

9th SFB-Symposium 2016

SFB 35 Transmembrane Transporters in Health and Disease
Scientific Sessions and Postersession

August 31 - September 2, 2016

Great Lecture Hall, University Clinic of Dentistry
Sensengasse 2a, 1090 Vienna

The Number of Participants is limited to 160.
We will award Student Travel Grants!

Randy Blakely, US
Piet Borst, NL
Olga Boudker, US
Ellen Closs, DE
Gerhard Ecker, AT
Michael Freissmuth, AT
Bruno Giros, CAN
Michael Gottesman, US

Baruch Kanner, IL
Karl Kuchler, AT
Claus Loland, DK
Hartmut Luecke, USA
Haley Melikian, US
Gary W. Miller, US
Amy Newman, US
Poul Nissen, DK

Gaia Novarino, AT
Manuel Palacin, E
Christian Piffl, AT
Walter Sandtner, AT
Harald Sitte, AT
Dirk Slotboom, NL
Thomas Stockner, AT
Kristian Strømgaard, DK

Kazumitsu Ueda, JP
Robert Vandenberg, AUS
Roxanne Vaughan, US
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Poster Presentations

Wednesday, August 31 / LUNCH
Thursday, September 1/ LUNCH
Friday, September 2

EVEN poster numbers
ODD poster numbers
free poster viewing

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Scientific Program

Wednesday, August 31, 2016

10:00 Registration

10:55 **Opening Remarks** (Harald Sitte, Medical University Vienna)

11:10 Session 1: Neurotransmitter transporters

(Chair: Margaret E. Gnegy, University of Michigan, USA)

11:15 Speaker 1: **Amy Newman** (National Institute of Drug Abuse, USA)
Translating the Atypical Dopamine Uptake Inhibitor Hypothesis from Structure to Function

11:40 Speaker 2: **Gaia Novarino** (Institute of Science and Technology, Austria)
Branched chain amino acid transport at the blood brain barrier: what for?

12:05 Speaker 3: **Harald Sitte** (Medical University Vienna, Austria)
Organic cation transporter 3: An unsuspected player in the actions of psychostimulant drugs

12:30 Lunch / Poster Session I

14:45 Session 2: ABC transporters

(Chair: Balazs Sarkadi, Hungarian Academy of Sciences, Hungary)

14:50 Speaker 1: **Karl Kuchler** (Medical University Vienna, Austria)
A structural model of human ABCG2 suggests a possible mechanism for drug export by a mammalian ABC drug efflux transporters

15:15 Speaker 2: **Kazumitsu Ueda** (Kyoto University, Japan)
Mechanism of action of ABCA1 and ABCB1 (MDR1)

15:40 Speaker 3: **Thomas Stockner** (Medical University of Vienna, Austria)
Do nucleotides control the conformation of the NDB dimer?

16:05 Coffee/Tea break

16:35 Session 3: Regulation of transporters

(Chair: Ulrik Gether, University of Copenhagen, Denmark)

16:40 Speaker 1: **Michael Freissmuth** (Medical University of Vienna, Austria)
Sleepless in Vienna: how to rescue a folding-deficient mutant dopamine transporter of Drosophila melanogaster by pharmacochaperoning

17:05 Speaker 2: **Haley Melikian** (UMASS Medical School, USA)
Dopamine Transporter Trafficking: From Molecules to Behavior

17:30 Speaker 3: **Roxanne Vaughan** (University of North Dakota, USA)
Reciprocal phosphorylation and palmitoylation regulate dopamine transporter kinetics and membrane mobility

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17:55 **Plenary Lecture 1:**

(Chair: Jeffrey Rothstein (Johns Hopkins School of Medicine, USA))

18:00 Plenary Speaker: **Mark. H. Wightman** (University of North Caroline, USA)

Monitoring the dynamics of monoamine transporters

19:30 **Reception Townhall of Vienna / Dinner**

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Thursday, September 1

09:00 Registration

09:30 Session 4: Amino Acid Transport

(Chair: Dimitrios Fotiadis, University of Bern, Switzerland)

09:35 Speaker 1: **Ellen Closs** (Johannes Gutenberg University Mainz, Germany)

Role of cationic amino acid transporters in human immune and tumor cells

10:00 Speaker 2: **Francois Verrey** (University Zurich, Switzerland)

Transporter cooperation for amino acid homeostasis

10:25 Speaker 3: **Manuel Palacin** (IRB Institute for Research in Biomedicine, Barcelona, Spain)

The structure of Heteromeric Amino acid Transporters: getting there

10:50 Coffee/Tea break

11:20 Plenary lecture 2:

(Chair: Gerhard Schütz, Technical University of Vienna, Austria)

11:25 Plenary Speaker: **Randy Blakely** (Vanderbilt Kennedy Center, Nashville, USA)

Mining Immune Serotonin Crosstalk for Insights into Novel Therapeutics in Autism

12:25 Lunch / Poster Session II

14:30 Session 5: Serotonin transporters and ancestors

(Chair: Steffen Sinning, Aarhus University, Denmark)

14:35 Speaker 2: **Kristian Strømgaard** (University of Copenhagen, Denmark)

Examining inhibitor and substrate binding to the human serotonin transporter

15:00 Speaker 3: **Claus Loland** (University of Copenhagen, Denmark)

Direct assessment of conformational changes uncovers functional regulation by K⁺ of a neurotransmitter:sodium symporter

15:25 Speaker 1: **Walter Sandtner** (Medical University Vienna, Austria)

Voltage dependent K⁺ binding pushes the catalytic cycle of the human Serotonin transporter into the forward direction

15:50 Coffee/Tea break

16:20 Session 6: Vesicular Transport

(Chair: Oliver Kudlacek, Medical University Vienna, Austria)

16:25 Speaker 1: **Bruno Giros** (University Quebec, Canada)

Deciphering the Functional role of the Vesicular Monoamine Transporter in defined monoaminergic systems

16:50 Speaker 2: **Christian Pifl** (Medical University Vienna, Austria)

Compromised vesicular dopamine transport activity in Parkinson disease

17:15 Speaker 3: **Gary Miller** (Emory University, USA)

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A role for the synaptic glycoprotein 2C (SV2C) in dopamine neurotransmission

17:40 **Plenary Lecture 3:**

(Chair: Peter Chiba, Medical University of Vienna, Austria)

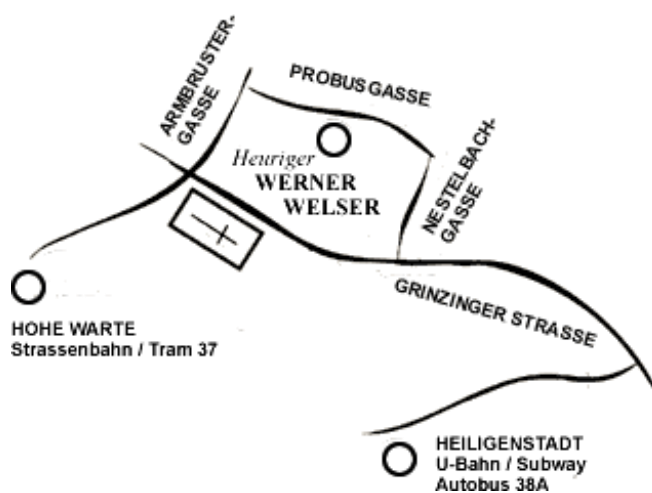
17:45 Plenary Speaker: **Dirk Slotboom** (University of Groningen, Netherlands)

Mechanisms of cobalamin uptake

19:30 **Heurigen Restaurant "Ing. Werner Welser"**

Probusgasse 12, 19th district Vienna, Tel: +43 (0) 1 318 97 97

Public transport: Please take tram line 37 from "Schottentor". Get off at the end station "Hohe Warte". 5 minutes walk to Probusgasse.



