. In case of 5-methylcytidine, the establishment of methods, such as bisulphite sequencing for RNA, made it possible to study RNA methylation at nucleotide resolution. m5C modifications in RNA can be installed by any of the seven proteins of the Nol1/Nop2/SUN domain (NSUN) family as well as the DNA methyltransferase family member DNMT2. Our previous data suggested that RNA methylation in mitochondria might be higher than in the cytoplasm. In a search for mitochondria-specific RNA cytosine methyltransferases (RCMTs), we targeted Nsun3, which was unstudied at that time and showed close sequence similarity to the mitochondrial enzyme Nsun4. Using CRISPR/Cas9 we introduced mutations in the presumptive catalytic domain of Nsun3 in embryonic stem cells (ESC) to study Nsun3's function. We show that Nsun3 methylates mitochondrial tRNAMet at position C34 in the anticodon loop. Nsun3 inactivation results in substantial impairment of mitochondrial translation and transcription and affects cellular glycolysis and respiration rates. Nsun3 mutant cells also exhibit compromised ESC differentiation. Together, these results suggest an important role for mitochondrial Nsun3 in ESCs even though these cells largely rely on glycolysis rather than oxidative phosphorylation for generating energy.

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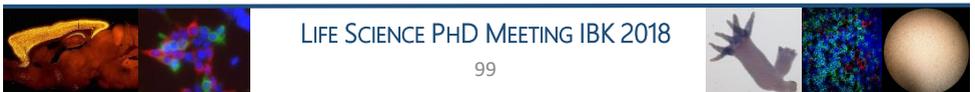
Combination of lentiviral gene ontology vectors and iPS-derived cerebral organoids to get insights into early human corticogenesis in a three-dimensional environment

Three dimensional structures derived from human suspension cell cultures have emerged as potent in vitro models of various organ systems as they display an extraordinary self-organizing capacity to form functional tissues which resemble whole organs. A recently published protocol enables the generation of cerebral organoids which to a certain extent recapitulate neural progenitor cell proliferation and neuronal differentiation processes in vivo. Human 3D cerebral organoid (CO) models are expected to close the gap between common two-dimensional in vitro human cell culture and 3D animal models, thus enabling sophisticated assays that will give new insights into early human brain development. According to current knowledge, cerebral organoids are limited in their growth and maturation because of insufficient nutrition supply due to lack of vascularization. Preliminary results indicate that while human induced pluripotent stem cells (hiPSCs) are able to form COs, multipotent neural stem cells (NSCs) fail to form stratified layers within these aggregates. This suggests that a yet unknown component is provided by hiPSCs but not by hNSCs. We hypothesize that hNSCs lack a mesodermal component for CO generation which cannot be compensated by ectodermal cells. It will be investigated how non-ectodermal components contribute to CO development and whether vasculogenesis (de novo formation of endothelial cells) can be mimicked by introducing mesodermal precursors (primitive streak, cardiovascular stem cells, and endothelium) into COs. Additionally, fluorescent proteins will be introduced via lentiviral gene ontology (LeGO) vectors for cell labeling studies. In 2007, one of the first multicolor systems called Brainbow was shown in mouse, followed by similar systems introducing more colors also in other species such as zebrafish and human primary cells. Introducing a multicolor labeling technique into a human 3D model system will allow identifying and characterizing neural, neuronal and glial subtypes that arise, migrate, interact and form networks within the COs. This study will provide more comprehensive insight into the potential of NSCs to form 3D brain-like structures.

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FAHD-1 effects on neuronal function in the nematode Caenorhabditis elegans

Many diseases emerge during old age, including neurodegenerative diseases, which are among the most deleterious. A strong correlation between neurological dysfunction and metabolism in the elderly has become apparent, however, the underlying mechanisms still remain elusive. In our lab, we discovered and described a protein of the FAH superfamily, named FAHD1, with the ability to decarboxylate oxaloacetate into pyruvate and by doing so, regulates the citric acid cycle. In Caenorhabditis elegans (C. elegans) the orthologous protein FAHD-1 was found. The characterization of a C. elegans fahd-1 deletion mutant allowed insight about the proteins function. Severe locomotion phenotype and egg laying impairment characterizes the fahd-1 deletion mutant. To identify the cause of the locomotion impairment tissue specific rescue strains (expressing fahd-1 in the mutant strain) were generated using myo-3 and rab-3 promoter for neurons and muscles respectively. Striking improvements of the behavioral defect were made by neuronal reexpression of fahd-1. Additionally, exposure to dopamine rescues the egg laying phenotype of the fahd-1 deletion mutant. According to our findings obtained with the nematode orthologue FAHD-1, the enzyme appears to link pyruvate metabolism and neuronal function. Further investigation of FAHD-1 function in C. elegans will help to understand its physiological function and the neuronal pathways influenced by its activity. Through our work we seek to contribute to a basic understanding of the development of neurodegenerative diseases in the light of mitochondrial dysfunction and thereby enable new therapeutic strategies.

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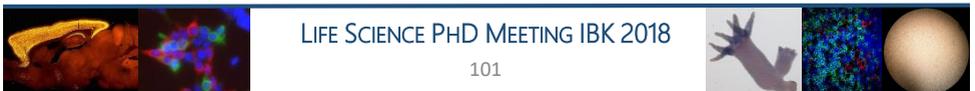
Generation of early human neural progenitors from fetal brain tissue for biomedical applications

Recently, major advances in cellular reprogramming for modeling neurological and neurodevelopmental diseases have been achieved. The differentiation of neural progenitor cells (NPCs) from iPSCs and the direct conversion of somatic cells into NPCs emerged into a promising strategy to obtain patient-specific neuronal and glial cell types. Early primary human NPCs from fetal brain tissue might serve an alternative cell source for neurodevelopmental as well as biomedical and comparative studies. Therefore, we assessed a chemically defined medium composition capable to modulate crucial signaling pathways such as SHH, WNT and FGF orchestrating early human neural development. Indeed, specific culture conditions containing compounds and growth factors enabled the formation of proliferating early neuroepithelial progenitor (eNEP) colonies of homogeneous morphology. Primary eNEPs were monoclonally expanded for more than 45 passages carrying a normal karyotype. The characterization by immunofluorescence, flow cytometry and quantitative RT-PCR revealed a distinct NPC profile including SOX1, PAX6, Nestin, SOX2 and CD133. NOTCH and HES5 upregulation in combination with a non-polarized morphology indicate an early neuroepithelial identity. Interestingly, eNEPs were detected to be of ventral midbrain/hindbrain regional identity. The validation of yielded cell types upon differentiation suggests a strong neurogenic potential. Nevertheless, astrocytes and putative myelin structures indicating oligodendrocytes were identified following targeted long-term differentiation. Electrophysiological recordings revealed functionally active neurons and immunofluorescence indicated GABAergic, glutamatergic, dopaminergic and serotonergic subtypes. Additionally, putative synapse formation was observed by immunostainings and ultrastructural examination. Notably, neurons are positive for the peripheral neuronal marker Peripherin as well as the sensory neuron-specific marker Nav1.7. Moreover, mesenchymal cells could be detected suggesting the potential of eNEPs to give rise to cells of neural crest origin. Taken together, the presented study demonstrates the derivation of novel early fetal-derived eNEPs which might help to elucidate mechanisms of early human neurodevelopment and serve as a comparative cell line for reprogramming studies. Moreover, eNEPs potentially represent a novel source for cell replacement, drug screening and neural tissue engineering.

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Advanced cellular models for Alzheimer's disease – inflammatory changes in cortical neurons and cerebral organoids

Alzheimer's disease (AD) is a chronic neurodegenerative disorder and is the cause of about 70% of dementia worldwide. There are two forms of AD defined by the age of onset. The familial or early-onset AD starts before the age of 65, and sporadic or late-onset AD starts in patients older than 65 years. The disease progress is associated with amyloiod-β plaques and neurofibrillary tangles in the brain. Overall, more than 70% of AD cases are caused by genetic risk factors. The disease progress is associated with amyloid-β plaques and neurofibrillary tangles in the brain. Despite decades of intensive investigations, the causative pathophysiological mechanisms of AD are still not well understood. The availability of induced pluripotent stem cells (iPSCs) derived from patient specific fibroblasts and 3D differentiation protocols have opened new possibilities to model diseases in vitro using human cells.

Here, we used human iPSCs derived from Alzheimer patients carrying a deletion in exon 9 of the presenilin 1 gene (PS1ΔE9), a known risk factor for early-onset AD, isogenic controls, where PS1ΔE9 mutation was repaired using CRISPRCas9 technology, and healthy subjects. IPSCs were differentiated towards cerebral cortical neurons and neural networks in an adherent 2D system and self-organizing 3D cerebral organoids to identify disease phenotype and new therapeutic targets in AD. In purified exosomes of late stages of both differentiation strategies we found elevated amounts of miRNA associated with inflammatory processes even prior to plaque formation. Thus, extracellular vesicles could be an appealing target in both diagnostics and therapeutic approaches in AD and other neurological disorders.

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Sprouty1: A Novel Major Regulator of Adipogenesis?

Sprouty1 (SPRY1) is a negative regulator of mitogen-activated protein kinase (MAPK) signalling and expressed in a tissue specific manner. Sprouty1 expression is stimulated by growth factors which bind to receptor tyrosine kinases (RTK) and trigger MAPK signalling. Thus, Sprouty1 induction constitutes a negative feedback loop. Recent studies revealed a major role of this protein in stem cell maintenance and aging. The importance of Sprouty1 in human adipose stem cells is unknown. To address the role of Sprouty1 in adipogenesis we use an ex vivo model, in which one differentiate primary human adipogenic stromal/progenitor cells (ASCs) into adipocytes. Monitoring of Sprouty1 during adipogenic differentiation revealed an increase of mRNA expression until day 3 after induction of adipogenesis while Sprouty1 protein levels were decreased at the same time point. We presently study the function of Sprouty1 in ASCs by RNA interference and CRISPR/Cas9 genome editing experiments.

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Human bone marrow adipocytes display distinct immune regulatory properties

The bone marrow (BM) is a specialized primary lymphoid organ of the human immune system where T cell precursors are generated. In addition, the importance of the BM in the maintenance of antigen-experienced adaptive cells has been documented. The BM has proven to be a major reservoir of resting memory T cells, capable of providing protection against recurrent infections. The survival and maintenance of these cells is mediated by cytokine and chemokine producing stromal cells and myeloid cell types, forming specific areas known as BM niches. However, with increasing age, a reduction in bone formation is accompanied by the accumulation of bone marrow fat. No information is yet available on the production of memory T cell survival factors by BM fat tissue and the interaction with adaptive immune cells in the BM. Using microarrays, we show that bone marrow fat significantly differs from subcutaneous fat regarding specific gene expression profiles including inflammatory responses and adipogenesis. Lower expression levels of the adipocyte-specific genes adiponectin (AdipoQ) and fatty acid translocase (FAT), and higher expression of the effector/memory T cell survival factors IL-7 and IL-15 were found in BM compared to subcutaneous adipocytes. The expression of the pro-inflammatory molecules TNFα and IL-6, which contribute to the low-grade inflammatory background known as "inflamm-aging" observed in elderly persons, was also higher in BM fat. With our data, we can show that the unique phenotype of BM adipocytes may support the production of survival molecules for the maintenance of effector/memory T cells.

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Checkpoint kinase 1 (CHK1) controls normal B cell development, lymphomagenesis and cancer cell survival

Checkpoint kinase 1 (CHK1) is critical for intrinsic cell cycle control and coordination of cell cycle progression in response to DNA damage. Despite its essential function, CHK1 has been identified as a target to kill cancer cells, especially those lacking functional p53. Studies using Chk1 haploinsufficient mice initially suggested a role as tumor suppressor in line with its function to halt cell cycle progression and orchestrate DNA repair in response to replication stress or when cells experience exogenous DNA damage. Yet, in cancer cells Chk1 appears to display pro-survival properties, suggesting context dependent activities. Here, we report on the key-role of CHK1 in normal B-cell development, lymphomagenesis and cell survival. Chemical CHK1-inhibition induces BCL2-regulated apoptosis in primary as well as malignant B-cells and CHK1 expression levels control the timing of lymphomagenesis in mice. Loss of Chk1 precludes the outgrowth of lymphomas in Eμ-MYC transgenic mice, while a reduction in gene-dose delays tumor onset. This effect was associated with an obvious increase in replication stress and hallmarks of DNA damage in premalignant MYC transgenic B cells. Moreover, total ablation of Chk1 in B-cells arrests their development at the pro-B cell stage, a block that, surprisingly, cannot be overcome by inhibition of mitochondrial apoptosis, as cell cycle arrest is initiated as an alternative fate to limit the spread of damaged DNA.

Together, these findings suggest that targeting CHK1 is a valid strategy to hit hard-to-treat blood-cancer with high apoptotic threshold or low apoptotic priming. A limitation of this strategy, however, might be that, in the absence of functional p53, or reduced cell cycle check-point proficiency, BCL2-controlled apoptosis resistance can trigger aneuploidy tolerance, increasing the chances for treatment failure.