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Friday, September 2

08:30 Registration

08:55 Plenary lecture 4:

(Chair: Gergely Szakacs, Medical University Vienna, Austria)

09:00 Plenary Speaker: **Michael Gottesman** (National Cancer Inst, Bethesda, USA)

Exploring the Complexity of Multidrug Resistance in Cancer

10:00 Coffee/Tea break

10:30 Session 7: Glycine & Glutamate transporters

(Chair: Elena Bossi, University of Insubria, Italy)

10:35 Speaker 1: **Robert Vandenberg** (University of Sydney, Australia)

An Allosteric Mechanism for Lipid inhibition of Glycine Transporter 2

11:00 Speaker 2: **Olga Boudker** (Cornell University, USA)

Linking structure, dynamics and function in a glutamate transporter homologue

11:25 Speaker 3: **Baruch Kanner** (The Hebrew University of Jerusalem, Israel)

Structure and Function of Excitatory Amino Acid Transporters

11:50 Session 8: Poster Bullets

(Chair: Sonja Sucic, Medical University Vienna)

11:55 3 Short Talks of Selected Poster Presenters (12' + 3')

12:40 Lunch / Poster Session III

14:05 Plenary lecture 5:

(Chair: Gary Rudnick: Yale University, USA)

14:10 Plenary Speaker: **Poul Nissen** (Aarhus University, Denmark)

Completing the NSS transport cycle - structure of an occluded return state of LeuT

14:55 Session 9: Computational

(Chair: Ivet Bahar, University of Pittsburgh, USA)

15:00 Speaker 1: **Harel Weinstein** (Cornell University, USA)

Tales of tails and membranes in the dynamics of transporter mechanisms

15:25 Speaker 2: **Gerhard F. Ecker** (Medical University of Vienna, Austria)

The power of linked open data - drug transporter profiling and beyond

15:50 Speaker 3: **Hartmut Luecke** (University of California, USA)

Structure, function, and inhibitors of the acid-gated Helicobacter pylori urea channel, an essential component for acid survival and chronic infection

16:15 Coffee/Tea break

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16:45 **Plenary lecture 6:**

(Chair: Bruno Stieger, University Hospital Zurich, Switzerland)

16:50 Plenary Speaker: **Piet Borst** (Netherlands Cancer Institute, Netherlands)

Pump and circumstance; drug-transporting ABC-transporters in health and disease

17:50 **Closing Remarks, End of meeting:**

Harald Sitte (Medical University of Vienna, Austria)

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Plenary Lecture

PL-01 Monitoring the dynamics of monoamine transporters

R. Mark Wightman

University of North Carolina, USA

Carbon fiber microelectrodes enable the concentration dynamics of monoamine to be tracked in the intact brain. Research has shown that the instantaneous concentration of these neurotransmitters is established by the balance between release and uptake. This was first unequivocally established for dopamine in the dopamine transporter knock-out mice generated in Marc Caron's laboratory. Subsequent research has shown that a similar balance exists for both the noradrenergic system and the serotonergic system. In all three monoamine systems, autoreceptor antagonists tend to increase extracellular monoamines as the inhibitors of the specific transporters. Despite these similarities, each monoamine system has unique components that regulate their specific activities.

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Plenary Lecture

PL-02 Mining Immune Serotonin Crosstalk for Insights into Novel Therapeutics in Autism

Randy Blakely

Vanderbilt Kennedy Center, Nashville, USA

Autism spectrum disorder (ASD) has been associated with changes in both serotonin (5-HT) homeostasis and elevated immune system activation, though the connections between the two have not been mechanistically linked.

Previously (Sutcliffe et al, 2005), we identified five rare coding variants in the presynaptic 5-HT transporter (SERT) and demonstrated that all five conferred elevated 5-HT uptake activity in subject lymphoblasts and transfected cells, either through enhanced transporter surface expression or elevated transport function. Independently, studies in our lab identified PKG and p38 MAPK linked pathways that could drive elevated 5-HT uptake of wildtype human and rodent SERT.

Additionally, we have found that a p38 MAPK pathway can be triggered in cells and mouse brain preparations via immune system activation, specifically involving the inflammatory cytokine IL-1 β . That these two lines of research were possibly related became apparent when we found that the most common of the ASD-associated SERT coding variants, Ala56, lacked sensitivity to both PKG and p38 MAPK activation, and displayed p38 MAPK-dependent hyperphosphorylation.

These findings support the hypothesis that the mutant transporter is locked in a state normally reserved for the transiently activated wildtype protein upon PKG/p38 MAPK activation. Subsequent studies with the SERT Ala56 knock-in mouse model (Veenstra-VanderWeele et al, 2012) revealed hyperserotonemia, elevated in vivo SERT activity, hypersensitive 5-HT_{1A} and 5-HT_{2A} receptors, elevated p38 MAPK-dependent SERT phosphorylation and behavioral changes suggestive of ASD-like perturbations. Additionally, 5-HT neuron-specific conditional elimination of p38 α MAPK (Baganz et al, 2015) eliminates the ability of innate immune system activation with LPS to increase SERT activity. In my presentation, I will review these studies and discuss how the dialog between inflammatory signaling pathways and SERT, specifically linked to IL-1 β /p38 α MAPK, may be modulated pharmacologically for therapeutic benefit in developmental and adult onset disorders linked to perturbed 5-HT signaling, including ASD, OCD and depression.

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Plenary Lecture

PL-03 Mechanisms of cobalamin uptake

Dirk J. Slotboom

University of Groningen, The Netherlands

All naturally produced cobalamin (vitamin B12) originates from a handful of bacterial species that have the capacity to synthesize the tremendously complex compound *de novo*, a process of approximately 30 steps. The large majority of bacteria cannot synthesize cobalamin even though many of them depend on the co-factor for survival. These auxotrophs must take up cobalamin from the environment. The ABC-importer BtuCD-F from *E. coli* is the only identified and characterized prokaryotic cobalamin transporter, but it is absent from many cobalamin auxotrophs. I will present the identification and characterization of alternative cobalamin transporters, and reveal an astonishing diversity in transporter types and mechanisms. One of the cobalamin transporters is the Energy Coupling Factor (ECF)-type ABC transporter CbrTUV. I will describe the transport mechanism used by ECF transporters in detail as it has more general implications for our understanding of membrane transporters.

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Plenary Lecture

PL-04 Exploring the Complexity of Multidrug Resistance in Cancer

Michael M. Gottesman

National Cancer Institute/NIH, USA

Our laboratory has been seeking molecular mechanisms to account for why some cancers are intrinsically resistant to anti-cancer drugs or acquire resistance to such drugs. Our own work and a review of the literature indicates that a variety of mechanisms play a role in such resistance including target alterations and bypass mutations, alterations in cellular pharmacology, such as expression of energy-dependent efflux pumps (ABC transporters), global changes in gene transcription such as occur during cell differentiation (e.g., EMT), and local cancer cell interactions with the microenvironment. Our own work has focused on the role of ABC transporters, such as ABCB1 (P-glycoprotein). Progress has been made based on structure-function studies in understanding how the multidrug efflux pumps are able to recognize so many different substrates, and in developing approaches to target P-glycoprotein for the treatment of multidrug resistant cancers. Current efforts include the development of alternatives to classical cell culture techniques to develop laboratory-based assays for the study of more complex mechanisms of drug resistance in cancer.

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Plenary Lecture

PL-05 Completing the NSS transport cycle - structure of an occluded return state of LeuT

Poul Nissen

Aarhus University, Dept. Molecular Biology and Genetics, Denmark

NSS structures obtained so far (LeuT, MhsT, dDAT, hSERT) represent intermediates of Na⁺/substrate, Na⁺/inhibitor binding or intracellular release, but not the inward-to-outward return transition. We have obtained crystal structures of wild-type *Aquifex aeolicus* LeuT in an outward-facing, Na⁺- and substrate-free state likely to be H⁺-occluded. We find a remarkable rotation of the completely conserved Leu25 into the empty substrate-binding pocket and rearrangements of the empty Na⁺ sites. Mutational studies of the equivalent Leu99 in the human serotonin transporter show a critical role of this residue on the transport rate. Molecular dynamics simulations show that extracellular Na⁺ is blocked unless Leu25 is rotated out of the substrate-binding pocket. We propose that Leu25 facilitates the inward-to-outward transition by compensating a Na⁺- and substrate-free state and acts as the gatekeeper for Na⁺ binding that prevents leak in inward-outward return transitions

Malinauskaite L, Said S, Sahin C, Grouleff J, Shahsavar A, Bjerregaard H, Noer P, Severinsen K, Boesen T, Schiøtt B, Sinning S, Nissen P (2016). A Conserved Leucine Occupies the Empty Substrate Site of LeuT in the Na⁺-free Return State. *Nature Comm.* **7**, 11673

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Plenary Lecture

PL-06 Pump and circumstance; drug-transporting ABC-transporters in health and disease Piet Borst

Netherlands Cancer Institute, Amsterdam, Netherlands (p.borst@nki.nl)

Cells are separated from their surroundings by a lipid membrane and their communication with the outside world requires proteins that can shuttle compounds through the membrane. In recent years we have studied 2 types of membrane proteins in Amsterdam: a channel, VRAC, that promotes uptake of the anti-cancer drugs cisplatin and carboplatin; and ABC-transporters of the Multi-Drug Resistance-associated Protein (MRP, ABCC) family. In my talk I shall focus on ABCC5 and 6 and notably why the absence of ABCC6 in the liver results in the generalized calcification disease Pseudoxanthoma elasticum.

References:

1. Jansen, R.S., et al., ABCC6 prevents ectopic mineralization seen in pseudoxanthoma elasticum by inducing cellular nucleotide release. *Proc Natl Acad Sci* 2013. 110(50): p. 20206-11.
2. Jansen R, A.R., R, Fish A, Mahakena S, Bleijerveld Altelaar M, L, Wanders R, Borst P. and Van de Wetering, N-lactoyl-amino acids are ubiquitous metabolites that originate from CNDP2-mediated reverse proteolysis of lactate and amino acids. *Proceedings of the National Academy Sciences of the United States of America*, 2015. 112(21): p. 6601 - 6.
3. Jansen, R.S., et al., ATP-binding Cassette Sub-family C Member 5 (ABCC5) Functions as an Efflux Transporter of Glutamate Conjugates and Analogs. *J Biol Chem.* 2015. 290:30429

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Session 1

S1-01 Translating the Atypical Dopamine Uptake Inhibitor Hypothesis from Structure to Function

Amy Newman

NIDA-Intramural Research Program, NIH, USA

The primary mechanism underlying the stimulant actions and abuse liability of cocaine and methamphetamine is binding to the dopamine transporter (DAT), wherein cocaine inhibits dopamine reuptake and methamphetamine serves as a substrate to ultimately release dopamine via the vesicular monoamine transporter and reverse efflux at the DAT. As such the DAT has been a primary target for psychostimulant abuse/addiction medication discovery. However predicted addictive liability and limited clinical evaluation has provided a formidable challenge for development of these agents for human use. The atypical (non-addictive) pharmacological profile of the benzotropine class of dopamine uptake inhibitors, both at the molecular level (Loland, 2008; Beuming, 2008) and in preclinical models of cocaine abuse (Newman and Katz 2009, for review), has encouraged further development of these agents. However, the inability to translate these preclinical findings into humans has thus far precluded testing the atypical dopamine uptake inhibitor hypothesis. R-Modafinil (armodafinil) is a clinically available drug used for treatment of narcolepsy and binds to the dopamine transporter, displaying a unique pharmacological profile (Cao, 2011; Loland 2012). The design and synthesis of novel modafinil analogues have provided additional tools that are currently being used to further investigate R-Modafinil's mechanism(s) of action that contribute to its therapeutic effects. Preclinical studies suggest that its abuse liability will be low and further support its clinical investigation for treatment of cocaine and methamphetamine abuse. As R-modafinil is available for human use, translation of the atypical DAT inhibitor hypothesis may be realized. Nevertheless, drug development efforts to further elucidate mechanistic underpinnings of R-modafinil have identified several high affinity DAT inhibitors that share the atypical DAT inhibitor profile and look highly promising in preclinical studies for treatment of cocaine and/or methamphetamine abuse disorders. Several novel drug candidates are currently being developed toward this therapeutic endpoint. Behavioral profiles and unique binding interactions at the DAT appear to be related providing the opportunity to develop novel and non-addictive DAT inhibitors for the treatment of psychostimulant abuse.

S1-02 Branched chain amino acid transport at the blood brain barrier: what for?

Gaia Novarino

IST, Austria

Autism spectrum disorders (ASD) are a group of genetic disorders often overlapping with other neurological conditions. Despite seemingly heterogeneous the great number of genes implicated in ASD converges along few biological pathways. We previously described abnormalities in the branched chain amino acid (BCAA) catabolic pathway as a cause of ASD. Here we show that the *solute carrier transporter 7a5* (*SLC7A5*), a large neutral amino acid transporter localized at the blood brain barrier (BBB), has an essential role in maintaining brain BCAA levels within a normal range. In mice, deletion of *Slc7a5* from the endothelial cells of the BBB leads to decreased levels of brain BCAA, abnormal cap-dependent translation and severe neurological abnormalities.

S1-03 Organic cation transporter 3: An unsuspected player in the actions of psychostimulant drugs

Harald H. Sitte

Medical University Vienna, Austria

Harald H. Sitte, Felix Mayer, Diethart Schmid, Anthony Owens, Georgianna G. Gould, Isabella Salzer, Stefan Böhm, Peter Chiba, Piper Williams, Mia Apuschkin, Hsiao-Huei Wu, Ulrik Gether, Wouter Koek and Lynette C. Daws

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Medications used to treat psychiatric disorders and psychostimulant abuse often fail to provide therapeutic benefit. For example, selective serotonin reuptake inhibitors (SSRIs), which target the serotonin transporter (SERT) and are the frontline treatment for depression, do not provide symptom relief for the majority of patients. In the case of psychostimulants such as amphetamine, which target the dopamine transporter (DAT), strategies targeting DAT have yielded little to no benefit in treating abuse of these drugs. A rapidly growing literature supports a prominent role for organic cation transporters, particularly the organic cation transporter 3 (OCT3) in regulating biogenic amine neurotransmission. Here we discuss recent findings pointing to these transporters as important players modulating the effects of psychostimulants.

We obtained functional measures of transporter activity using uptake assays in cell-based systems *in vitro* by radioactive and fluorescent ligand flux measurements; we determined monoamine clearance *in vivo* by high-speed chronoamperometry. We examined transporter phosphorylation sites by mass spectrometric analysis. Locomotor activity was assessed using beam break measurements. Radioligand binding and western blotting using specific antibodies were used to examine transporter expression.

The lecture will discuss results that point to OCT3 as a promising target for the development of novel drugs with improved therapeutic outcome for psychostimulant abuse.

These studies were supported by FWF/Austrian Science Fund grant F3506 & W1232 (HHS), DOC stipend to FM, NIH R01MH093320 (LCD and WK), MH64489 (LCD) and MH106978 (LCD).

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Session 2

S2-01 A structural model of human ABCG2 suggests a possible mechanism for drug export by a mammalian ABC drug efflux transporters

Narakorn Khunweeraphong¹, Thomas Stockner², Karl Kuchler³

¹ Medical University Vienna, Austria

² Institute of Pharmacology, Medical University of Vienna, Austria

³ Medical University of Vienna, Max F. Perutz Laboratories, Dr. Bohr-Gasse 9/2, 1030 Vienna, Austria

ATP-binding cassette (ABC) proteins form one of the largest transport protein families with several thousand genes operating in all living cells from bacteria to yeast, and of course humans. Notably, eukaryotic ABC transporters mediate anticancer drug resistance, limit efficacies of anti-infective therapies, and several human genes are also intimately connected to prominent genetic diseases. Although the domain organization of ABC proteins is conserved in evolution, the molecular basis for their broad yet distinct drug substrate specificities and their catalytic cycle has remained an unsolved mystery. At present, we even have a limited molecular understanding of their structure or transport mechanisms, which is, however, a mandatory prerequisite to combat ABC-mediated diseases or drug resistance phenomena, respectively.

The phenomenon of multidrug resistance often observed in cancer cells is arguably conferred by membrane transport proteins, including among others P-glycoprotein, BCRP/ABCG2 or MRP. We have thus pursued comparative structural modeling analysis of the human ABCG2 efflux transporter with the aim to discover mechanistic aspects of drug recognition and transport. We used the coordinates of the ABCG5/G8 crystal structure to generate a high resolution homology model of the human ABCG2 transporter implicated in anti-cancer resistance as well as in genetic disorders such as gout. Moreover, we have carried out extensive molecular-genetic structure function analysis and validation of the predictions deduced from the model. The iterative cycle of modeling and mutational analysis yielded data that suggest a possible transport mechanism used by ABCG2 for drug extrusion and may explain the broad substrate specificity. In my talk, I will present the refined and validated ABCG2 homology model, and discuss the possible transport mechanism which may be generally applicable for medically relevant mammalian ABC transporters.

Our work is supported by a grant from the Austrian Science Foundation (SFB035-20).

S2-02 Mechanism of action of ABCA1 and ABCB1 (MDR1)

Kazumitsu Ueda

Kyoto University, Japan

ABCA1 and apoA-I are essential for nascent HDL (nHDL) formation. However, the mechanism is still controversial. nHDL is a discoidal molecule composed of 2-4 molecules of apoA-I and several hundreds of phospholipids, mainly phosphatidylcholine (PC), and cholesterol. Because ABCA1 is a transporter which moves its substrate one by one in an ATP-dependent manner, it is hard to imagine that ABCA1 can directly generate such a large complex.