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Special genome editing strategies to study human gene functions in in vitro differentiating stem cells as disease models

Studying human gene function in developmental and metabolic diseases resembles an important but sometimes difficult challenge in today's research. The development of protocols to differentiate pluripotent stem cell into various somatic cell types together with the ability to mutate genes of interest by CRISPR/Cas9 technology opened up completely new possibilities for such approaches. However, low efficiencies to generate specific cell populations or complex gene functions that require conditional interference with gene activity frequently posses limitations for functional studies. Here we describe genome-editing strategies that aim to solve some of these limitations. Experiments were done to define pancreatic functions of the neonatal Diabetes gene MNX1. Complications for corresponding studies arose from (i) independent MNX1 requirements in early pancreas morphogenesis and during later beta-cell formation, (ii) lack of in vitro differentiation protocols to generate pure human pancreatic cell populations, (iii) lack of ChIP-grade MNX1 antibodies that are required for all molecular approaches. To bypass these problems we generated two types of MNX1 knock-in pluripotent human stem cell lines by using CRISPR/Cas9 technology. Both lines utilized MNX1-specific expression of in vivo tags to allow sorting (FACS) of relevant cell populations. In one line, we used a 'FLEx-Switch'-based recombination system to combine tissue and cell-type specific conditional MNX1 gene inactivation with the labeling of relevant mutant cells by live-cell reporter expression. These second constructs aimed at introduction the same live reporter together with the previously well-established ChIP-grade TY1 epitope tag into the MNX1 locus without interfering with MNX1 function. All of these complex constructs were cloned by a novel strategy called In-vitro Assembly (IVA) and successfully tested in hES and hiPS cells. In the light of our work, we hope to contribute to genome editing strategies for clonal-generation of mammalian stem cell models to study gene function in in vitro differentiating cells more elaborately.

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Metabolic switch in human induced neurons derived from Alzheimer Patients

Alzheimer's Disease (AD) is a neurodegenerative disease, which presents a huge health burden for the elderly and still lacks drugs for disease prevention or cure. One reason for this difficulty to find appropriate drug targets is the lack of suitable model systems for studying the underlying age-related disease mechanisms. In this project we use a novel in-vitro method to convert patient-specific fibroblasts into induced neurons (iNs), which retain key features of aging in their epigenome. iNs open up new possibilities to study age-related diseases such as AD. Our previously obtained transcriptomic data indicate that iNs generated from AD patients' fibroblasts exhibit a metabolic switch from oxidative phosphorylation to glycolysis. Here, we will present preliminary data on the further characterization of this metabolic switch including measurements of lactate and NAD+ levels in AD iNs. Further, we aim at identifying other potential cellular consequences of this metabolic switch and explore means to restore the balance between oxidative phosphorylation and glycolysis in AD neurons as a proof of concept for novel treatment strategies for AD.

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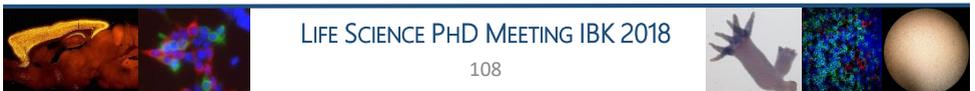
Gpr161 plays an essential role in vertebrate development by regulating Hedgehog signaling

The Hedgehog (Hh) signaling pathway is of fundamental importance in the regulation of cell fate specification, cell proliferation, stem cell homeostasis, and has also been linked to various cell biological processes such as axon guidance and cell migration. In the adult organism, strict regulation of Hh signaling remains critical, as misregulation of several components of the pathway can lead to severe forms of human cancer.

Although the Hh pathway has been the focus of extensive studies over the last three decades, a comprehensive molecular and biochemical understanding of Hh signal transduction remains elusive. Additionally the understanding of which components of the signaling cascade allow the diverse tissue specific outcomes of different Hh activity states is still limited.

It was recently shown that, in mouse, the orphan G-protein coupled receptor Gpr161 acts as a negative regulator of Hh signaling by modulating downstream signaling components of the Hh pathway. Gpr161 has not only been shown to be essential for mammalian development but also was linked to the development of severe forms of Hh-driven cancers such as medulla blastoma.

To deepen our understanding of the functional importance of Gpr161, we used CRISPR/Cas9 technology to knock out the two Gpr161 paralogs in the vertebrate model system zebrafish (Danio rerio). Here we present a first functional characterization of a loss of Gpr161 model that, because of its ex utero development, allows a thorough analysis of various affected tissues beyond early embryonic development.

Zebrafish gpr161 mutants show that the role of Gpr161 as a negative regulator of Hh signaling is conserved among vertebrates. Interestingly many Hh related processes such as craniofacial development, brain patterning and eye development are affected in those mutants, however not all Hh related processes during embryogenesis seem to be affected equally, which leads us to believe that Gpr161 might modulate tissue specific outcomes of Hh signaling.

This newly created zebrafish line will help to complete the understanding of the consequences of a loss of Gpr161, while rescue experiments with different mutant forms of Gpr161 promise to be a useful tool to investigate the role of Gpr161 on a more cell biological level.

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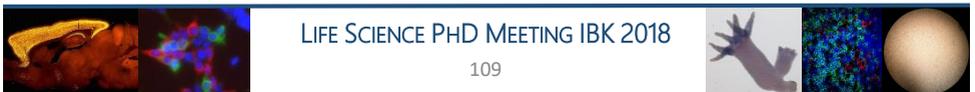
In vivo life cell imaging in zebrafish as a novel tool in diabetic research

Development of diabetes has a strong hereditary component with more than 80 risk factors showing robust disease association in whole genome association studies. While the majority of the affected genes appear to be active in the Insulin-secreting β-cells, very little is known about their roles in beta-cell formation and/or function.

Here, we introduce transgenic zebrafish expressing genetically-encoded in vivo sensor for addressing such question in native pancreatic islet of living animals. Using a zebrafish line encoding a membrane localized beta-cell specific GCaMP6s Ca2+ sensor, we demonstrate in vivo and ex vivo assays to examine Ca2+ fluxing in intact islets or in individual beta cells in response to glucose, free fatty acid or amino acid challenges. Wild-type zebrafish pancreatic beta cells show rapid fluorescence signal alteration of GCaMP6s sensor in response to intraperitoneal injection of D-glucose to zebrafish larvae. Fluorescence signal alteration was not detected neither before injection of the component, nor after injection of L-glucose. CaV1.2 channel mutant in combination with the GCaMP6s imaging line serve as a prove of principle to assure the physiological usefulness of GCaMP6s sensor in relation with disease phenotypes. Furthermore, in vivo imaging and ex vivo physiological studies done on islets from Cav1.2 mutant will help to determine requirements of these channels in glucose-induced insulin secretion.

This approach to monitor the intracellular Ca2+ dynamics in the pancreatic beta cells for the first time in native pancreas, in vivo will expand the understanding of beta cell physiological function and the disease state.

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LCMV-GP Pseudotyped Vesicular Stomatitis Virus shows strong oncolysis in syngeneic lung cancer model

The therapeutic effect of oncolytic virotherapy is largely based on a two-pronged approach - the direct action of tumor-selective infection, viral replication, and cell killing and the associated activation of innate and adaptive immune responses with the potential of long-lasting tumor remission. Using a chimeric vesicular stomatitis virus variant VSV-GP, we addressed the direct oncolytic effects and the role of antitumor immune induction in the syngeneic mouse lung cancer model LLC1. In vitro, VSV-GP was found to efficiently infect and lyse LLC1 cancer cell-lines. Exogenously applied interferon type 1 revealed a dependence of the oncolytic effect on defects in the IFN response of cancer cells. Using a matched pair of LLC1 wildtype and interferon receptor knockout tumors (LLC1-IFNAR-/-) in vivo, interferon insensitivity of cancer cells correlated with prolonged intratumoral viral replication and therapeutic outcome. Luciferase imaging revealed successful tumor-to-tumor spread of viral progeny in bilateral tumor models. Histological analysis confirmed widespread and rapid infection and cell killing within the tumor. To analyze the role of the adaptive immunity, we compared efficacy of LLC1-IFNAR-/- tumor treatment in the presence or absence of cytotoxic T-cells using immune-incompetent nu/nu hosts or CD8-depleting antibody regimen. In the absence of CD8 T-cells, LLC1-IFNAR-/- tumors were efficiently lysed by VSV-GP, even showing a mildly enhanced treatment effect. In addition, surviving mice showed little protection from tumor rechallenge. Together, these studies present a case for a lytic-dominant treatment effect of VSV-GP in a syngeneic mouse lung cancer model. Future studies will focus on improving the balance between facilitating viral oncolysis and enhancing anti-tumor immunity.

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HIV neutralizing antibodies induced by native-like envelope trimers incorporated into a rhabdoviral vector vaccine

Question: The vesicular stomatitis virus pseudotyped with the glycoprotein of the lymphocytic choriomeningitis virus, VSV-GP, is a potent viral vector vaccine, which overcomes several of the limitations of wild-type VSV. Here, we evaluated the potential of VSV-GP as vaccine vector against HIV infection.

Methods: We incorporated antigens from HIV or marker genes into the genome of VSV-GP and generated infectious viruses via reverse genetics. These viruses were analyzed for transgene in vitro expression, infectivity, localization and conformation of the antigen. In mice distribution and kinetics of infected cells, antigen-specific and vector-specific immune responses were analyzed. In rabbits HIV neutralizing antibodies were characterized.

Results: We found that the addition of the Env antigen did not attenuate VSV-GP replication. All HIV Env variants were expressed in VSV-GP infected cells and some were incorporated very efficiently into VSV-GP particles. Crucial epitopes for binding of broadly neutralizing antibodies against HIV such as MPER (membrane-proximal external region), CD4 binding side, V1V2 and V3 loop were present on the surface of VSV-GP-env particles. Binding of quaternary antibodies indicated a trimeric structure of VSV-GP incorporated Env. We detected high HIV antibody titers in mice and showed that vectors expressing membrane-anchored Env elicited higher antibody titers than vectors that secreted Envs. In rabbits, Tier 1A HIV neutralizing antibodies were detectable after prime immunization and titers further increased after boosting with a second immunization.

Conclusion: Taken together, VSV-GP is a promising candidate as a viral vector vaccine against HIV infection.

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Reinforcing dendritic cells for cancer immunotherapy: diverse ways and means to target antigens to human skin

Dendritic cells (DC) are essential for the induction of primary immune responses, and hence preferred targets for immunization against cancer. Skin DC express C-type lectin receptors such as Langerin or DEC-205 for recognition of (pathogen-derived) antigens. In situ, Langerin is expressed mainly on Langerhans cells (LC), whereas DEC-205 is expressed by dermal DC and LC. We aim to load skin-resident DC (i) with antibody-antigen fusion proteins directed against these C-type lectin receptors or (ii) with antigens encapsulated in liposomes coated with a Langerin ligand.

(i) Monoclonal antibodies (mAb) were injected intradermally into human skin explants for targeting of skin DC subtypes corresponding to their C-type lectin receptor expression. Langerin mAb was detected exclusively in LC, whereas DEC-205 mAb targeted both dermal DC and LC. A model antigen (EBNA1) fused to DEC-205 mAb elicited EBNA1-specific T cell responses.

(ii) Liposomes coated with a Langerin ligand showed exclusive binding to LC in cell suspensions obtained from healthy human skin. These liposomes were rapidly incorporated into LC as visualized by confocal microscopy.

In summary, this study will provide a deeper insight into DC-targeted cancer vaccines, their uptake, intracellular trafficking and antigen processing in skin DC. Furthermore, liposomes provide a flexible platform that will allow us to encapsulate antigens to investigate their potential for targeted delivery. Those antigen:anti-DC antibody constructs or LC-specific liposomes loaded with antigens will allow to boost patient´s pre-existing immunity. Ultimately, this DC-based immunotherapy can be used to increase the response rates when used in combination with immune checkpoint inhibitors.

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Fine-tuning of DC functions by complement-opsonized HIV-1

Recognition and phagocytosis of pathogens by dendritic cells (DCs) is enhanced by complement (C) opsonization. During transmission via mucosal surfaces HIV-1 spontaneously activates the classical complement pathway by direct binding of C1q to the viral envelope glycoproteins and is therefore surrounded by covalently linked C3 fragments like a halo. DCs then capture HIV-1 via abundantly expressed complement receptor 3 (CR 3, CD11b/CD18) and 4 (CR 4, CD11c/CD18) leading to improved HIV-1-specific DC function and antigen-presenting capacity.

Our group recently highlighted that C-opsonized HIV-1 (HIV-C) overcomes restriction in immature DCs (iDCs), resulting in a superior antiviral immune response (Posch et al., 2015). Therefore, we are now interested in unraveling the specific binding partner and processing route of HIV-C in DCs and generated CD11b, CD11c and CD18 knock-out DCs using the CRISPR-Cas9 system. The monocytic cell line THP-1 was used for knocking out complement receptor single chains (CD11b, CD11c) or dimer (CD18), since knock out of the receptors in primary cells by transfection resulted in low DC survival rates. To establish THP1-WT and k.o. DCs we compared their expression of cell surface molecules, phagocytosis and HIV-1 infection rates to primary DCs and found that they represent a good model to study the single functions of CR3 and CR4. Binding, fusion and phagocytosis assays revealed significantly lower HIV-C entry and internalization in CD11c-k.o.-DCs compared to their wild-type and CD11b-k.o.-DC counterparts, while these processes were not altered in DCs exposed to non-opsonized HIV-1. Knock-out of CD18, the common ⊠-chain of CR3 and CR4, slightly enhanced the reduced binding, fusion and phagocytosis of HIV-C or C-opsonized beads. In accordance to decreased entry and internalization of HIV-C in CD11c- and CD18-k.o.-DCs we found significantly lower productive infection of these cells. Our data reveal that THP-1-k.o. DCs represent a good cell model to study the specific functions of CR3 and CR4 with respect to improvement of the antiviral capacity of DCs. Following detailed characterization of complement receptor functions and pathways in DCs, targeting CR3 or CR4 specifically will provide an efficient vaccine adjuvant to strengthen antiviral immune responses also during the chronic phase of HIV-1 infection.

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Pro-coagulant effect of blood-borne tissue factor on the endothelium in inflammation

Introduction:

Systemic inflammation triggers higher levels of tissue factor (TF) contributing to dysregulated haemostasis in patients with inflammatory syndromes. Microparticles (MP) can transfer their surface markers to cells remote to their cell of origin. Tissue factor (TF) can be detected on the surface of endothelial cells without the corresponding mRNA upregulation, suggesting that TF is conveyed by MP.

Aim:

The aim of this project is to test MP derived from human primary blood cells and cell lines concerning their ability to induce functionally active TF on endothelial cells (EC) and identify the cellular source of the most potent MP.

Methods:

MP were isolated from red blood cells, platelets, peripheral mononuclear cells and granulocytes of healthy donors. Endothelial MP were obtained from human umbilical vein EC (HUVEC). To model inflammation, MP from untreated cells versus TNFalpha stimulated cells were isolated after three hours. To test FX activation, HUVEC were co cultured with cell specific MP for six hours. Total TF levels of MP, supernatants and cell lysates were tested.

Results:

MP isolated from TNFα treated cells had higher pro coagulant activity. Functional TF was detected on both MP and treated EC. TF-specific FX activation was inhibited by anti CD142. FX activation on EC was elevated when incubated with MP from stimulated cells compared to MP from untreated cells (mean relative activity 166,5 ±74,91 vs 459,6 ±293,5; n=7;). Total TF levels were higher in isolated MP derived from TNFα treated cells and total TF concentration was elevated in EC lysates and supernatants after stimulation with MP of stimulated cells (mean 247,26 ±74,4 vs. 307,48 ±121,59 pg/mL; n=6).

Conclusion:

TF-specific FX activation on HUVEC was induced by pro-inflammatory stimulation and further increased by co-incubation with MP from TNFalpha conditioned cells compared to MP from untreated cells. Total TF levels increased after incubation with MPs. MP of TNFα treated EC and leucocytes show the highest TF activity.

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Impact of bile leakage on perioperative and long-term oncological outcome

Background: Bile leakage is a frequent and severe complication following liver surgery. This study aims to evaluate risk factors and related outcome parameters.

Methods: All hepatic resections performed at the Medical University Innsbruck between 2005 and 2016 were retrospectively analysed. Perioperative risk factors related to postoperative bile leakage were identified using univariate and multivariate analysis. Kaplan-Meier method was used for survival analysis.

Results: Bile leakage (according ISGLS definition) occurred in 52 of 498 liver resections (10%), excluding bilioenteric-anastomosis (7%). The incidence of bile leakage was higher in patients after major hepatectomy (15 vs. 6%, p<0.001), whereas there was no difference between benign and malignant diseases (7 vs. 12%, p=0.193). Postoperative 90-day-mortality (11 vs. 4%, p=0.411) and 90- d-morbidity excluding bile leakage (37 vs. 29%, p=0.231) was not significantly different within both groups. Portal vein embolization, bilioenteric-anastomosis, regional lymphadenectomy, vascular reconstruction and intraoperative T-drainage were significant factors in univariate analysis for developing bile leakage. Only lymphadenectomy (HR:2.43) and bilioenteric-anastomosis (HR:4.99) remained significant in multivariate analysis. Postoperative renal failure and surgical site infections were more common in patients with bile leakage. Median survival in the entire cohort (42 vs. 65mo, p=0.14) and among malignant disease (39 vs. 54mo, p=0.23) was not statistically affected by bile leakage. Moreover, disease-free survival (12.0 vs. 10.0mo, p=0.88) was comparable in both groups.

Conclusion: Bile leakage is a frequent complication following major liver resection, whereby bilioenteric-anastomosis and regional lymphadenectomy are significant risk factors. However, neither short-term postoperative mortality nor long-term oncological outcome is affected by this specific complication.

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Antifungal susceptibility patterns of rare yeasts

Introduction: Nosocomial yeast infections are a major public health concern, majority of them are caused by Candida albicans, C. glabrata, C. parapsilosis and C. tropicalis. But over 20 species are known to cause human infections. Among the rare agents (causing less than 1% of the infections) are Diutina rugosa, Turolopsis inconspicua, Stephanoascus ciferrii and C. pararugosa. Their rareness limits comprehensive studies on antifungal susceptibility patterns and therefore epidemiological cut-off values and clinical breakpoints have not been established.

Methods: Our objective was to generate species-specific susceptibility profiles for D. rugosa (N=34), T. inconspicua (N=164), C. pararugosa (N=49) and S. ciferrii (N=5) for all available azoles, echinocandins and amphotericin B using broth-microdilution according to EUCAST guidelines and E-test®. Species ID was confirmed with MALDI-TOF.

Results: Results demonstrate that these species have on average a higher resistance level than species such as C. albicans as showcased by the most prominent species in our collection T. inconspicua (N=164), D. rugosa (N=34) and C. pararugosa (N=56). The EUCAST broth microdilution MIC50 values for isavuconazole, itraconazole, posaconazole, and voriconazoles in T. inconspicua ranged between 0.125–0.5µg/ml, while fluconazole MIC50 value was high with 32.0µg/ml. Echinocandins MIC50 values ranged from 0.03–0.5µg/ml and for amphotericine B MIC50 was 0.5µg/ml. MIC50 values for Diutina rugosa and C. pararugosa ranged for azoles from 0.25 to 0.5µg/ml, but were again high for fluconazole 16 µg/ml. MIC50 for amphotericin was 0.5 µg/ml for both species. Echinocandin MIC50 values ranged from 0.25-1.0µg/ml for D. rugosa and were higher for C. pararugosa (0.5-4.0µg/ml).Method agreement was generally low (<90%) for E-test® and EUCAST broth microdilution method. Lowest method agreement was seen for C. pararugosa in all tested drugs (<70%).

Conclusion: Highest MIC50 values were seen for fluconazole in all tested rare yeast species. Amphothercin B showed good in vitro activity against all tested yeasts, azoles and echinocandins varied in activity. Depending on the species the MIC50 of azoles or echinocandis was over the clinical breaking point of C. albicans which is the cause of the negative treatment outcome of these species. Method agreement between EUCAST and E-test® was limiting the comparability of data.

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The role of the PIDDosome in hepatocyte polyploidization and tumorigenesis

The PIDDosome is an activation platform for Caspase-2 consisting of the proteins PIDD1 and RAIDD. It functions as a sensor for supernumerary centrosomes which occur upon cytokinesis failure or endoreduplication. Failed cell division promotes genomic instability which is considered to be a hallmark of cancer. In such a tetraploid cell, the PIDDosome activates Caspase-2, starting a signaling cascade which triggers cell cycle arrest via cleavage of Mdm2, p53 stabilization and p21 induction. Therefore, in many cell types the PIDDosome can contribute to prevention of tumorigenesis. In the liver, however, polyploidization of hepatocytes is part of the normal organogenesis and is found in mice to occur especially postnatally and during regeneration.

Experiments in mice revealed that hepatocytes specifically upregulate caspase-2 when proliferating and that caspase-2 and the PIDDosome function as a break for excessive hepatocyte polyploidization. However, the mechanisms by which these cells control caspase-2 expression are still elusive. In the course of this project, we are investigating transcriptional regulation of caspase-2 and the responsible transcription factors as well as the protein stability in human hepatocyte cell lines.

In the second part of the project we focus on the role of caspase-2 in hepatocellular carcinoma. Recently, it was reported that in contrast to other cell types, increased hepatocyte ploidy is protective against hepatocellular carcinoma (HCC), since it can prevent loss of heterozygosity and therefore reduce tumor development. We plan to investigate the role of the PIDDosome-caspase-2 pathway in tumorigenesis in wildtype and PIDDosome knockout mice in chemically induced hepatocellular carcinoma. We aim to answer the question whether caspase-2 and the PIDDosome are directly involved in HCC development and how PIDDosome-deficiency and therefore increased hepatocyte ploidy affect tumorigenesis and cancer cell ploidy.

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Transcriptional integrator p300 in human prostate tumor-initiating cells

Introduction

p300 is a well-known coactivator of the androgen receptor (AR) and has been shown to play an important role in prostate cancer (PCa). The expression of p300 has been correlated to tumor volume and PCa progression. Therefore, we hypothesize that p300 may play an important role in tumor-initiating cells and therapy resistance. Due to its multifunctional role p300 may serve as a promising new therapeutic target for treatment of PCa.

Material & Methods

The tumor-initiating capacity of the AR-negative PCa cell lines PC3 and Du145, as well as primary prostate epithelial cells was characterized by seeding a limiting number of cells per flask. Colony formation efficiency, colony type and detailed morphological information about each colony were determined by an automated pipeline that we have developed. Stable cell lines with doxycycline inducible p300 sh RNAs were generated by lentiviral transduction. Knockdown of p300 was verified by Western Blot and reduced p300 activity was confirmed by measuring histone h3 acetylation.

Results

In concordance with the literature we identified three colony types (holo-, mero- and paraclones) in PC3 and Du145 cell lines as well as primary PCa cells. Serial clonogenic assays confirmed that holoclones show the highest colony formation potential and maintain their tumor-initiating capacity over numerous rounds. In PC3 we detected 10% and in Du145 30% holoclones. Under optimal growth conditions p300 was detected in all colony types, however no differences in protein expression of p300 as well as on mRNA level were observed. Knockdown of p300 did not alter colony formation efficiency or the relative distribution of colony types.

Conclusion

Taken together, we confirmed the presence of tumor-initiating holoclones in PCa cell lines and primary prostate epithelial cells and established stable cell lines with inducible knockdown of p300. In contrast to several other types of malignancies, p300 has no specific role in regulation of stemness in our PCa models at the tested conditions. As a next step we will determine the effects of p300 knockdown during chemotherapy to evaluate if p300 depletion may enhance the treatment efficacy and which role p300 plays in therapy resistance.

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Novel 68Ga-radiolabelled FSC-based tetrazine derivatives for improved pretargeted PET imaging

Introduction: The bioorthogonal inverse-electron-demand Diels-Alder (IEDDA) reaction between radiolabelled tetrazines (Tz) and trans-cyclooctene (TCO) has attracted increasing interest in particular for antigen targeting with modified antibodies, immunoPET respectively. The extraordinary chemoselectivity enables this click reaction even in complex biological systems. Furthermore, extremely fast reaction kinetics make this approach feasible for positron emission tomography (PET) imaging with short-lived radionuclides such as gallium-68. Based on a Fusarinine C (FSC) chelating scaffold, suitable for gallium-68, we synthesized mono- and multimeric tetrazine derivatives for potentially improved targeting and here we present the evaluation of its targeting efficiency.

Methods: FSC with three primary amines attached to the chelating core was partially acetylated and resulting Monoacetyl- (MAFC), Diacetyl- FSC (DAFC) as well as non-acetylated FSC were functionalized with Tz by conjugation of the remaining amines with NHS-PEG5-Tz. The resulting DAFC-PEG5-Tz (monomer), MAFC-(PEG5-Tz)2 (dimer) and FSC-(PEG5-Tz)3 (trimer) were radiolabelled with gallium-68 and characterized in vitro - including protein binding, log P, stability studies, binding studies with TCO-modified Rituximab (Rtx) on CD20 expressing Raji cells- and in vivo -including biodistribution studies in normal BALB/C mice and imaging studies using a novel non-tumour based model.

Results: Mono- and multimeric Tz-FSC conjugates could be quantitatively radiolabelled with gallium-68 revealing good stability in PBS solution and serum. Distribution coefficient expressed as log P was around -1 indicating a hydrophilic character and protein binding resulted to be intermediate (~50%). In vitro binding to CD20 expressing Raji cells incubated with Rtx-TCO was 4.01 ± 0.24% for DAFC-PEG5-Tz, 7.75 ± 0.56% for MAFC-(PEG5-Tz)2 and 15.9 ± 0.88% for FSC-(PEG5-Tz)3 revealing significantly enhanced binding of multimeric constructs compared to the monomeric counterpart. Biodistribution studies showed a similar profile with rapid renal excretion and moderate uptake in liver and kidneys (<10% ID/g), blood levels increased from the mono to the trimer from 1%-3%ID/g 1h p.i.

Conclusion: Our preliminary results show that the preparation of polyvalent Tz-conjugates based on FSC chelating scaffold is feasible and the grade of multimerization is accompanied by enhanced binding to the TCO-modified counterpart. Furthermore, fast blood clearance and low accumulation in non-targeted tissue, favourable biodistribution, respectively is highly promising for these radioligands. Proof of tumour targeting in vivo in respective animal models is currently ongoing.

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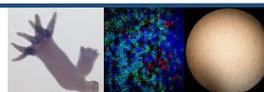
Xenoantigen dependent complement-mediated neutralization of LCMV glycoprotein pseudotyped VSV in human serum

Neutralization by antibodies and complement limits the therapeutic potential of oncolytic viruses after systemic application. We and others previously showed that pseudotyping of oncolytic rhabdoviruses like the maraba virus and the vesicular stomatitis virus (VSV) with lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) results in only a weak induction of neutralizing antibodies. Moreover, LCMV-GP-pseudotyped VSV (VSV-GP) is significantly more stable in normal human serum (NHS) than VSV. Here, we demonstrate that depending on the cell line used for virus production, VSV-GP showed different complement sensitivities in non-immune NHS. Neutralization of VSV-GP in NHS was dependent on the activation of the classical complement pathway by IgM antibodies. Xenoantigens like galactose-α-(1,3)-galactose or N-glycolylneuraminic acid expressed on mouse, monkey or hamster derived production cell lines were recognized by such IgM antibodies and subsequently induced complement-mediated lysis of VSV-GP. In contrast, VSV-GP produced on human cell lines was stable in NHS. However, GP-specific antibodies induced complement-mediated neutralization of VSV-GP independent of the producing cell line. Thus, our study points to the importance of a careful selection of cell lines for viral vector production in human and animal studies.