ABCB1 (MDR1) exports a wide variety of hydrophobic compounds. It is not yet clear how ABCB1 recognizes a wide variety of substrates and how ABCB1 exports them out of cells. ABCB4 (MDR2), highly homologous to ABCB1, exports PC and cholesterol in the presence of bile salts. Interestingly, ABCA1 also exports PC and cholesterol in the presence of bile salts. ABCA1, ABCB1 and ABCB4 are inhibited by the same inhibitors. Therefore, the mechanisms of action of ABCA1 and ABCB1 (MDR1) are expected to be similar. The mechanism of human ABC proteins will be discussed.

References

- 1) Ueda K et al. Expression of a full-length cDNA for the human MDR1 gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc. Natl. Acad. Sci. USA*, 84: 3004-3008 (1987)

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- 2) Nagao K ATP hydrolysis-dependent conformational changes in the extracellular domain of ABCA1 are associated with apoA-I binding. *J. Lipid Res.* 53, 126-136 (2012)
- 3) Nagao K et al. Cyclosporine A and PSC833 inhibit ABCA1 function via direct binding. *Biochim Biophys Acta*, 1831, 398-406 (2013)
- 4) Nagata K O et al. ABCA1 dimer-monomer interconversion during HDL generation revealed by single-molecule imaging. *Proc. Natl. Acad. Sci. USA*, 110, 5034-5039 (2013)
- 5) Kodan A et al. Structural Basis for Gating Mechanisms of a Eukaryotic P-glycoprotein Homolog. *Proc Natl Acad Sci U S A.* 111, 4049-4054 (2014)

S2-03 How nucleotides control the conformation of the motor domains of ABC transporters

Daniel Szöllösi¹, Yaprak Doenmez-Cakil¹, Imran Sohail², Gabor Szaloki³, Katalina K. Goda³, Gergely Szakacs⁴, Peter Chiba², **Thomas Stockner**¹

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⁴ Institute for Cancer Research, Medical University of Vienna, Borschkegasse 8A, 1090 Vienna, Austria, Institute of Enzymology, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Magyar Tudósok körútja 2, H-1117 Budapest, Hungary

The human genome code for 44 genes of ABC membrane transporter. At least 12 of them have been causally related to disease. The multidrug resistance transporter P-glycoprotein (ABCB1) is expressed in barrier tissues and transports an extraordinarily diverse range of structurally unrelated drugs, xenobiotics and endogenous substrates. The bile salt export pump BSEP (ABCB11) shows high sequence homology to ABCB1, but its expression is limited to the canalicular membrane of hepatocytes. It has a narrow substrate specificity, transporting essentially only bile salts. The bacterial transporter Sav1866 was the first exporter to be structurally resolved. ABC exporters have now been crystallized in several conformations, but uncertainty remains regarding physiological conformations, because not all biochemical evidence can be explained by crystal structures.

Several models of the transport cycle have been proposed and it is well established that binding and subsequent hydrolysis of ATP drives substrate transport. However, details of the mechanism remain enigmatic. Two ATP molecules bind at the two nucleotide binding sites (NBS) at the interface of the two nucleotide binding domains (NBD). ABCB1 has two canonical NBS, while ABCB11 (49% sequence identity to ABCB1) has only one NBS that harvest energy from significant ATP hydrolysis rates.

We made use of similarity and differences of the two active transporters ABCB1 and ABCB11 to identify how the chemical energy stored in ATP is converted into conformational motion that ultimately result in directional substrate transport. We find that canonical and degenerate NBS have different conformational preferences and we discovered that both, binding as well as hydrolysis of ATP have a large impact on the free energy profile of NBD dimerization, thereby leading to change in the geometry of NBD dimer, which energizes the transport cycle.

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Session 3

S3-01 Sleepless in Vienna: how to rescue a folding-deficient mutant dopamine transporter of *Drosophila melanogaster* by pharmacochaperoning

Michael Freissmuth

Medical University of Vienna, Austria

Folding-defective mutants of the human dopamine transporter (DAT) cause a syndrome of infantile dystonia/Parkinsonism. We provide a proof-of-principle that the folding-deficit is amenable to correction *in vivo* by two means, the cognate DAT ligand noribogaine and the HSP70 inhibitor, pifithrin- μ : a mutation in the gene encoding dDAT was identified in the Zuker collection of *Drosophila melanogaster*, which leads to a sleepless phenotype in flies harboring the mutation dDAT-G108Q. We examined the structure of dDAT-G108Q by molecular dynamics simulations using the published crystal structure of dDAT as a starting point. These simulation provided evidence for structural instability of dDAT-G108Q consistent with a folding defect. We verified this conjecture by visualizing heterologously expressed dDAT-G108Q and the human equivalent hDAT-G140Q in the endoplasmic reticulum and by showing that it was found in a complex with endogenous folding sensors (calnexin and HSP70-1A). Incubation of the cells with noribogaine (a DAT ligand selective for the inward facing state) and/or pifithrin- μ (an HSP70-inhibitor) restored folding of, and hence dopamine transport by, dDAT-G108Q and of hDAT-G140Q. The mutated versions of DAT were confined to the cell bodies of the dopaminergic neurons in the fly brain and failed to reach the axonal compartments. Axonal delivery was restored and sleep time increased to normal length (from 300 to 1000 min/d), if dDAT-G108Q expressing flies were treated with noribogaine and/or pifithrin- μ . Rescuing misfolded versions of DAT by pharmacochaperoning is of therapeutic interest: it may provide opportunities to remedy disorders arising from folding-defective mutants of human DAT and of other related SLC6 transporters, e.g. of the human creatine transporter-1, which gives rise to mental retardation when mutated at the equivalent glycine residue.

S3-02 Dopamine transporter trafficking: New mechanisms and insights into behavior

Haley Melikian

UMASS Medical School, USA

Dopamine (DA) neurotransmission is requisite for numerous CNS functions including movement, working memory and reward. Following vesicular release, DA signaling is temporally and spatially constrained by presynaptic DA clearance, facilitated by the plasma membrane DA transporter (DAT). Compromised DAT function, by either genetic or pharmacologic means, significantly increases extracellular DA concentrations and results in enhanced DAergic responses in both mammalian and invertebrate animal models. Moreover, recently identified DAT coding variants in ADHD, Infantile Parkinsonism, and Autism Spectrum Disorder illustrate DAT's critical role in DA homeostasis, and demonstrate the severe physiological consequences that ensue when DAT function is perturbed. DAT expression at the plasma membrane is not static; endocytic trafficking dynamically fine-tunes the available DAT pool at the cell surface. An endocytic brake stabilizes DAT at the plasma membrane, and is released in response to acute protein kinase C (PKC) activation or exposure to amphetamine, thereby decreasing DAT surface expression and function. However, the molecular mechanism(s) that comprise the DAT endocytic brake have not been well-defined. Moreover, it is entirely unknown whether DAT trafficking contributes to normal dopaminergic transmission and related behaviors. This presentation will discuss recent findings from our laboratory that define the molecular basis of the DAT endocytic brake and its potential as a mechanism to rescue DAT coding variants with inherent membrane instability. Additional new findings that reveal the physiological impact of DAT trafficking *in vivo* will also be discussed.

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S3-03 Reciprocal phosphorylation and palmitoylation regulate dopamine transporter kinetics and membrane mobility

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The dopamine transporter mediates reuptake of dopamine and is the major determinant of transmitter availability in the brain. Transport capacity is regulated by protein kinase C, and we have investigated the roles of transporter post-translational modifications in this response. Our findings show that Serine 7 phosphorylation and Cysteine 580 palmitoylation undergo reciprocal regulation that mediates transporter kinetic capacity and lateral membrane mobility. The modifications are affected by amphetamines and by polymorphisms identified from ADHD and bipolar disorder patients, linking their dysregulation to dopaminergic disease conditions. These findings reveal novel mechanisms for control of reuptake that may represent targets for therapeutic intervention in dopamine imbalance disorders.

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Session 4

S4-01 Role of cationic amino acid transporters in human immune and tumor cells

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Tumor as well as immune cells are highly proliferative and have therefore a high demand on nutrients, including cationic amino acids. These are mainly imported into the respective cells by cationic amino acid transporters (CATs), members of the solute carrier family (SLC) 7. However, some tumor cells may also use the system b^{0,+} amino acid transporter (b^{0,+}AT: SLC7A9) or the amino acid transporter B^{0,+} (ATB^{0,+}: SLC6A14) for this purpose. Export of cationic amino acids occurs via system y⁺L amino acid transporter (y⁺LATs: SLC7A6 and 7). We are currently exploring these transport pathways as possible tumor targets. My talk will give a short overview over transporters of cationic amino acids and then focus on the expression pattern and function of individual transporters in tumor and immune cells.