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Predicting drug combinations for precision immuno-oncology in colorectal cancer

Colorectal cancer (CRC) is one of the most lethal cancer diseases worldwide. CRC is a very heterogeneous cancer type and exhibits very diverse prognosis and drug responses. Targeted agents have shown considerable clinical effects in different cancer types and are currently object of several ongoing clinical trials. However, only a subgroup of CRC patients benefit from this treatment and drug resistance remains a major issue. A promising approach to overcome drug resistance is to combine different targeted drugs and thereby overcome resistance mechanisms of escape. However, the identification of optimal drug combinations is very challenging due to different escape mechanisms, different tumour mutational profiles, and limited power of experimental drug screening systems. Therefore, a combined computational-experimental approach holds promise to identify optimal and patient-specific drug combinations.

A novel computational-experimental approach was applied to identify optimal drug combinations for individual CRC patients. The concept is based on perturbation experiments using patient derived CRC tumour-organoids. Whole-exome and RNA sequencing of the obtained organoids was performed. The organoids were pertubed with approved kinase inhibitors and targeted phosphoproteomic profiling was carried out. Patient-specific dynamic models using differential equations and parameter identification was performed.

Furthermore, simulation experiments of combinatorial regimens with these personalized models will enable the selection of optimal drug combinations. The method developed in this study and the obtained results will build the basis for the development of a computational-experimental platform for precision oncology in CRC.

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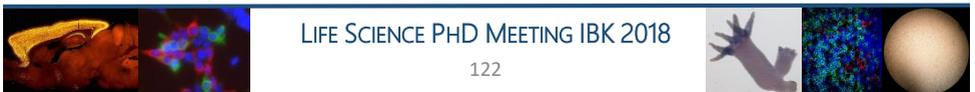
Loss of skin dendritic cells in a spontaneous melanoma mouse model and its consequences in antitumour immune responses

A decrease in the percentage of skin dendritic cells (DCs) has been reported for various types of skin cancers, including melanoma. We used the tg(Grm1)EPv melanoma mouse model to understand the mechanism responsible for the loss of skin DC. We here show that total DCs (CD11c+ cells) are gradually lost as the tumor progresses. Mainly the dermal DCs were affected, including mostly CD11b+ DCs, whereas epidermal Langerhans cells remained unchanged. We hypothesized that extrinsic cell death of the DCs may be induced within the growing tumor. Indeed, the tumor tissue upregulated the expression of Fas ligand (FasL) that can induce extrinsic apoptosis in immature DCs that express Fas (CD95). Fas-mediated apoptosis depends on activation of caspase-8 and mice in which the autoproteolytic cleavage site D387 is mutated to alanine (casp8D387A/D387A mice) show strong resistance to Fas-mediated death. By injecting intradermally agonistic anti-CD95 antibody in WT and in casp8D387AD3987Amice, we saw that WT CD11b+ dermal DCs are susceptible to Fas-mediated apoptosis. In addition, tumor development and growth was accompanied by a decreased expression of Flt3L, suggesting that growth factors that are critical for the survival of DCs are being downregulated in the skin. Treatment of tg(Grm1)EPv mice with Flt3L resulted in a higher percentage of CD11b+ dermal DCs within the tumor and in the induction of better cytotoxic T cell responses both in the tumor and in the tumor-draining lymph node. Our data suggest that the loss of DCs in melanoma depends on induction of apoptosis and reduction of survival signals within the tumor. At the same time we show that treatments that increase DC numbers and activation can be efficient therapeutic options against melanoma.

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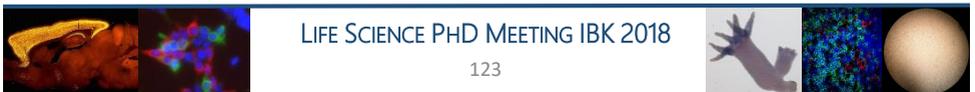
miR-17~92 mediated Bim repression is essential for MYC-driven lymphomagenesis

In lymphomas, deregulation of MYC results in high expression of the miR-17~92 microRNAs (miRNAs). miRNAs modulate the levels of their respective target mRNAs by 3'UTR binding, causing translational repression or degradation. According to bioinformatical predictions, one miRNA can potentially regulate hundreds of targets. However, determining functionally relevant targets as well as distinguishing direct from indirect targets remains a key challenge. The oncogenic miR-17~92 cluster encodes for six miRNAs whose target recognition specificities are determined by their distinct sequence. The pro-apoptotic Bcl2 family member Bim is one of the top predicted combinatorial targets of miR-17~92. According to literature MYC-driven lymphomas are addicted to miR-17~92 and miR-17~92-mediated Bim repression has been suggested to be one potential mechanism.   To test this hypothesis, we utilize a conditional Bim allele allowing in vivo exchange of the wild type Bim 3'UTR against a miR-17~92 seed-match mutated counterpart. Indeed, we found that Bim 3'UTR seed match mutations are of vital importance: they inhibit miR-17~92:Bim interactions thus relieving Bim repression, promoting apoptosis of transforming/malignant B cells and thereby delaying/preventing lymphomagenesis.

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Combination therapies to improve Dendritic Cell based treatment of melanoma

Dendritic cells are the most potent antigen-presenting cells of the immune system with the ability to induce both CD4+ and CD8+ T cell responses. However, the tumor microenvironment presents multiple obstacles to prevent proper tumor immunity. The aim of this project is the development of combination therapies that allow control of melanoma growth. For this purpose, tumor antigens will be delivered directly to DC by antibody-mediated uptake and these DC-vaccines will be combined with pharmacological or immune checkpoint inhibition to further boost anti-tumor immune responses.

For the generation of DC-vaccines, the expression of three melanoma-associated antigens (gp100, TRP-2 and MAGE-A2) were examined by RT-PCR. In transplantable models, the expression of tumor antigens is high in B16 melanoma, while low levels were observed in the BRAF mutant SM1WT1 and D4M tumors. Still, the expression of gp100 in D4M tumors was sufficient to drive the proliferation of gp100-specific T cells in the tumor draining lymph nodes. Additionaly, we observed a significant upregulation of the gp100 and TRP-2 antigens when the D4M tumors were treated with a BRAF-specific inhibitor, as has been previously reported.

We are currently cloning the melanoma antigens gp100, TRP-2 and MAGE-A2 into the DC targeting antibody DEC-205. With the resulting antibody-antigen fusion proteins we will deliver the antigenic peptides to skin DC. In a second step, we will clone the antigens also into a Langerin antibody that allows specific targeting of Langerin+ skin DC subsets. We are also in the process of testing the optimal delivery route for these DC-vaccines, either intradermal injection or laser-assisted epicutaneous application.

The first combination therapy will be a BRAF inhibitor together with DC-vaccines. So far, we observed that the transplantable D4M melanoma model is very sensitive to BRAF inhibition and the prolonged exposure to this targeted therapy results in resistance, as observed in melanoma patients. Moreover, the immune infiltrate in D4M tumors changed during BRAF inhibitor treatment. These changes will be exploited in our vaccination scheme in order to achieve optimal anti-tumor immune response. With optimal timing of DC-vaccines we aim to improve tumor immunity and prevent development of resistance to targeted therapy.

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The role of glucocorticoids on B cell development and function

Glucocorticoids play an important role in many feedback pathways of the immune system. They are involved in dampening inflammatory processes and are therefore widely used in many therapeutic approaches. Recent studies have focused on the role of glucocorticoids on development and function of different immune cells. B cells, as key players of the adaptive immune system, have been reported to express high levels of the glucocorticoid receptor and several reports show that glucocorticoid treatment both in vitro and in vivo impacts B cell physiology in terms of development, differentiation and cell death. However, the role of endogenous glucocorticoids in modulating B cell development and function has not been characterized so far and will be subject of this research. The role of glucocorticoids during B cell development, activation, function and antibody production will be studied.

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Studying atherosclerosis using large-scale data

Background: Atherosclerosis is believed to be a major determinant of life expectancy ('vascular age'). The study of atherosclerosis markers is limited to single studies with limited statistical power. A large individual-participant data meta-analysis would clarify uncertainties and could assess differences and communalities of atherosclerosis parameters and their effect on clinical outcomes, such as myocardial infarction, stroke, and cardiovascular mortality. The Prospective Studies of Atherosclerosis (Proof-ATHERO) Consortium aims to combine results of various studies focusing atherosclerosis to gain insight into the complexity of the disease process.

Methods: Data of the Proof-ATHERO Consortium are centrally collected on a secure server at the Department of Neurology of the Medical University Innsbruck. Data are checked for inconsistencies and any queries are resolved via direct correspondence with individual study collaborators. Statistical analyses will involve calculating results within each study separately, and then combining study-specific results using random-effects meta-analysis.

Key aims: The objective of the Proof-ATHERO consortium is to understand and predict atherosclerosis and its consequences. Its main scientific aims are threefold: (1) to better characterize the natural history, communalities and differences of atherosclerosis measures, (2) to study atherosclerosis determinants and (3) to investigate clinical consequences of atherosclerosis. To examine these topics, large scale data is strongly needed to reach adequate statistical power. Therefore, individual-participant meta-analysis approach can be applied to combine results of single study cohorts and provide overall estimates. Since the number of studies focusing atherosclerosis is constantly growing, Proof-ATHERO aims to combine results of individual studies and tries to use worldwide available data to deliver most reasonable scientific evidence.

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Satb2-driven changes in neuronal nuclear morphology depend on Vps4/ESCRT III complex

SATB2 (Special AT-rich sequence binding protein 2) is a risk locus for schizophrenia and is associated with human intelligence. It encodes a highly conserved DNA-binding protein that regulates higher-order chromatin configuration. Satb2 ablation in adult mouse forebrain causes long-term memory deficits. Loss- and gain-of-function studies in primary neuronal cultures have revealed that Satb2 is both necessary and sufficient for synaptic activity-triggered changes in neuronal nuclear shape referred to as nuclear infoldings (NIFs). In addition, co-immunoprecipitation / mass spectrometry analysis has demonstrated specific interactions of Satb2 with inner nuclear membrane proteins (e.g. Lem2, Lem3, and Lap2) as well as with members of the ESCRT complex (endosomal sorting complex required for transport), known to play a major role in the endosome-to-multivesicular body trafficking and membrane remodeling. Given that, Lem2 is known to recruit components of the ESCRT III complex to mediate nuclear envelope closure in non-neuronal cell types, we hypothesized that Satb2, Lem2 and the ESCRT III machinery work together in mediating NIF formation.

To test this hypothesis, we employed a siRNA-mediated knock-down of Lem2 and Vps4 protein expression in primary hippocampal and cortical neurons. In cultures, transfected with scrambled siRNA, AAV-mediated Satb2 overexpression resulted in a robust 2-fold increase in the number of nuclei bearing infoldings, similar to our previous findings with neurons not treated with siRNA. In contrast, in Lem2- or Vps4-depleted neurons Satb2 overexpression did not lead to an increase in the number of infolded nuclei, indicating that Lem2 and Vps4 are both necessary for the Satb2-driven nuclear infolding formation.

Based on our results we suggest that Satb2 regulates nuclear shape by binding to inner nuclear membrane proteins that in turn recruit Vps4/ESCRT machinery to drive nuclear envelope remodelling. Further studies will be required to elucidate the role of Satb2-mediated nuclear invaginations in gene expression programmes underlying memory consolidation and synaptic plasticity.

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Development of an AAV based gene therapy in rodent models of temporal lobe epilepsy

Introduction

Temporal lobe epilepsy (TLE) is one of the most frequent neurological diseases and current pharmacotherapies cause severe side effects; moreover, 30% of the patients cannot achieve seizure freedom and a better quality of life with available medications. Neuropeptide receptors are considered modulators of neuronal activity and may represent valuable novel drug targets to treat epilepsies. In recent years, our lab provided proof of principle that permanent over-expressing of preprodynorphin (pDyn) in the epileptogenic focus through a viral vector reduces seizures in a mouse model of TLE. However, many promising therapies proved efficient in mouse models but failed to translate to other species. Therefore, the aim of this study is to further validate the AAV-based expression of pDyn as gene therapy in rats and to optimize the gene product to minimize the risk of side effects.

Methods

Self-sustained status epilepticus (SSSE) was induced in rats through lateral amygdala electrical stimulation. An AAV based pDyn expressing (AAV-pDyn) vector was infused via guide cannula in both ipsi- and contralateral hilus. Focal seizure-like abnormalities in both hippocampi were analyzed using EEG before and after treatment. The pentylenetetrazole model in pDyn KO mice was used to screen for optimized gene products.

Results

One month after AAV-pDyn infusion the SSSE-induced EEG abnormalities observed in ipsi- or contralateral hippocampi were almost completely suppressed. Modified pDyn derived peptides displayed altered potency and efficacy in restoring the reduced seizure threshold of pDyn KO mice, suggesting potential for optimized therapy vectors.

Conclusions

Replenishing pDyn in the epileptogenic focus reduces EEG abnormalities in mice and rats. Further optimization of the gene product may lead to a more efficient and safer therapy for TLE.

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Implications of the ablation of the metabotropic glutamate receptor 5 (mGluR5) on social behavior

Amongst the many neurotransmitters systems causally linked to the expression of social behavior, glutamate appears to play a pivotal role. In particular, mGluR5 received particular attention as its altered function was reported in multiple mouse models of autism. Systemic suppression of mGluR5 activity improved the deficit in sociability in these models. However, in normal wild type mice, pharmacological manipulations yielded inconsistent results.

The aim of our study was to investigate the actual contribution of mGluR5 in sociability and explore the impact of mGluR5 deletion on the pattern of brain activation upon social interaction.