S4-02 Transporter cooperation for amino acid homeostasis

François Verrey

University of Zurich, Institute of Physiology, Switzerland

Transmembrane transport of amino acids is central for the control of amino acid concentration in the extracellular fluid and in cells. The 20 proteinogenic amino acids and many related substances are transported by the approximately 50 human amino acid transporters that present overlapping selectivity, differential affinities and also different transport modes (uniporters, antiporters and symporters). About half of the proteinogenic amino acids are essential for mammals such that they need to be taken up with the nutrition. Whereas the intracellular concentration of essential amino acids is often similar to their extracellular concentration, many of the non-essential amino acids are highly concentrated in many cells. Obligatory amino acid exchangers (antiporters) play here an important gate keeper role by equilibrating the concentration of specific amino acids without mediating an overall net amino acid flux. One of these exchangers is the heterodimeric neutral amino acid antiporter Lat1-CD98 (Slc7a5-Slc3a2) that plays both an important role for the transfer of amino acids across the blood-brain barrier endothelial cells and is necessary for the uptake of essential amino acids in specific growing cells, in physiology and many tumors. In contrast, the related neutral amino acid antiporter Lat2-CD98 (Slc7a8-Slc3a2) appears less involved in cell growth but plays a major role for transepithelial amino acid transport. A few essential amino acid transporters with narrow substrate ranges, e.g. Lat4 (Slc43a2) and Tat1 (Slc16a10), function as low affinity facilitated diffusion pathways (uniporters) and thus can equilibrate the concentration of their substrates across membranes. Thereby such essential amino acid uniporters may control the flux of a broad range of other amino acids via antiporters that in exchange recycle these essential amino acids. To complete this overview, the sodium-amino acid symporter ATB^{0,+} (Slc6a 14) needs to be mentioned. It imports all proteogenic neutral and cationic amino acids with high affinity and next to its narrow expression range is expressed in several cancers. Examples illustrating the differential, complementary and cooperative function these amino acid transporters will be presented.

S4-03 The structure of Heteromeric Amino acid Transporters: getting there

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Heteromeric amino acid transporters (HATs) are relevant targets for structural studies. On the one hand, HATs are involved in inherited and acquired human pathologies (aminoacidurias, cancer, cocaine addiction), being relevant targets for drug development in cancer and schizophrenia. On the other hand, HATs are the unique example known of solute transporters composed of two subunits (heavy-SLC3A members, and light-SLC7A members or LAT transporters) linked by a disulphide bridge. Unfortunately, structural knowledge of HAT is scarce and is limited to the atomic structure of the ectodomain of a heavy subunit (human 4F2hc-ED) and distant prokaryotic homologues of the light subunits (APC transporters AdiC, ApcT and GadC) that share a LeuT-fold. Recent data at nanometer resolution of human 4F2hc (SLC3A2)/LAT2 (SLC7A8) revealed 4F2hc-ED situated on top of the external loops of the light subunit LAT2. Crystal structures of LAT transporters and structural models of HAT at least at subnanometric resolution become essential to establish the structural bases for light subunit recognition and evaluate the functional relevance of heavy and light subunit interactions on the transport cycle. Advances towards the resolution of the structure of the first close prokaryotic homologue of the light subunits (a prokaryotic LAT transporter) and a vertebrate HAT heterodimer will be presented.

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Session 5

S5-01 Examining inhibitor and substrate binding to the human serotonin transporter

Kristian Stromgaard

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Despite the well-established role of the human serotonin transporter (hSERT) in the treatment of depression, the molecular details of antidepressant drug binding are still not fully understood, especially examining the S1 and S2 binding sites. We have used a number of different approaches including a combination of comprehensive mutational studies, modeling studies and structure-activity relationship (SAR) studies to further elucidate these details. Recently, we applied the amber codon suppression methodology to encode photocrosslinking unnatural amino acids (UAAs) into hSERT and mapped the binding sites of antidepressants. In addition, we designed and employed bivalent substrate ligands to potentially address differences in S1 and S2 binding sites.

S5-02 Direct assessment of conformational changes uncovers functional regulation by K⁺ of a neurotransmitter:sodium symporter

Claus Loland

University of Copenhagen, Denmark

Neurotransmitter:sodium symporters (NSSs) are responsible for Na⁺-dependent reuptake of neurotransmitters and represent key targets for antidepressants and psychostimulants. LeuT, a prokaryotic NSS protein, constitutes a primary structural model for these transporters. Here, we show that K⁺ competitively inhibits Na⁺-dependent binding of substrate to LeuT, promotes an outward-closed/inward-facing conformation of the transporter and increases uptake of [³H]alanine into proteoliposomes. To assess K⁺-induced conformational dynamics we measured fluorescence resonance energy transfer (FRET) between fluorescein site-specifically attached to inserted cysteines and Ni²⁺ bound to engineered di-histidine motifs (transition metal ion FRET). The measurements supported K⁺-induced closure of the transporter to the outside, which was counteracted by Na⁺ and substrate. Mutational locking of LeuT in the outward-open conformation abolished the K⁺-effect. The K⁺-effect depended on an intact Na1-site and mutating the Na2-site potentiated K⁺ binding by facilitating transition to the inward-facing state. The data reveal an unrecognized ability of K⁺ to regulate the LeuT transport cycle.

S5-03 Voltage dependent K⁺ binding pushes the catalytic cycle of the human Serotonin transporter into the forward direction

Walter Sandtner

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Transporters of the LeuT superfamily are essential in the physiology of prokaryotic and eukaryotic organisms. Crystal structures of several members of this family have provided snapshots of intermediates that occur during substrate translocation. However, understanding the structure-function relationship of these reactions requires information on their time-dependence. Here, we report a comprehensive electrophysiological investigation of intracellular solute-binding to the human serotonin transporter (SERT). We found that intracellular Na⁺ release is highly electrogenic, rendering transport by SERT vulnerable to changes in membrane potential. However, rapid electrogenic binding of intracellular K⁺ or H⁺ can antagonize voltage dependent Na⁺ binding. This warrants voltage independent transport. In addition, our data suggest that Cl⁻ stays bound to SERT during the entire transport process. We elaborated a kinetic model that accounts for our data and provides a mechanistic framework for SERT function and maybe also for the function of other related transporters.

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Session 6

S6-01 Deciphering the Functional role of the Vesicular Monoamine Transporter in defined monoaminergic systems

Bruno Giros

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Monoaminergic neurotransmitters, namely dopamine (DA), noradrenaline (NE), serotonin (5HT) and Histamine (HA) extensively modulate cognitive, affective, neuroendocrine and motor functions in the brain. The functional subdivision of the monoamine systems indicates a disparity in cerebral distribution, receptor subtypes and mechanisms of action. Unraveling the independent roles of the monoaminergic systems in neuropsychiatric disorders remains a challenge that is complicated by their large anatomical, pharmacological and functional overlaps.

Two types of transporters primarily control the intra- and extra-cellular concentrations of monoamines. The plasma membrane transporters (norepinephrine transporter, NET; serotonin transporter, SERT; and dopamine transporter, DAT) control the clearance and recycling of monoamines via uptake from the extracellular space, and the vesicular monoamine transporter (VMAT) accumulates monoamines in synaptic vesicles and is essential for their release. Two VMAT genes have been identified, VMAT1 is primarily expressed in the peripheral nervous system and VMAT2 is primarily expressed in the neurons of the central nervous system. To better understand the physiological and behavioral roles of monoaminergic signaling, the constitutive knock-out (KO) model for VMAT2 has been created. However, animals did not survive and dye at the age of 2-3 days. Given that the depletion is observed on all systems simultaneously, it is not possible to specifically associate any behavioural phenotype to a given monoamine. To understand how each single monoamine can regulate various superior brain functions, it is essential to have a comprehensive knowledge of the processes involved in establishing and controlling the synaptic fluxes, and to be able to individually study the various pathways that are using each neurotransmitter.

In order to reach this objective, we generated a conditional KO for VMAT2, allowing studying the specific role of each monoamine. The floxed VMAT2 gene could be specifically spliced in the DAergic, 5HTergic or NAergic neurons by the cre recombinase expressed under the control of the DAT, SERT or DBH genes respectively. In a first set of studies, we have been able to study the heterozygous mice in each of these three systems, and compare it to the full constitutive heterozygous KO of VMAT2. This study allowed us to decipher the respective role of each neurotransmitter system within the global phenotype (*Isingrini et al. 2016a*).

We then investigated the role of NE transmission using VMAT2lox/lox-DBHcre mice. We were first able to conduct an exhaustive behavioral study to precisely investigate how NE could be involved in many cognitive and learning tasks. Then, we could show for the first time how NE in the *locus coeruleus* can directly control the electric activity of dopamine neurons in the Ventral tegmental Area (VTA). Using optogenetic control of NE release in the VTA of control mice produced an opposite effect, i.e. decreasing the functional activity of DA neurons in the VTA. This new neural circuit, from the LC to the VTA, directly underlies resilience against chronic emotional stress, providing a rationale for the use of NE releaser in depression or PTSD and future direction to develop therapeutic treatments (*Isingrini et al., 2016b*).

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Session 6

S6-02 Compromised vesicular dopamine transport activity in Parkinson disease

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Idiopathic Parkinson disease (iPD) is a neurodegenerative brain disorder in which the most affected neurons are the dopaminergic neurons of the nigrostriatal pathway. The cause of dopamine neuronal death in iPD is unclear and even in the rare cases of familial Parkinson disease there is no clear connection between the cause, a pathological protein variant, and dopamine neuronal death since these proteins (alpha-synuclein, LRRK2, parkin...) are not only expressed in dopamine neurons. What makes then dopamine neurons prone to neurodegeneration? A reason might be the neurotransmitter dopamine itself which is easily (auto)oxidizable and also metabolized by monoamine oxidase to reactive molecules as intermediates. The resulting "toxic-dopamine-cascade" is prevented by the uptake of dopamine into synaptic vesicles driven by the vesicular monoamine transporter 2 (VMAT2), therefore a VMAT2 defect might promote mechanisms leading to dopamine neuronal death. VMAT2 activity was determined in synaptic vesicles isolated from the striatum of autopsied brains of iPD patients. The about 90% VMAT2 reduction as compared to controls was beyond the loss of VMAT2 molecules due to loss of dopamine nerve endings as determined by the VMAT2-selective label [³H]dihydrotetrabenazine. Thus, the calculated dopamine uptake per VMAT2 transport site was significantly reduced by 53 % in caudate and 55% in putamen of iPD but unchanged in MPTP monkeys with a similar degree of nigrostriatal neurodegeneration. Since several potential explanations, tested in cellular models, transgenic mice, and levodopa-treated MPTP monkeys, could be ruled out, elucidation of this specific defect of striatal vesicular transport, might shed light on the pathogenesis of dopamine cell death in Parkinson disease.

S6-03 A role for the synaptic glycoprotein 2C (SV2C) in dopamine neurotransmission

Gary Miller

Emory University, USA

The synaptic vesicle glycoprotein 2 (SV2) family of proteins are involved in synaptic function within the brain and are members of the Major Facilitator Superfamily of transporters. The ubiquitously expressed SV2A has been widely implicated in epilepsy, though SV2C with its restricted basal ganglia distribution has no known function. Here we identify SV2C as a mediator of dopamine function and report that disrupted expression of SV2C within the basal ganglia is a pathological feature of Parkinson's disease. Genetic deletion of SV2C leads to reduced dopamine release in the dorsal striatum as measured by fast-scan cyclic voltammetry, disrupted alpha-synuclein expression, and deficits in motor function. Further, SV2C expression is dramatically altered in postmortem brain tissue from PD cases, but not in Alzheimer's disease, progressive supranuclear palsy or multiple system atrophy. This disruption was paralleled in mice overexpressing mutated alpha-synuclein. These data establish SV2C as a novel mediator of dopamine neuron function and suggest that SV2C disruption is a unique feature of PD that likely contributes to dopaminergic dysfunction.

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Session 7

S7-01 An Allosteric Mechanism for Lipid inhibition of Glycine Transporter 2

Robert Vandenberg

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The glycine transporter, GlyT2, is the target for a number of drugs that can alleviate neuropathic and inflammatory pain. We have identified a series of lipids that inhibit GlyT2 and also show promise as leads for the development of a novel class of analgesics. Three lipids that show high affinity and efficacy are N-Arachidonyl-Glycine (NAGly), N-Oleoyl-Glycine (NOGly) and Oleoyl-L-Carnitine (OLCarn). All three lipids are non-competitive inhibitors and are unlikely to bind at the substrate binding site. We have used site-directed mutagenesis to identify potential lipid binding sites and to understand allosteric communication between the lipid binding site and inhibition of transport. Mutations in four distinction regions of the protein influence the potency and extent of inhibition. Extracellular loop 4 undergoes considerable conformational changes in the transport cycle, and mutations in the loop disrupt inhibition by all three lipids. This site is unlikely to form a lipid binding site but it may influence the conformational changes of the protein. We reasoned that transmembrane domains that flank EL4 may also influence the conformational changes required for lipid inhibition. Mutations on the membrane-exposed external surface of TM8 differentially reduce the potency of the three lipids and suggest potential head and tail group interactions of the lipids with the transporter. Mutations of additional residues in TM8 that face the opposite direction and point towards the substrate binding site also reduce the potency of lipid inhibition. Finally, S479 forms part of the substrate binding site of GlyT2 and the S479G mutation relaxes the substrate selectivity to allow sarcosine to be transported, and also reduces the potency of all three lipids to inhibit glycine transport. No other mutations in the substrate binding site, that alter substrate selectivity, have any effects on lipid inhibition. We propose a model where the lipids bind to the external membrane-exposed surface of the transporter, and alter the conformational changes in extracellular loop 4 and also the substrate binding site to disrupt glycine transport. We are currently using this information to develop novel lipid-based inhibitors that may have therapeutic value in treating chronic pain.

S7-02 Linking structure, dynamics and function in a glutamate transporter homologue

Olga Boudker

Cornell University, USA

Glutamate transporters are responsible for clearance of the neurotransmitter glutamate from the synaptic cleft following rounds of neurotransmission. They maintain steep concentration gradients of glutamate by coupling its uptake to movements of ions, primarily sodium. We have been investigating the molecular mechanism of this family of transporters using an archaeal homologue GltPh as a model system. We have focused on determination of atomic resolution structures of the key states of the transporter along its functional cycle and on the dynamic relationships between them. We now investigate how the transition rates between the states determine the overall rate of transport and how these rates might have been optimized in evolution to fit the physiological needs.

S7-03 Structure and Function of Excitatory Amino Acid Transporters

Baruch Kanner

The Hebrew University of Jerusalem, Israel

Excitatory amino acid transporters remove synaptically released glutamate and maintain its concentrations below neurotoxic levels. EAATs also mediate a thermodynamically uncoupled substrate-gated anion conductance that may modulate cell excitability. Based on the Crystal structures of the archeal homologue Glt_{Ph}, we address two aspects of the function of these transporters.

1. Substrate specificity: Whereas mammalian glutamate transporters can translocate both glutamate and

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aspartate, Glt_{Ph} is only capable of aspartate transport. Most of the amino acid residues that surround the aspartate substrate in the binding pocket of Glt_{Ph} are highly conserved. However, in the brain transporters, Thr-352 and Met-362 of the reentrant hairpin loop 2 (HP2) are replaced by the smaller Ala and Thr, respectively. Therefore we have studied the effects of T352A and M362T on binding and transport of aspartate and glutamate by Glt_{Ph}. Both mutants exhibit a markedly elevated affinity for glutamate binding. However, only the T352A mutant gained the ability to transport glutamate. The differential impact of mutation on binding and transport of glutamate suggests that HP2 not only plays a role in the selection of the substrate but also in its translocation.

2. Anion conductance: We have introduced two cysteine residues in the neuronal glutamate transporter EAAC1 at positions predicted to be close enough to form a disulfide bond only in outward-facing and early intermediate conformations of the Glt_{Ph} homologue. Our studies of the effect of reducing and oxidizing agents on radioactive transport and transport currents by double mutant expressed in *Xenopus* oocytes support the idea that the anion conducting conformation of the neuronal glutamate transporter is associated with an early step of the transport cycle.

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Session 9

S9-01 Tales of tails and membranes in the dynamics of transporter mechanisms

Harel Weinstein

Cornell University, USA

Secondary active transporters couple the unfavorable process of solute transport against its concentration gradient to the energetically favorable transport of one or several ions. The process is now known to be much more complex than the alternating access model would suggest, and involves complex allosteric mechanisms that are progressively being revealed in atomistic detail. The new mechanistic findings in the presentation will focus on two sodium-coupled symporter families - the mammalian synaptic neurotransmitter transporter in the LeuT-fold family, DAT, which exhibits “gated pore” characteristics (1,2), and the prototype for the “elevator” mechanism in the mammalian excitatory amino acid transporters, GltPh (3,4). In the course of establishing the nature of allostery in these transporter families in a quantitative formalism that can reconcile biochemical and biophysical data (1,5), we identified the roles played by the properties of their diverse structural elements, and especially by intramolecular loop regions (e.g., the “elevator door”) (4) and the N- and C-terminal regions (6). From uptake kinetics to efflux efficiency, physiologically important processes of these transporters are dependent on specific behaviors of various functional microdomains of the molecules such as the Na binding sites (7,8) and especially the terminal regions (9), which in turn are modulated by phosphorylation, by modes of protein-membrane interaction (4, 10), and by specific membrane components (e.g., PIP2) (8,11). These molecular mechanisms, which are not well described by movements of levers or rocking bundles, require rigorous representations capable of predicting and interpreting, not just agreeing with, detailed experimental results.