Using the 3-chamber test to assess sociability, adult male germline mGluR5-knockout (mGluR5-KO) mice were found to spend a significantly higher proportion of time in the chamber with a novel conspecific than in the one with a novel object, compared to age-matched C57BL/6J mice. The c-Fos mapping following exposure to a novel conspecific or object revealed, upon the 28 brain areas analyzed, an over-activation of the prelimbic prefrontal cortex and paraventriular thalamic nucleus in mGluR5-KO mice, in response to both conspecific and object. Network analyses revealed an important role of the medial prefrontal cortex (mPFC) in coordinating brain activity upon social exposure and suggested a reduced inhibitory activity in the mPFC of mGluR5-KO mice.

In conclusion, this work reveals a complex contribution of mGluR5 in sociability and social interaction and points out mPFC inhibitory circuits as primary sites of regulation of social behavior by these receptors.

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Nogo Receptor (NgR) - signaling is required for the maintenance of the structural integrity of mouse fungiform taste buds.

Background and aim: The integrity of taste buds (TB) in the lingual epithelium is intimately dependent on an intact gustatory innervation, yet the molecular nature of this dependency is still unknown. Nogo receptors (NgRs) are crucial for axon regeneration in the CNS and function in spine maturation and axon arborization in the developing brain. Because NgRs are expressed in geniculate ganglion (GG) and trigeminal ganglion neurons throughout development, we postulated loss of NgRs might regulate gustatory innervation, indirectly impacting taste buds.

Results: Genetic elimination of NgR1/2 caused severe perturbation in TB structure. While total number of TB are unchanged, TB volume and taste cell number of p5 - p21 fungiform papillae in the anterior tongue are significantly reduced by 31% and 35%, respectively. At p21, 82% of NgR1/2- TB exhibit distorted morphology with no visible taste pore. Despite the diminished TB size, their peripheral sensory innervation density as revealed by P2X3-staining did not differ between mutants and controls. Similar, no apparent changes in GG size and neuronal survival were detectable in E14.5 mutant embryos. Further studies are performed to investigate whether the reduced TB size is caused by an enhanced taste cell apoptosis or decreased proliferation rate of gustatory basal cells in NgR1/2- mice.

Conclusion: Our preliminary data suggest that NgR-signaling plays critical roles in maintaining structural integrity of the peripheral gustatory system.

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Therapeutic efficacy of GDNF loaded collagen scaffolds in organotypic brain slices with rotenone-induced Parkinson´s disease

Parkinson`s disease (PD) is the second most common neurodegenerative disorder, affecting 10 million people worldwide. The main pathophysiology in PD is selective degeneration of dopaminergic neurons in the substantia nigra (SN), which causes motor deficiencies such as tremor, bradykinesia, rigidity and postural instability. Current treatment methods in PD aim to provide symptomatic treatment rather than protection strategies. Glial cell-line derived neurotrophic factor (GDNF) was shown to provide neuroprotection of the dopaminergic neurons in in vitro and in vivo models; however GDNF cannot cross the blood-brain barrier and has a very short half-life in blood circulation. The use of injectable hydrogels that release GDNF in a localized and controlled manner for a prolonged time period could be an ideal solution in order to overcome these issues.

In this study, we aim to test the neuroprotective effects of GDNF encapsulated in collagen scaffolds on dopaminergic neurons of SN and ventral tegmental area with an ex vivo model. We are using organotypic brain vibrosections from wild type postnatal day 8-10 mice and evaluate the survival of tyrosine hydroxylase (TH) positive dopaminergic neurons by immunohistochemistry, as well as qRT-PCR and Western Blotting. We have established that the pesticide rotenone (300 nM, 3 days) causes selective degeneration of dopaminergic neurons in SN, providing a good model to study PD in such slices. GDNF (10 ng/ml) in the culture media significantly prevents toxic effects of rotenone. Collagen scaffolds cross-linked with polyethyleneglycol (PEG), which were optimized in our project, show a good GDNF release profile over 14 days in culture conditions. We are on the way to assess the protective effects of collagen scaffolds loaded with GDNF on the survival of dopaminergic neurons against the toxicity of rotenone.

To conclude, the use of injectable collagen scaffolds loaded with GDNF for the protection of dopaminergic neurons may become a useful approach in the treatment of PD in the future by preserving degenerating neurons.

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Do L-type calcium channels share a common mechanism determining voltage sensitivity?

Voltage-gated calcium channels regulate fundamental functions of muscle, nerve and endocrine cells. They are organized in four homologous repeats (I-IV) of six transmembrane domains (S1-S6). The four S5-S6 domains form the channel pore, whereas S1-S4 of each repeat forms separate voltage sensing domains (VSD). Among the members of CaV channel family the skeletal muscle CaV1.1a is atypical because of its low voltage sensitivity, low current density, and its slow activation kinetics. Alternative splicing of exon 29, located in the extracellular IVS3-S4 linker, results in dramatically increased voltage sensitivity and current density, demonstrating the importance of VSD IV for controlling CaV1.1 gating properties. Using structural modeling combined with mutagenesis and electrophysiology, our team recently discovered that inclusion of exon29 in CaV1.1a disrupts the interactions between the two outermost gating charges (R1, R2) in IVS4 and a countercharge (D4) in IVS3. Our model predicts that in the absence of exon 29 these interactions facilitate the transition from intermediate state into the activated state, resulting in increased voltage sensitivity, and stabilize the activated state, leading to an increased opening probability. Although the countercharge D4 as well as alternative splicing of the S3-S4 linker in VSD IV are conserved in all L-type calcium channels, our results show that, while in CaV1.1 this mechanism determines its characteristic gating properties, in CaV1.2 and CaV1.3 D4 is involved in fine-tuning voltage sensitivity. Applying patch-clamp analysis of CaV1.1 with and without exon 29, we characterized the single-channel properties of CaV1.1a and CaV1.1e and refined our mechanistic model of the VSD IV transition into the activated state.

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Functional imaging during presentation of high and low-caloric food in adolescents with Anorexia Nervosa

Anorexia nervosa (AN) is an eating disorder (ED), typically with the onset in adolescence. According to different studies, even up to 4% of adolescents suffer from anorexia nervosa. Although AN is from 6 to 10 times more common in girls than in boys, the growing number of male patients is observed in the last years. As AN has the highest mortality rate of any psychiatric illness, it seems essential to develop a program of prevention, early detection and effective treatment.

There are some functional magnetic resonance imaging (fMRI) studies in adult AN, but only rare in adolescents. During watching images of high-caloric food in a state of hunger an enhanced activation of the posterior cingulate cortex can be observed; whereas the anterior cingulate cortex, frontal and prefrontal cortex and insula are activated in healthy controls. Studies about differences in fMRI in satiety and hunger in AN and healthy controls show higher activation in attentional processing area. Visual presentation of food to anorectic patients – even without consumption – is often described by them as aversive, and can even cause satiety. Also gustatory stimuli and their effect on brain processing in AN patients show differences compared to control groups.

The hypothesis of our study is that activation patterns in fMRI in the AN group will change after therapy. Our experiment is designed as an event-related fMRI setting with conditions of alternating high- and low-caloric images and images of fixation. Patients are interviewed in advance with questionnaires specific for personality (SCID II) and eating disorders (EAT and EDI).

So far a group of 10 female patients attended pre- and post-therapy fMRI examination. Preliminary results show a tendency of enhanced activation in orbitofrontal and anterior cingulate cortex. Half of the group can be characterised as depressive and non-assertive. We are planning to examine 20 female patients, comparing them to similar group of healthy participants. Gustatory stimuli (cold chocolate milk versus water) in those groups will be also performed and analysed. We are planning to compare fMRI studies in both sexes.

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Chemical compositions of traditional alcoholic beverages and consumers' characteristics, Ethiopia

Commercially available home brewed alcoholic beverages of Areki and Tej from Addis Ababa and other regional cities of Ethiopia were analysed for alcohol concentration, methanol level and other additives. Surveys were also carried out on the rate of alcoholism and the socio-demographic characteristics of the consumers. The chemical properties of the sampled beverages showed that home distilled Areki and fermented Tej drinks can pose health threats due to their high alcoholic strength and undesirable additives. Methanol concentration was found significantly below the highest limit to causing harm to human health. Close to a third of the observed Tej and Areki users have exhibited symptoms of alcoholism. Factors related to gender and reasons for drinking were significantly associated with alcohol abuse. The introduction of community-based intervention to reduce the rate of alcoholism in Addis Ababa is strongly suggested. Commercial vending houses should be subjected to acceptable regulations in their mode of production and delivery mechanisms. Applicable strategies for effective management and supervision of traditional alcohol consumption and to reduce alcoholism and risks of health menace are recommended. Further studies on other health influencing substrates deserve supporting.

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Mu-opioid receptor activation and signaling exploration of opioid ligands emerging as new antinociceptive drugs

Aims: Opioids are the cornerstone drugs for effective pain control. The mu-opioid receptor (MOR) is the primary target for analgesia, but also unwanted effects of opioids. Accumulated evidence indicates that MOR-mediated antinociception results from G-protein-mediated signaling, while β-arrestin2 signaling pathways promote adverse effects. One long-standing focus of drug discovery is the pursuit for opioids exhibiting a favorable beneficial/side effects index. Herein, activation and signaling at the MOR of oxymorphone analogues, namely 14-O-methyloxymorphone (14-OMO), 14-methoxymetopon (14-MM) and their newly developed N-phenethyl substituted derivatives is presented.

Methods: Radioligand binding, [35S]GTPγS functional and β-arrestin2 recruitment assays were used for binding and activation of neuronal and recombinant MOR. Immunocytofluorescence with primary cultures of neurons natively expressing MOR fused to mcherry was employed for internalization studies. Antinociception was evaluated in a mouse model of acute thermal nociception.

Results: Binding studies showed that N-phenethyl analogues of 14-OMO and 14-MM retained the subnanomolar affinities at the MOR of the parent molecules. They activate G-protein with high potency as full MOR agonists, while displaying much lower potencies in inducing β-arrestin2 recruitment. The MOR selectivity was demonstrated using brains from MOR-knockout mice. Agonists did not promote MOR-mcherry internalization in mouse neurons up to 1 μM. In vivo, the N-phenethylmorphinans produced marked antinociception in the tail-flick test in mice after subcutaneous administration, with comparable potencies to 14-OMO and 14-MM.

Conclusions: Analysis of the MOR activity profile of targeted oxymorphone analogues provides important insights into ligand-MOR interaction and signaling pathways, with the prospect to identify more effective and safer opioid analgesics.

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Mesial temporal lobe epilepsy: HS666, a selective kappa-opioid receptor partial agonist, showing antiseizure/anticonvulsant effects without causing aversion after systemic administration in mice

Aims: Mesial temporal lobe epilepsy (mTLE) is one of the most common and severe types of epilepsy. The high incidence of resistance and side effects of currently available drugs highlight the need of new mechanism-based and safer treatments. Accumulated evidence established the importance of endogenous dynorphin, and its primary target, the kappa-opioid receptor (KOR) in epileptogenesis and seizure control. Modulation of the dynorphin/KOR system emerges as a prominent avenue in the pursuit of novel therapies for epilepsy. Along with promising antiepileptic effects of full KOR agonists, dysphoria and sedation limit their potential clinical use. A behavioral study on the antiseizure/anticonvulsant efficacy and potential for adverse effects of a new selective KOR partial agonist, HS666, after systemic administration in mice is presented.

Methods: Pentylenetetrazole (PTZ)-induced seizures were used to model acute seizures in prodynorphin-knockout mice. The intrahippocampal injection of kainic acid (KA) in C57BL/6N mice was used as a model for drug-resistant TLE. Conditioned place aversion and locomotor activity were assessed in C57BL/6N mice.

Results: Intraperitoneal administration of HS666 showed dose-dependent (0.3-10 mg/kg) and significant increase in the threshold for PTZ-induced seizures, and reduced paroxysmal activity in the KA model. The central site of action and specific KOR-mediated antiseizure/anticonvulsant actions were demonstrated using selective KOR antagonists. Moreover, HS666 did not induce aversion or affected locomotor activity at therapeutic doses.

Conclusions: These findings indicate that HS666 has the prerequisite pharmacological characteristics of an effective drug in experimental epilepsy, by activating central KOR to produce antiseizure/anticonvulsant effects with reduced liability for adverse effects.

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Generation of neurons from patient-derived cells to model the metabolome in Schizophrenia

Schizophrenia (SZ) is a devastating psychiatric disorder that appears in late adolescence with a prevalence of 0.5-1% worldwide. SZ is characterized by a loss of contact with reality and a disruption of thought, perception, mood and movement.

The neurobiological basis of SZ is not well understood, however, causes of the disease include environmental and genetic factors. Notably, studies on monozygotic (MZ) twins have shown that, although sharing the same genome, around 50% of SZ patients have a non-affected twin, suggesting an environmental contribution to the disease. Investigations on the metabolome of SZ patients have shown that aberrations in biosynthetic pathways may contribute to the mechanisms of the disease. Due to the inaccessibility of live human brain tissue, most studies on SZ have been limited to post mortem analyses or transgenic animal models that, however, reveal limitations regarding transferability to human physiology. The differentiation to neurons of patient-specific pluripotent stem (iPS) cells provide a promising cell source to overcome these challenges.

With a cohort of discordant MZ twins, of which one twin suffers from SZ and the other one does not, iPS models are employed to gain an in-depth understanding of neuronal metabolic dysregulation in SZ. Whole and targeted metabolome analyses with differentiated neurons is performed to gain comprehensive unbiased insight into the metabolic components of the SZ brain and differences between the discordant MZ twins. Markers such as CD24, CD56, and CD133 are validated to sort out a pure population of neurons for the metabolic analysis and pilot experiments with undifferentiated neural stem cells are employed for the bioinformatical analysis of metabolites. iPS cells from MZ twins are differentiated to subtype specific neurons such as cortical neurons. These neurons are phenotypically assessed using imaging techniques and sorted to get a pure population of neurons for metabolic analyses. Moreover, structural and functional disorganization of the developing SZ brain are investigated in cortical 3D models. Comparative analysis of metabolic mechanisms in SZ cell models will be presented to enable insight into the etiology and pathophysiology of SZ.

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The neuropeptidergic PACAP/PAC1 receptor system modulates behavioral and neuroendocrine stress reactions of rats within different forebrain areas

Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) is a neuropeptide with neurotransmitter/neuromodulator, properties which has been implicated in the regulation of emotional processes such as stress and anxiety reactions. However, despite the evidence of an implication of PACAP in stress mechanisms, there has been no direct functional evidence for an action of endogenous PACAP in distinct forebrain area on stress responses under ethologically relevant conditions. The aim of the present study was to investigate the role of the PACAP/PAC1 receptor system on neuroendocrine and behavioral stress reactions. We administered a PACAP agonist (PACAP-38) ICV and bilaterally into the PVN, LS or BNST of male Sprague-Dawley rats (200-250g at surgery) and tested animals in a stress behavioral task such as the modified forced swim test with simultaneous stress hormone measurements in plasma samples. In addition, we compared c-Fos expression as a marker of neural activation after central PACAP administration. We found that ICV administration of PACAP-38 increased c-Fos expression in PVN and LS. Moreover, ICV as well local administration of PACAP-38 in these brain areas increased the immobility time and reduced active coping behavior during the forced swim stress exposure as PACAP-38 treated animals showed enhanced floating and reduced struggling behavior compared to controls. Furthermore, administration of PACAP-38 into the LS and PVN significantly increased ACTH stress response without changing basal ACTH levels. Thus, our data show that the PACAP/PAC1 receptor system mediates the modulatory effects on neuroendocrine and behavioral stress function within distinct forebrain areas such as PVN and LS. (funded by the FWF - P28146-B21)

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Sleep alterations as a predictive biomarker for PTSD? – Lessons from an animal model

Posttraumatic stress disorder (PTSD) is a psychiatric disorder that may occur after exposure to a traumatic event. However, it is still unclear why only about 10-20% of trauma victims develop the disease. Sleep disturbances constitute a hallmark symptom of PTSD. Interestingly, impaired sleep has also been proposed to play a role in in disease development, as it may interfere with fear memory processing.

To address this theory, we evaluated circadian sleep/wake behavior of C57BL/6J (BL6) mice prior to and following cued fear conditioning (FC), as well as after a later fear retrieval (RET) session. Electroencephalogram and electromyogram activities were recorded chronically over 23h and animals were assessed for freezing behavior.

We performed k-means clustering based on freezing scores during FC and RET, which allowed assignment of the animals into two subgroups of 'high freezers' and 'low freezers'. Behavioral divergence was accompanied by dissimilarities in baseline sleep/wake patterns, especially in the inactive period. Here, high freezers displayed significantly lower amounts of non-rapid-eye-movement sleep (NREMS) and rapid-eye-movement sleep (REMS) and spent more time awake. Further analyses also revealed differences in REMS latency and spectral power.

Our findings suggest that the identified alterations in baseline sleep might interfere with fear memory processing and facilitate development of a high-fear, PTSD-like phenotype in susceptible animals. Evaluation of such sleep alterations may have potential prognostic and early diagnostic value in disorders such as PTSD.

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The presynaptic calcium channel subunit α2δ-2 regulates postsynaptic GABAA-receptor abundance and axonal wiring by a trans-synaptic mechanism

Auxiliary α2δ subunits modulate voltage-gated calcium channels and have been implicated in synapse formation. By employing a cellular α2δ triple knockout model in cultured hippocampal neurons, we could recently identify α2δ isoforms as redundant key regulators of glutamatergic synaptogenesis (Schöpf et al., submitted). Here we show that the specific expression of a single isoform, α2δ-2, in presynaptic glutamatergic terminals induces a mismatched localization of postsynaptic GABAA-receptors (GABAARs). This puzzling observation may be explained by (1) a compensatory upregulation of postsynaptic GABAARs, (2) an active participation of presynaptic α2δ-2 in the trans-synaptic anchoring of postsynaptic GABAARs, and (3) aberrant axonal wiring induced by presynaptic expression of α2δ-2. In order to distinguish between these hypotheses we analyzed the consequences of presynaptic α2δ-2 expression on glutamatergic and GABAergic synapse composition and synaptic transmission. Using immunofluorescence analysis we show that presynaptic α2δ-2 increases postsynaptic GABAARs both in glutamatergic and GABAergic synapses. This effect is even stronger in hippocampal α-neurexin triple knockout cultures, suggesting that α-neurexins are not needed for recruiting GABAARs. Most importantly, employing high- and super-resolution (gSTED) microscopy we demonstrate that presynaptic expression of α2δ-2 induces aberrant wiring of glutamatergic axons to GABAergic postsynaptic positions, resulting in altered synaptic transmission and plasticity. Finally, using structure homology modeling and immunofluorescence analyses we identify a single splice region in α2δ-2 mediating the trans-synaptic effect on GABAARs. Taken together, our findings provide novel insights into trans-synaptic mechanisms and are particularly interesting considering neuropsychiatric diseases such as autism spectrum disorders, which are associated with axonal wiring defects.

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Neural circuits underlying cocaine preference versus social interaction in conditioned place preference

The central part of the brain reward system can be considered the ventral tagmental area (VTA) and its dopaminergic projections to the nucleus accumbens (Acb), the so-called mesolimbic pathway. Acb is the main converging ground for projections from various cortical and midbrain structures. It is composed in its majority from GABAergic projecting neurons termed medium spiny neurons (MSN). VTA contains two major neuronal populations; 1-dopamine (DA) neurons which play a crucial role in reward dependent behaviors, and 2-local GABAergic population which inhibits the DA neurons.

One of the pathways which are not investigated yet is the glutamatergic projections from anterior insular cortex to MSN neurons in Acb core and its disinhibiting effects (INS→Acb→VTA) on VTA-DA neurons. The insular cortex (INS), a brain area involved in the representation of interoceptive information, gained attention in the addiction field due to the study by Naqvi and colleagues, in which they showed that patients with insular lesions quitted smoking and did not present any craving for cigarretes.

We believe that this network is implicated in affective and motivational components of addictive behaviors associated with craving. Moreover, repeated exposure to cocaine is associated with insular dysfunction, to properly attribute salience to social versus drug- associated context. Recent studies showed that optogenetic stimulation of GABA population of the interneurons located in the VTA decrease the rewarding effects and promoted conditioned place aversion.

Anatomical studies displayed a glutamatergic projection from anterior INS to ventral striatum (Acb core). In fact, insula glutamatergic inputs to the Acb core are necessary for aversion-resistant alcohol consumption in rats. Also, the efferent GABAergic projections from Acb to the VTA and its role in controlling cocaine's effects have been described. In particular, the inhibitory architecture in the VTA is a target of afferent projections from D1-containing MSNs. Thus, the existence of this neural circuit would suppose a way to explain the disinhibition of VTA-DA neurons and DA release that is produced when addicts/animals are exposed to drug or drug cues.

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Receptor tyrosine kinase trafficking in response to Sprouty2 regulation

INTRODUCTION: Receptor tyrosine kinases (RTKs) control a variety of processes in the nervous system. The Sprouty (Spry) proteins belong to a group of evolutionary conserved modulators of RTK signaling. Spry2 inhibits extracellular signal-regulated kinase (ERK) signaling in response to fibroblast growth factor (FGF) or nerve growth factor (NGF). In contrast, Spry2 promotes ERK signaling induced by epidermal growth factor (EGF) and inhibits the transfer of EGF receptor (EGFR) from early to late endosomes.

OBJECTIVES: The purpose of this study is to determine how different Spry2 levels influence internalization, trafficking and degradation of FGF receptor 1 (FGFR1) in comparison to EGFR in glioma cells, and of FGFR1 and tropomyosin receptor kinase A (TrkA) in adult sensory neurons. Thus, we will analyze the effects of Spry2 on trafficking of different RTKs in the nervous system.

METHODS: U251 glioma cell lines with stable overexpression or downregulation of Spry2, transiently transfected with EGFR-EGFP or FGFR1-EGFP and treated for 30 minutes with fluorescently labeled EGF or FGF1, were analysed to assess the colocalization of overexpressed RTKs, their ligands and lysotracker. Images were acquired by confocal microscopy and colocalization analysis was performed with Imaris software.

RESULTS: Colocalization analysis showed that downregulation of Spry2 promotes the colocalization of EGF with lysosomes whereas overexpression of Spry2 reduces this colocalization. By contrast, downregulation of Spry2 reduces colocalization of FGF1 with lysosomes and overexpression of Spry2 increases the colocalization with FGF1.

OUTLOOK: Our data reveal a difference in the modulation of FGFR1 and EGFR trafficking by Spry2 in U251 glioma cells. Therefore, we will compare trafficking of the two receptors in another glioma cell line, SF126, with higher endogenous Spry2 levels and with stable overexpression or downregulation of Spry2. Furthermore, we will investigate the effect of different levels of Spry2 on FGFR1 and TrkA trafficking in adult sensory neuron cultures of Spry2 heterozygous and homozygous mutant mice. The outcome of this study will provide new insights into the role of Spry2 in trafficking of different RTKs in the nervous system.

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PTEN downregulation enhances axon elongation of Spry2 deficient DRG neurons

Activation of neuronal receptor tyrosine kinases (RTKs) by growth factors stimulates major signaling pathways required for axon growth including the Ras/extracellular signal-regulated kinase (ERK) and the phosphatidylinositol-3-kinase (PI3K)/Akt pathway. Sprouty2 (Spry2) is an intracellular negative regulator of the Ras/ERK pathway. Recent reports of our group indicated that shRNA against Spry2 promotes elongative axon growth of adult dorsal root ganglia (DRG) neurons. Moreover, DRG neurons obtained from Spry2 global knockout mice also revealed enhancement in axon growth. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is an important endogenous inhibitor of the PI3K/Akt pathway. Knockdown of PTEN increases neurite outgrowth of adult DRG cultures. Since the single inhibition of Spry2 and PTEN promotes axon regeneration, it is the aim of the present study to analyze the effect of PTEN downregulation on axon growth of adult DRG neuron cultures from Spry2 heterozygous and homozygous knockout mice.

DRGs were removed from wild-type (WT), heterozygous Spry2 and homozygous Spry2 deficient mice and dissociated into single neuron cultures. After 2h of plating, neurons were transfected with Accell PTEN siRNA (Dharmacon) and PTEN mRNA and protein levels were analyzed after 24, 48 and 72h by qPCR and immunostaining. Neurons were fixed and processed for βIII-tubulin staining to study the effects of PTEN down regulation on axon growth after 72h.

PTEN siRNA significantly reduced PTEN mRNA expression after 24, 48 and 72h with the strongest downregulation after 72h. As revealed by immunocytochemistry, inhibition of PTEN mRNA translation by PTEN siRNA also significantly reduced PTEN protein levels in a time dependent manner compared to non-targeting siRNA group. The reduction of PTEN protein levels was almost 50% after 72h of siRNA transfection. Endogenous PTEN protein levels of both, Spry2 deficient and WT DRG neurons, were decreased after 48 and 72h in culture. After 72h DRG cultures from Spry2 knockout mice exhibited a branching phenotype. Downregulation of PTEN enhanced axon elongation of Spry2 deficient DRG neurons and reduced axonal branching.

In summary, our data indicate that downregulation of both, Spry2 and PTEN, promotes axon elongation of adult DRG neurons.

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α-synuclein in the retina of a multiple systems atrophy mouse model

Multiple System Atrophy (MSA) has been shown to be associated with visual symptoms. Recently published data immunohistochemical studies have reported that retinal ganglion cells (RGCs) were significantly reduced in MSA patients' peripheral retinae. In our study we aimed to investigate whether such abnormalities can be related to exceeding aggregation of α-synuclein (α-SYN), a 140aa presynaptic protein that exerts toxic function in case of dysregulation and has been shown to be a disease hallmark in Parkinson's disease. We therefore investigated homozygous transgenic mice overexpressing human α-SYN under the proteolipid protein (PLP)-promoter (PLP-α-SYN) compared to wild type (WT) animals of two different age groups (two months, one year). Using RBPMS as a specific global marker for RGCs we found a significant reduction of RGCs in the ventral retina of PLP-a-SYN mice but without pronounced expression of α-SYN in those cells. However stainings of the optic nerve showed that α-SYN signal was approaching the optic nerve/retina junction. The staining was stronger in more distant PLP expressing axonal regions suggesting that α-SYN is taken up into the ganglion cells axon where it might be spreading retrogradly towards the retina. By performing immunohistochemical analyses on vertical retinal sections we discovered that distinct human α-SYN signal occurred also in different retinal cell layers of PLP-α-SYN mice as well as in one specific retinal cell type, but not in WT mice. This is remarkable as the PLP promotor driving the α-SYN expression in oligodendrocytes was previously reported to be inactive in the retina. By using a quantitative PCR approach we are currently testing whether PLP mRNA could still be expressed in the retina, albeit at a much lower level than in the brain, because we did not observe specific PLP staining in PLP-α-SYN retinae. The questions whether ectopic expression of human α-SYN is upregulating endogenous α-SYN expression and/or whether particular types retinal show defective clearance mechanisms will be in the focus of future investigations. A marker against the glial fibrillary acidic protein, however, clearly showed an increased signal in the peripheral retina indicating neuroinflammatory events that might coincide with the pronounced occurrence of α-SYN in this part of the retina. Our findings suggest the PLP-α-SYN mice can serve as interesting biological model to investigate α-SYN spreading mechanism in the retinal pathway.