S9-02 The power of linked open data - drug transporter profiling and beyond

Gerhard F. Ecker

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With the public availability of large data sources such as ChEMBL and the Open PHACTS Discovery Platform, retrieval of data sets for certain protein targets of interest measured under consistent assay conditions is no longer a time consuming process. Especially the use of workflow engines such as KNIME or Pipeline Pilot allows to submit complex queries and enables to simultaneously search for several targets. Data can then directly be used as input to e.g. MOE for ligand- and structure-based studies.

Within this talk we will present case studies for the exploitation of linked open data for P-gp/BCRP and DAT/SERT profiling, as well as for predicting complex in vivo endpoints such as hyperbilirubinemia, DILI, and cholestasis.

We acknowledge financial support provided by the Austrian Science Fund (F3502) and the Innovative Medicines Initiative (Open PHACTS, 115191)

S9-03 Structure, function, and inhibitors of the acid-gated *Helicobacter pylori* urea channel, an essential component for acid survival and chronic infection

Hartmut Luecke

UC Irvine, United States

Half the world's population is chronically infected with *Helicobacter pylori*, causing gastritis, gastric ulcers and an increased incidence of gastric adenocarcinoma. Its proton-gated innermembrane urea channel, *HpUreI*, is essential for survival in the acidic environment of the stomach. The channel is closed at neutral pH and opens at acidic pH to allow the rapid access of urea to cytoplasmic urease. Urease produces NH₃ and CO₂, neutralizing entering protons and thus buffering the periplasm to a pH of roughly 6.1 even in gastric juice at a pH below 2.0. Here we report the structure of *HpUreI*, revealing six protomers assembled in a hexameric ring surrounding a central bilayer plug of ordered lipids. Each protomer encloses a channel formed by a twisted bundle of six transmembrane helices. The bundle defines a previously unobserved fold comprising

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a two-helix hairpin motif repeated three times around the central axis of the channel, without the inverted repeat of mammalian-type urea transporters. Both the channel and the protomer interface contain residues conserved in the AmiS/UreI superfamily, suggesting the preservation of channel architecture and oligomeric state in this superfamily. Predominantly aromatic or aliphatic side chains line the entire channel and define two consecutive constriction sites in the middle of the channel. Mutation of Trp153 in the cytoplasmic constriction site to Ala or Phe decreases the selectivity for urea in comparison with thiourea, suggesting that solute interaction with Trp153 contributes specificity. The previously unobserved hexameric channel structure described here provides a new model for the permeation of urea and other small amide solutes in prokaryotes and archaea. Follow-up microsecond-scale unrestrained molecular dynamics studies now provide a detailed mechanism of urea and water transport by *HpUreI*.

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Poster Presentation

PP-01 Exploiting the serendipitous natural cysteine portfolios of *Arabidopsis thaliana* PIN transporters to investigate oligomerisation

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IST Austria, Austria

In plants, PIN proteins are involved in regulating the hormone auxin. PINs are predicted to have 10 transmembrane domains (TMDs), and phylogenetic evidence strongly suggests they are transporters, but there is no experimental structural data available. They are found in all plant species as a family of closely related homologs, e.g. *Arabidopsis thaliana* has eight PINs. I have analysed PINs from native *A.thaliana* plant tissue and found that all homologs form homo-oligomers, but the size and stability of the oligomers varies considerably between the homologs as well as the tissue in which they are expressed (e.g. root, shoot, cell culture). To investigate why the oligomeric capacity varies so much between the different PINs, I analysed their cysteine residues (CYS) because the oligomers were destabilised by reducing conditions, suggesting a role for inter- or intra-molecular disulfide bonds. PINs contain very few CYS (two to five CYS out of about 650 amino acids), which also suggests an important role for the few that are present. Most of the CYS sites are conserved between the PINs, but each PIN has a unique complement, e.g. PIN1, PIN2, PIN3 and PIN4 have four, two, five and three CYS respectively. I took advantage of this natural variation as a basis for a comparative study, i.e. to investigate how each CYS may contribute to homo-oligomer formation and to see if the different CYS portfolio of each PIN could explain the different oligomerisation capacity of that particular PIN. I used a range of CYS labelling and crosslinking techniques to analyse endogenous PINs from plant tissue, and combined the results with a predicted structure (from homology modelling) to come up with a structural model for oligomer formation. As all the CYS are predicted to be on the cytosolic side, the fact that disulfide bonds can persist is intriguing, given the reducing environment of the cytosol. This suggests that plant cells could potentially use cytosolic redox control to manipulate the oligomerisation status and thus the stability, activity or abundance of PIN proteins.

PP-02 Pharmacological characterization of [³H]ATPCA – a novel radioligand with selectivity for the betaine/GABA transporter 1 (BGT1)

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The betaine/γ-aminobutyric acid (GABA) transporter 1 (BGT1) is one of the four GABA transporters (GATs) that have been identified in mammals to date. The GATs belong to the solute carrier family 6A (SLC6A) of the Na⁺/Cl⁻-dependent neurotransmitter transporters and facilitate the termination of GABAergic signaling. Although hypothesized to be involved in diseases such as epilepsy, the exact role of BGT1 in the brain under physiological and pathological conditions is not yet determined. This is partly due to the low expression levels and lack of specific and potent pharmacological tools that can be used to elucidate its function.

We have recently reported the identification of a BGT1-selective substrate inhibitor, 2-amino-1,4,5,6-tetrahydropyrimidine-5-carboxylic acid (ATPCA) that displays ~400 times higher potency at BGT1 (IC₅₀ 2.5 μM) than at GAT1 (IC₅₀ >1,000 μM).¹

We here report the characterization of a newly synthesized tritiated analogue of ATPCA, [³H]ATPCA as a novel radioligand with BGT1-selectivity.

Using traditional radioligand uptake assays in Chinese hamster ovary cell lines stably expressing one of the four recombinant human GAT subtypes, the uptake parameters of [³H]ATPCA were characterized. BGT1-selective uptake of [³H]ATPCA (20 and 100 nM) was observed, which was both temperature- and time-dependent with linear uptake up to ~15 minutes. At BGT1, the Michaelis-Menten kinetic parameters were determined to be K_m 13 μM and V_{max} 3.5.

Guided by computational docking studies, we also examined the uptake of [³H]ATPCA at two single-point mutated transporters, BGT1 Q299L and GAT3 L314Q, in an attempt to probe the

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molecular determinants for the BGT1 subtype-selectivity. The activity of ATPCA was not affected at the BGT1 mutant compared to the wild-type transporter, but interestingly, a regain of activity of ATPCA and an analogue was seen at GAT3 L314Q compared to wild-type GAT3.

With the development of a BGT1-selective radiolabeled substrate, we now have a tool that can aid to elucidate the physiological role and therapeutic potential of BGT1 as a drug target. Studies are underway to determine native BGT1 uptake and binding in cultured astrocytes and neurons.

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PP-03 Exocyst modulates dopamine transporter (DAT) function by regulating its trafficking

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Uptake through the dopamine transporter (DAT) represents the primary mechanism used to terminate dopaminergic transmission in the brain. Intracellular membrane trafficking of dopamine transporter at presynaptic membrane is critical for synaptic function. However, little is known about the specialized trafficking events occurring at the presynaptic membrane. Here, we tested the hypothesis that DAT requires the exocyst for reaching the cell surface. The exocyst is a multiprotein complex required by many membrane proteins for delivery to and insertion into the plasma membrane. DAT requires an intact C-terminal PDZ-binding motif to reach the cell surface, whereas the closely related serotonin transporter SERT does not.

HEK 293 (a cell line of fibroblast origin) or CAD (a Cath.a-cell-derived line of neuronal origin) cells were transiently co-transfected with plasmids encoding the wild-type dopamine transporter (DAT) and serotonin transporter (SERT) along with different components of the exocyst using jetPRIME (Polyplus). Radioligand uptake, confocal laser scanning microscopy and immunoprecipitation experiments were performed 48 h after transfection to study the effect of exocyst components on trafficking of DAT and SERT

Overexpression of the C-terminal fragment of EXO70 reduced dopamine uptake in HEK293 and CAD cells, which heterologously expressed DAT. Confocal microscopy verified that this was due to the retention of DAT in the endoplasmic reticulum (ER). ER retention was confirmed by immunoblotting for core glycosylated DAT. This effect was phenocopied by Overexpression of EXO70, SEC6 and SEC8. In contrast, surface expression of SERT was not affected by overexpression of any component of the exocyst, regardless of whether the experiments were performed in HEK 293 cells or in CAD cells.