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Dopaminergic mechanisms in the medial prefrontal cortex in deficient und successful fear extinction

Although exposure-based behavioral therapy (EBT) is an efficacious psychotherapeutic intervention for the treatment of many anxiety- and trauma-related disorders, its success is restricted by the occurrence of fear relapse after initial response. One reason for that is that in patients the central process underlying EBT, namely the formation of a new, fear inhibitory memory in the course of fear extinction, is often impaired. We have recently shown that enhancing dopaminergic signalling by L-DOPA treatment facilitates fear extinction in extinction-deficient 129S1/SvImJ (S1) mice (Whittle et al. 2016). Here, we provide evidence that increasing dopamine availability selectively in the medial prefrontal cortex (mPFC) is sufficient to rescue fear extinction in S1 mice in the long-term suggesting that the mPFC is a critical substrate in mediating extinction-promoting effects of L-DOPA. On the other hand, this finding raises the interesting hypothesis that a dopaminergic dysfunction may contribute to deficient fear extinction. In order to study dopaminergic neurotransmission in extinction-deficient S1 vs. extinction-competent C57Bl6 mice, we combined in-vitro and in-vivo techniques including microdialysis, Multi-electrode array (MEA), qPCR, quantitative receptor autoradiography and functional imaging. We have evidence of a reduced mPFC network response to dopamine in S1 as compared with controls despite similar dopamine release during fear extinction. Overall, these data suggest that in extinction-deficient individuals with dysfunctional mPFC dopaminergic neurotransmission targeted approaches to increase local dopamine is a powerful strategy to overcome extinction resistance and to protect from spontaneous recovery of fear.

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Rewiring of bipolar cells in mouse models for congenital stationary night blindness type 2

Cav1.4 voltage gated L-type calcium channels are predominantly expressed at photoreceptor (PR) terminals at the outer plexiform layer (OPL) and also in bipolar cells. They are localized at specialized ribbon synapses where they allow sustained calcium influx and ensure neurotransmitter release. Mutations in the CACNA1F gene which is encoding Cav1.4 channels are associated with human congenital stationary night blindness type 2 (CSNB2). We compared the retinal morphology of two Cav1.4 mutant mouse models - a gain-of-function Cav1.4 (Cav1.4-IT) and a Cav1.4 deficient (Cav1.4-KO) - with wild-type inbreed controls (WT) (11-14 weeks). Immunostaining with anti-PSD-95 showed that rod spherules were mislocated in the outer nuclear layer (ONL) in Cav1.4-IT mice in contrast to WT where they were located in the OPL and to Cav1.4-KO retinas which totally lacked PSD-95 staining. In Cav1.4.IT mice, some rod bipolar cell (BC) dendrites approached displaced rod PR terminals in the ONL but invaginating contacts were also observed. Mislocated rod PR terminals of Cav1.4-IT retinas contained mostly elongated synaptic ribbons but also mature ribbons. Rod BC sprouting also occured in Cav1.4-KO retinas but their ribbon structures never matured. Differences similar to those seen in two mouse models may also explain subtle variations in the clinical manifestation of human CSNB2. In both mouse models immunostaining with cone BC markers revealed also cone BC sprouting supporting the fact that also the cone pathway is affected in CSNB2. Preliminary analyses showed changes in the morphology of BC axonal ending. Whether this is an effect of the mutant Cav1.4 channels expressed in BC is currently investigated. Rod BC dendrites were lacking in the peripheral retina in both models at the age of 28 weeks, likely due to the absence of PRs in the corresponding area. These data might tell us to approach the disease from a new perspective and investigate whether Cav1.4 mutations can cause a rather progressive, retinitis pigmentosa like retinal phenotype. Funding : ITN-Switchboard 674901 to AK, ES, FWF P26881, P29359 to AK, LFUI and CMBI.

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Functional neuroanatomy of prodynorphin.

Dynorphins (Dyn) and kappa opioid receptors (KOR) are abundantly expressed throughout limbic brain areas and were shown to be involved in the regulation of emotion and stress control. In line with this, the Dyn/KOR system is implicated in the pathophysiology of depression and addiction. Understanding the highly complex organization of the Dyn/KOR system is a prerequisite for potential therapeutic intervention.

To gain deeper insight into the functional neuroanatomy of the Dyn/KOR system, we implemented independent, yet complementary strategies based on restricted pDyn knock-out or pDyn re-expression within the extended amygdala. Such mice were tested in paradigms related to anxiety and stress-coping behaviour and cocaine-induced conditioned place preference.

Stress induced reinstatement of the conditioned place-preference was observed in wild-type animals and several control groups. By contrast, no reinstatement was observed in animals deficient for pDyn in the central amygdala, or neurokinin B-positive neurons. Still these animals re-expressed place preference upon cocaine challenge. Interestingly, no differences in trait anxiety or stress coping behaviour was observed applying standard tests.

Our findings suggest critical involvement of specific populations of dynorphinergic neurons in stress-induced relapse of drug abuse.

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Cellular populations activated by hunger and fear extinction in basolateral amygdala and periaqueductal grey

Anxiety disorders are the most frequent brain disorders imposing a significant burden to affected individuals and the society. Eating disorders, on the other hand, are often associated with altered emotional processing. However, how food intake affects fear and anxiety is not known. The focus of our project is to investigate a possible interaction between hunger and fear and to identify the underlying neuronal circuitries.

We subjected fed and fasted mice to fear conditioning, in which a specific context was paired with an aversive stimulus (foot shock, US), resulting in increased freezing behavior to the context. Prolonged exposure to the context alone without US resulted in fear extinction. To characterize the neuronal ensembles activated by hunger and/or fear extinction, we combined neuronal tract tracing with dual immunohistochemistry for the immediate early gene c-Fos and established markers for neuronal subclasses.

Interestingly, in fasted mice contextual fear extinction was facilitated when compared to non-fasted controls, suggesting a direct relation between feeding and fear. Fasting during extinction increased neuronal activation in several brain areas, including different amygdala nuclei, which are central for fear processing. Furthermore, we found significant activation of periaqueductal grey and raphe nuclei. These brain areas are important for generating an adaptive behavioral response and for synchronization of emotional states with visceral stimuli.

In summary, our experiments identified several brain structures as possible interaction nodes for hunger and fear and created a base for further analysis and manipulation of involved neuronal ensembles.

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Hyperpolarization-activated and Cyclic Nucleotide-Gated Channels in the Mammalian Inner Ear

Hyperpolarization-activated and cyclic-nucleotide-gated (HCN) channels are voltage-gated cationic channels. Unlike other voltage-gated channels that open following membrane depolarization HCN channels do so following hyperpolarization, cyclic nucleotides such as cAMP facilitate activation.

HCN channels play a prominent role in setting the pace of biological oscillations ("pacemaker" channels). In primary auditory neurons a hyperpolarization activated current (Ih) may shorten excitatory postsynaptic potentials (EPSPs) keeping these signals brief and preventing temporal summation. There are four different subunits (HCN1-4) arranged to form homo- or heterotetramers with different biophysical characteristics. Little is known about function and cellular location in the mammalian inner ear, especially in human. We describe the expression pattern and semi-quantitative distribution of HCN isoforms with immunohistochemical techniques in human, three inbred mouse strains, cat and guinea pig inner ear tissue.

HCN2 localizes in the afferent and efferent innervation of the inner and outer hair cells as well as in the membrane of spiral ganglion neurons (SGNs). Likewise HCN1 was present in perisomatic SGN membrane. HCN4 protein resided in the organ of Corti and in the SGNs membrane, with an intense staining in neuronal cell clusters. HCN3 was not detected in human or any other mammalian cochleae. HCN1 and HCN2 showed an age-dependent change in protein expression level.

HCN2 may help setting firing properties due to its high level of expression in neurons and innervation in the organ of Corti. HCN4 could be the most important channel in SGN clusters with a modulating function between the neurons and controlling temporal information important for binaural sound localization with lower frequencies. Fastest kinetics, low modulation effect by cAMP or tyrosine phosphorylation qualifies HCN1 for preserving temporal information and amplitudes in high frequency neurons. Formation of various hetero-tetramers may open possibilities to finetune timing information of EPSPs in SGNs with different spontaneous spiking rates and modulate information of sound signals at the level of the soma and axon initial segment.

The description of different subpopulations of neurons for the human hearing organ is relevant to further analyze pathological changes and deterioration with age and may help to fine tune electrical stimulation with an implant in man.

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Calcium influx and release control where neuromuscular junctions are formed

As the first step of neuromuscular junction (NMJ) formation, muscle fibers are intrinsically pre-patterned for correct motor nerve innervation by clustering acetylcholine receptors (AChRs) in the prospective central synaptic region. This AChR pre-patterning was proposed to require calcium influx through skeletal muscle L-type calcium channel CaV1.1 (DHPR), but not SR calcium release through ryanodine receptors (RyR) (Chen et al., 2011). Here we demonstrate that pre-patterning can be established in mice expressing non-conducting CaV1.1 (DHPRnc/nc) and mice lacking SR calcium release (RyR1-/-). However, by crossing these two mouse lines and thus genetically abolishing both calcium sources, we show that pre-pattering fails and motor nerve branching is excessive in double-mutants (RyR1-/-; DHPRnc/nc), indicating that activity-dependent calcium signaling in developing skeletal muscle is necessary for pre-patterning and sufficient calcium for this process can be supplied by either one of these two sources. Late in fetal development, NMJs are typically located in the muscle center and each muscle fiber contains a single NMJ. However, in mice lacking muscle calcium, CaV1.1-/- and RyR1-/-; DHPRnc/nc mice, NMJs are scattered across the muscle and individual muscle fibers contain up to eight NMJs. Although mice lacking calcium influx show normal NMJ phenotype, mice lacking SR calcium release display collateral overgrow of motor axons and a second wave of NMJ formation in the muscle periphery. These late stage NMJ defects in RyR1-/- mice suggest that although calcium influx through Cav1.1 is sufficient to maintain pre-patterning to the muscle center in early NMJ development, this is not the case in late development. We hypothesized that overgrowth of motor axons and ectopic NMJ formation in RyR1-/- mice are due to the progressive switch of channel splice variants from highly-conducting embryonic CaV1.1e to poorly-conducting adult CaV1.1a during development. To test this hypothesis, we crossed RyR1-/- and CaV1.1ΔE29/ΔE29 mice. In these double-mutants the motor nerve growth territory was narrower and number of peripheral synapses was significantly reduced compared to RyR1-/- mice. Thus, the continued expression of the highly-conducting CaV1.1e splice variant rescued the NMJ phenotype in RyR1-/- mice. Together these data provide genetic evidence that either calcium influx or SR calcium release are necessary and sufficient to confine the patterning of NMJs and the motor axon outgrowth to muscle center.

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Electrically imaging retinal neurons using high-density multi-electrode arrays

Multielectrode arrays (MEAs) have been widely used in the study of neuronal physiologies. The advantages of high sampling rate, multiple recording sites and transparency make it an ideal tool to study not only single neuronal electrophysiology but also the networks of neural tissues. Here with the CMOS-based high-density microelectrode array (CMOS MEAs), we were able to further increase the spatial resolution of the MEAs to a level that enable us to electrically imaging retinal neurons, which will lead us to a better understanding of the networking signal transduction in the retina.

In this study, we adapted isolated healthy C57BL/6 retinae in vertical slices onto poly-l-lysine coated CMOS MEAs with 1 mm2 sensor area and record with 20 k sampling rate. We provided light stimulus with LED setup and record the light response. Recordings from these retinae were further analyze and visualized in the customized python-based software SOMA and Matlab.

In the vertical slices from healthy mice retinae, we were able to electrically image the vertical signal transduction of light response from photoreceptor side to retinal ganglion side. In the same preparation, we also observed the horizontal signal propagation within the vertical slices. With longer light stimulation and pharmacological treatment, we further revealed the ON and OFF pathways separately.

Our results prove that with high-density CMOS MEAs, it is possible to electrically image retinal neurons in healthy retina to reveal the functional circuits. This technology will provide a fast and convenient way for neurological study in the future.

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The 129S1 mouse strain displays robust, extinction-insensitive social fear which is unrelated to social preference.

Fear and anxiety-related disorders are highly prevalent conditions which seriously impair lives of the ones who suffer them. Among them, Social Anxiety Disorder (SAD) is the most prevalent. In this condition, patients are supposed to have difficulties in extinguishing aversive memories related to social experiences. The available treatment options include pharmacotherapy, extinction-based psychotherapy and a combination of both approaches. Nonetheless, the available treatment options seem to be far away from full efficiency. Animal models of SAD are scarce and it would be of great interest to be able to develop new models in order to screen for new therapeutic options. A recently developed rodent behavioral paradigm, the Social Fear Conditioning (SFC) paradigm, allows to measure the development and extinction of aversive memories related to social experiences. The 129S1 mouse strain, which presents cued and contextual fear extinction deficits and is considered as a relevant strain for fear and anxiety-related disorders, may be ideally suited for developing a relevant model of SAD if tested with the SFC paradigm. Therefore, the goal of this preliminary work was to ascertain whether the 129S1 strain develops social fear, its sensitivity to standard extinction procedures and the relationship between social fear and its extinction to social preference. Results show the 129S1 strain develops a robust social fear, which was not sensitive to standard fear extinction procedures, and neither social fear nor (lack of) social fear extinction was related to the previously measured social preference levels. These results prove for the first time that the 129S1 strain develops extinction-insensitive social fear, supporting this could be a useful animal model of SAD. Additionally, the lack of relationship between social preference and social fear or its extinction suggest that SAD-like phenotypes can develop even in the absence of any other overt social impairment.

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Disinhibitory amygdala microcircuits for aversive learning

The basolateral amygdala (BLA) is a cortical-like structure known to be involved in simple forms of emotional learning such as fear conditioning. It is the main entry site for sensory information to the amygdaloid complex and local plasticity in the BLA is crucial for fear memory formation. BLA principal neurons (PN) excitability is under tight control of different subclasses of inhibitory interneurons (IN). However, the contribution of each subtype of IN to sensory processing and fear learning is still poorly understood.

Using a deep brain calcium imaging approach in freely behaving mice, we aim to understand how different IN subtypes control fear learning. We further apply novel intersectional viral tools and optogenetic approaches, together with rabies-based retrograde trans-synaptic tracing to analyse how different IN subgroups interact with each other, and how this disinhibitory interplay could affect plastic changes of BLA PNs and thus gate memory formation.

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Characterization of Novel Bumetanide Derivatives with Improved Pharmacological Profiles for Epilepsy Treatment

Despite the introduction of several novel antiepileptic medications to clinical practice during the last decades, the proportion of temporal lobe epilepsy (TLE) patients not achieving seizure-free status is still considerably high. As deranged cellular chloride homeostasis seems to be involved in disease pathogenesis, bumetanide, a selective antagonist to the Na-K-Cl cotransporter isoform 1 (NKCC1), gains increasing interest as potential therapeutic option. However, bumetanide induces a strong diuretic effect and displays poor penetration across the blood-brain barrier, two adverse features for antiepileptic treatment.

As bumetanide derivatives with increased lipophilicity and reduced diuretic effect are promising compounds to overcome these current treatment limitations, we investigated the anticonvulsant activity as well as effects on anxiety-related behaviour of two novel bumetanide derivatives, CSTS1 and CSTS2, in mice. Neither of the compounds alone altered the threshold of acute seizures induced by tail-vein infusion of the GABAA receptor antagonist pentylenetetrazole (PTZ), but CSTS2 potentiated the anticonvulsant effect of the established antiepileptic drug phenobarbital, which acts as GABA mimetic. Testing both substances in paradigms related to anxiety (open-field, elevated plus maze, light-dark test) did not reveal significant behavioural alterations.

Our data suggest that bumetanide derivatives with improved pharmacological profiles have potential for the treatment of intractable TLE when used in combinatorial drug regimes. In a further study the most promising compound will be evaluated in the kainic-acid (KA)-induced model of TLE.

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Genome-editing induced neural stem cells for therapy of vanishing white matter disease.

Vanishing white matter disease (VWMD) is a fatal genetic leukoencephalopathy caused by mutations in the eukaryotic translation initiation factor 2B (eIF2B). VWMD leads to a failure of proper white matter development and myelination. Both, astrocytes and oligodendrocytes are affected, however, astrocytes seem to be at the base of the disease.

In this study we aim to develop cell based therapy for VWMD. We generated induced neural stem cells (iNSCs) from patient-specific and healthy control fibroblasts. VWMD iNSCs show characteristic properties of neural stem cells such as self-renewal, differentiation potential into neurons and glial cells, and expression of several key markers such as SOX1, SOX2 and PAX6. Single point mutations in the eIF2B gene of iNSCs are currently genetically corrected through CRISPR/Cas9 technology. Recently, we established this technology in iNSCs. This rapid and robust protocol results in more than 30% corrected iNSCs. Efficiency can be further increased by flow cytometry-assisted sorting for transiently expressed fluorophores. Subsequently, monoclonal iNSC lines can be obtained, e.g. by serial dilution assays.

Affected and genome-edited iNSCs as well as control iNSCs will be differentiated in vitro towards the glial fate. Potential deficits in maturation of VWMD patient derived astrocytes might have impact on many processes that are regulated by astrocytes, like oligodendroglial maturation, synaptic transmission and scar formation. In a proof-of-principle study, iNSCs predifferentiated into glial precursor cells will be transplanted into a mouse model of VWMD. This study will provide a preclinical basis to cure patients from rare neurological disorders by transplanting genome-edited reprogrammed cells.

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Microglial alterations in a mouse model of innate hyperanxiety and depression.

Neuroinflammation has not yet been systematically investigated in 'trait' anxiety and depression, but is crucial as this abundant patient population shows increased treatment-resistance, symptom severity and suicide risk. As current anxiolytic drugs are often ineffective and possess a significant side-effect profile and discontinuation rate, environmental interventions have high translational relevance and can advance treatment options. The current project investigates whether a mouse model of innate anxiety and depression (HAB), in comparison to normal anxiety/depression (NAB), shows neuroinflammatory imbalances and normalization of these pathological events in response to successful treatment. HAB and NAB mice were housed in enriched environment (EE) or standard cage for 28d, and cell counts for microglia/myeloid marker 'Iba1', and co-expression of markers 'CD68' or 'CD163', were analysed in various brain regions.

HAB hyperanxiety was significantly correlated with increased Iba1+ cells in the dentate gyrus (DG), compared to NABs. EE attenuated Iba1+ cell count in the DG of HABs toward that of NABs, in association with successful normalization of anxiety behavior. HABs showed increased microglial phagocytosis (CD68+Iba1+) in the amygdala, and EE attenuated this increase. There was a significant increase in microglial alternative activation (CD163+Iba1+) in the DG and amygdala of HABs, which EE reduced specifically in the amygdala. These results suggest that EE-induced anxiolysis may involve modulation of microglial state, in a region-specific manner.

Overall, the data here support the role of neuroinflammation in innate anxiety/depression, and further suggest that microglia could serve as a therapeutic target in some forms of hyperanxiety/depression.

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BMP/SMAD pathway promotes neurogenesis of midbrain dopaminergic neurons in vivo and in human induced pluripotent and neural stem cells

Significant progress has been made in the generation of stem cell-derived midbrain dopaminergic (mDA) neurons owing to their importance for modeling of Parkinson's disease, drug screening and cell replacement therapy. However, essential parameters are still not fully controllable including consistency between experiments, phenotypic identity of progenitors and purity of mDA neurons. Progress in the ability to determine these parameters are essential, since they are directly linked to graft outcome, dyskinesia side effects and tumour formation after transplantation. Current in vitro differentiation protocols are based on the activation of the three signalling pathways, SHH, WNT and FGF, which regulate the formation of mammalian mDA neurons in vivo. Therefore, the discovery of additional signalling pathways that determine mDA development in vivo could critically advance the abilities to manipulate in vitro conditions to achieve desired outcomes.

Bone Morphogenetic Proteins (BMPs) belong to the transforming growth factor beta superfamily. Phosphorylated SMAD 1, -5 and -8, are the major intracellular BMP signalling pathway components. The BMP/SMAD pathway regulates a wide array of neurodevelopmental processes, including progenitor proliferation, apoptosis and differentiation. A significant progress in the directed neural differentiation of human pluripotent stem cells was the discovery that blocking the BMP/SMAD pathway in initial steps of the protocol lead to highly efficient neural conversion. However, the role of BMPs during later stages of mDA specification and maturation in vitro is unclear. Similarly, the in vivo role of BMP/SMAD signalling in the formation of mammalian mDA neurons is unknown. In the current study, we investigated the function of BMP5/6/7 and SMAD1 in the formation of mDA neurons in vivo. Moreover, we explored the potential of BMP5/7 in the directed differentiation of human stem cells to mDA neurons.

We identified BMP/SMAD signalling as novel essential pathway regulating the development of mammalian mDA neurons in vivo and provided insights into the molecular mechanisms of this process. Importantly, BMP5/7 increased robustly the differentiation of human induced pluripotent and induced neural stem cells to mDA neurons.

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Plasticity of Amygdala Intercalated Cell Microcircuits in Fear Learning

The amygdala plays a crucial role in attaching emotional significance to environmental cues. Its intercalated cell masses (ITC) are tight clusters of GABAergic neurons, which are distributed around the basolateral amygdala complex (BLA), and appear to be involved in the acquisition as well as extinction of conditioned fear responses. As their ablation results in a deficit of the expression of fear extinction, which is associated with anxiety disorders, ITC have been the subject of intense investigations. The aim of our study is to characterize neuron subtypes and plasticity properties of pre- and postsynaptic partners of ITC neurons in the medial paracapsular cluster (mpITC). Tracing of recorded and filled mpITC neurons has confirmed a variety of postsynaptic partners, with axons projecting not only to the main ITC, but also to the BLA, striatum and central amygdala. A detergent-digested freeze-fracture replica labelling approach was used to elucidate the spatial distribution as well as density of ionotropic glutamatergic receptors from thalamic inputs within postsynaptic specializations. Our results indicate that AMPA receptors are homogeneously distributed and show a positive correlation with the synaptic area. In contrast to these findings, NMDA receptors are inhomogeneously distributed within the postsynaptic area of mpITC neurons and vary highly in their density, with a positive correlation only onto mpITC spines. Currently, we investigate whether changes in AMPA and NMDA distribution and density correlate with functional synaptic changes observed during different fear states, and define neuron types of postsynaptic partners in target regions of the mpITC. Together, our results further a circuit-based understanding of how ITC activity can contribute to high and low fear states. Funded by grants from the FWF (F.F.) and DFG (I.E.)

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Genetic targeting of the canonical Wnt signaling pathway in human neural stem cells

The three identified Wnt signaling pathways are essential signal transduction pathways in embryonic development, involved in the regulation of cell fate specification, proliferation, cell migration and axis formation. It has been previously shown that activation of Wnt-signaling accelerates proliferation and supports maintenance of pluripotency in pluripotent stem cells. Moreover, it has been demonstrated that depletion of c-Myc and N-Myc, the major Wnt target genes, causes biosynthetic dormancy in pluripotent stem cells, in which cells keep their differentiation potential, but stop proliferation.

This phenotype appears to be conserved in hematopoetic stem cells, however, the role of Wnt/b-catenin signaling in neural stem cells is poorly investigated. Here we assess the impact of canonical Wnt signaling on neural stem cell proliferation by generating a functional knockout of the b-catenin-destruction-complex component APC employing CRISPR/Cas genome editing technology directly in neural stem cells. For control we use pharmacological inhibition of Wnt-signaling by small molecules in mutant and control cells. We used a double gRNA strategy to induce two double strand breaks in the APC-gene, resulting in a small deletion and frameshift. Transfection of NSCs with Nucleofector™ technology resulted in isolation of 12 clones currently analyzed.

Since self renewal of NSCs is dependent on continuous application of Wnt agonists we aimed at selecting APC-deficient colonies using Wnt-less media. We show that a loss of function mutation in the APC gene seems sufficient to sustain self renewal in NSCs in Wnt-agonist-free conditions. Further analyses regarding differentiation potential, cell cycle regulation and proliferation are currently carried out.

A deeper understanding of Wnt-signaling in NSCs might allow for more stable cultivation and improvement of (existing) differentiation protocols for use in disease modelling and on the way to therapeutic use of patient-specific neural stem cells.

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Assessment of maturation and functionality of human stem cell-derived neurons

The reprogramming of patients' skin cells to either induced pluripotent stem (iPS) or induced neural stem cells (iNSCs), followed by in vitro differentiation, serves as a model for human-specific disease modelling. In order to study neurodegenerative diseases, it is crucial to confirm that stem cell-derived neurons display physiological functionality. This can be assessed through the study of voltage-gated calcium channels (CaVs), which are involved in the regulation of various neuronal functions in the brain, such as postsynaptic signal integration and neuronal plasticity.

The aim of this study was to analyze maturation and functionality of artificial neurons derived from different stem cell sources (fetal, iPS-derived and iNSCs) by means of dendritic spine morphology, synapse formation, electrophysiological characteristics, and Cav subunit expression.

CaV subunit expression was investigated using qPCR to generate an expression profiling over the course of differentiation from iPSCs to cortical. Moreover, functionality of calcium channels was assessed by live cell calcium imaging and microelectrode array recordings. Immunostaining and super high resolution microscopy served to study synapse formation by partial co-localization of pre- and post-synaptic markers and dendritic spine morpholog.

We observed that maturation characteristics, such as synapse as well as dendritic spine formation, increase with time in culture and correlate with the developmental stage of the neural stem cell sources. We expect that the modification of Cav expression could counteract disturbances as they occur in several diseases and could constitute a basic approach for therapeutic treatment of neurodegenerative disorders.

This study provides the first comprehensive calcium channel expression profile of human iPS/iNS-derived neurons and allows insights into the impact of calcium signaling on functionality of artificially derived human neurons.

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

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.a. ITCs and central amygdala), suggesting a substantial influence of DAergic signaling on amygdaloid information processing and output.

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. 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.

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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Independent effects of kidney function and cholesterol efflux on cardiovascular mortality

Background

Impaired renal function is associated with cardiovascular and all-cause mortality. In the general population, HDL-cholesterol is associated with cardiovascular events, which is not true in patients with chronic kidney disease (CKD). This has been attributed to abnormal HDL function in CKD.

Methods

In this study, we analyzed cholesterol efflux capacity as one of the major HDL functions in 2469 patients of the Ludwigshafen Risk and Cardiovascular Health Study who all underwent coronary angiography. Patients were followed for a median of 9.9 years. Primary and secondary end points were cardiovascular and all-cause mortality, respectively.

Results

We found a strong association between cholesterol efflux capacity and kidney function. Cholesterol efflux capacity was associated with cardiovascular mortality, with the highest hazard ratio in the lowest quartile of cholesterol efflux. A genetic score of 53 SNPs associated with GRF and the uromodulin SNP rs12917707 was correlated inversely with cholesterol efflux. However, adjustment for eGFR and uromodulin as markers of kidney function did not affect the relationship between cholesterol efflux and mortality.

Conclusions

We are suggesting that impaired renal function lowers cholesterol efflux, but that this is not mediating the effects of impaired kidney function on cardiovascular mortality. Other mechanisms of low cellular cholesterol efflux may causally be involved in adverse cardiovascular outcomes.

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