Trafficking of DAT - but not of SERT - in the secretory pathway requires assembly of the exocyst at an early stage. This may explain why the PDZ-motif of DAT must be present for DAT to reach the cell surface.

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PP-04 In vivo verification of efflux transporter interactions at the blood-brain barrier in rodents.

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The toxic events caused by membrane transporter -associated drug-drug interactions came in the focus of pharmacokinetic/pharmacodynamic studies during the last decade. One of the most important target for drug therapy is the central nervous system itself. But sometimes the delivery of the drug to the brain and to cross the blood-brain barrier is challenging. In some cases the drug-drug interaction by co-treatment with a transporter inhibitor can help to reach the appropriate central efficiency of the drug, but in other cases it can result in unwanted central side effects.

The aim of our study was to investigate the drug-drug interactions at the two major efflux transporter proteins (P-gp and BCRP) at the blood-brain barrier. The P-gp interactions were studied

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by quinidine and PSC-833 as a reference substrate/inhibitor combination using triple probe microdialysis technique in rats. One probe was implanted in the striatum, the second one in the lateral ventricle and the third one in the jugular vein. The BCRP interactions were studied by teriflunomide and Ko-143 substrate/inhibitor combination using dual-probe microdialysis in mice. The central probe was placed into the frontal cortex and the peripheral probe into the jugular vein.

First the microdialysis systems had to be validated under in vitro conditions for their applicability to measure quinidine and teriflunomide in dialysate samples. Then the optimized methods were used in vivo in animal experiments. The pretreatment with the transporter inhibitors remarkably increased the brain exposure to substrate molecules both in rats and in mice. However, the features of quinidine and P-gp interactions at the blood-brain barrier (striatum) and the blood-cerebrospinal fluid barrier (lateral ventricle) were different. To clarify the orientation of the transport and the consequent drug concentrations in brain extracellular fluid and cerebrospinal fluid, further experiments are needed.

In summary, an in vivo assay system was validated in our laboratory for testing and evaluating drug-transporter interactions at the blood brain barrier. Our in vivo microdialysis techniques are appropriate tools to study the influence of P-gp and BCRP on drug penetration in rodents. By increasing the number of elements, further statistical analysis, mathematical modeling and prediction of drug distribution in human subjects can also be performed based on the microdialysis data.

PP-05 Measuring transport activity in *Xenopus Laevis* Oocyte by fluorophore quenching

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Membrane proteins are involved in many physiological functions and they are target of several drugs. To understand their function, extensive studies analyzed their activity by using different methods, among them the *Xenopus laevis* oocytes (XLO) that provide a powerful heterologous expression system to study these proteins.

Typical experiments investigate transporters function in XLO using electrophysiology and radiolabelled uptake.

Two-electrode voltage clamp (TEVC) is suitable only for electrogenic proteins and uptake measurements require the existence of radiolabelled substrates and of adequate laboratory facilities. Moreover, many researchers have chosen radioactive free experiments for their costs and for low availability of labelled compounds.

Recently, we characterized *ddNRAMP1* and 2 from *Dictyostelium discoideum* using multidisciplinary approach. While *ddNRAMP1* is electrogenic, *ddNRAMP2* is not, and its activity was investigated in XLO by acquiring confocal images of the quenching of injected fluorophore calcein (a divalent metal indicator)¹. In another work, we used a similar method to determine nanoparticles (NPs) interaction with XLO membrane with a conventional fluorescence microscope².

These procedures appear adequate for measuring the variation in emitted fluorescence and consequently the transport activity, but they require long experimental procedure to collect statistically consistent data. Considering that the best expression of heterologous membrane proteins last for only 48-72 hours, slow acquiring process requires to use more batches of XLO increasing the data variability.

In this work, we present a novel approach to determine the transporter function by measuring the substrate uptake using a fluorophore indicator and a microplate reader. The oocytes expressing or not the transporter after the injection with fluorophore, were incubated in the presence or in the absence of substrate for one hour and their transport activity was measured, evaluating the quenching of the fluorescent probe.

This protocol, called XLOS (*Xenopus laevis* oocytes smoothie), allows to test tens of oocytes in different experimental condition at the same time by reading fluorescence of oocytes extracts using microplate reader and it permits to collect significant statistical data for each batch, saving time and animals.

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The method was tested with several transporters (DMT1, c-NRAMP1, c-NRAMP2 and PepT1), with different fluorescence indicators. Each experimental condition tested with XLOS was verified by comparison with traditional methods (uptake, TEVC) and microscopy fluorescence methods, demonstrating its efficacy. In conclusion, the method is particularly suitable for fast screening electrogenic or non-electrogenic transporter function. The increasing availability of fluorescence indicator for several substrates permit to envisage a new life for XLO in transporters study.

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PP-06 Capacitance measurements of HEK293 cells expressing serotonin transporters

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Serotonin transporters (SERT) curtail the signal transmission of serotonergic neurons by clearing the extracellular space from serotonin. Some partial reactions of the transport cycle of SERT can be measured utilizing the substrate-induced capacitive current. However, other important reactions such as the binding of co-substrates (Na⁺ and Cl⁻), cannot be assessed by this method.

We therefore performed capacitance measurements before and during fast application of serotonin and observed that substrate binding to SERT reduces the membrane capacitance (ΔC).

A possible reason for the reduction of the capacitance could be surface charge screening by the ligand. In this scenario the substrate screens a charge in the ligand-binding site. Indeed, the substrate-binding site of SERT comprises a charged aspartate-residue that forms a bond with the amine group of serotonin. Omitting co-substrates, we observed that serotonin binding is the primary cause of ΔC . Moreover, the substrate affinity is lowered at sodium-free conditions, reflected by a smaller ΔC . Our findings suggest random order binding reactions of substrate and co-substrates and that serotonin and Na⁺ bind in a cooperative manner.

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PP-07 A tamoxifen analog is an asymmetric dopamine transporter modulator and reduces amphetamine *in vitro* and *in vivo* effects

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The reinforcing properties of amphetamine (AMPH) stem from its reversal of the dopamine transporter (DAT) which greatly increases extracellular dopamine (DA) in the brain. Inhibition of protein kinase C (PKC) reduces these AMPH effects. The CNS-permeant estrogen receptor (ER) modulator, tamoxifen, inhibits PKC and reduces AMPH effects. Therefore, revisiting the tamoxifen scaffold may fill the unmet need for a therapeutic against AMPH abuse.

We aim to utilize reported structure-activity relationship studies to create CNS-permeant tamoxifen analogs with increased selectivity for PKC inhibition and reduced ER affinity. Here we evaluate reduction in AMPH-stimulated neurochemical and behavioral effects in rats by our lead compound, CCG-215103. We also investigated more thoroughly the effects of CCG-215103 at DAT.

CCG-215103 inhibits PKC activity (IC₅₀, 0.2 μ M) but does not bind to ER α at ≤ 3 μ M. Further, CCG-215103 did not inhibit other kinase modulators of DAT, namely Ca²⁺/calmodulin-dependent protein kinase II and protein kinase B. We find that CCG-215103 is an asymmetric DAT modulator because it more potently blocks dopamine efflux than uptake. Treatment of rat striatal synaptosomes with 0.3 μ M to 3 μ M CCG-215103 robustly reduces DA efflux by 10 μ M AMPH. However, only 3 μ M CCG-215103 blocks [³H]DA uptake. CCG-215103 does not displace [³H]WIN 35428 binding showing that it does not bind near the active site of DAT. Injection of 1 μ M CCG-215103 into the nucleus accumbens reduces by ~40% both DA release and locomotion induced by

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2 mg/kg i.p. AMPH measured with microdialysis in freely-moving rats. Yet, CCG-215103 does not increase basal DA or locomotion *in vivo*, suggesting it will not have an abuse potential.

The novel compound CCG-215103 reduces both AMPH *in vitro* and *in vivo* effects with a bias towards efflux. CCG-215103 action could be due to allosteric modulation of DAT and/or its interaction with a mediator of DAT functioning, such as PKC.

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PP-08 Study on interaction of antiretroviral drugs lamivudine, emtricitabine and etravirine with placental transporters ABCB1 and ABCG2

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Lamivudine and emtricitabine are antiretroviral drugs used for prevention of mother-to-child transmission (MTCT) in HIV positive women. Etravirine is rather novel antiretroviral drug not yet recommended for its use in pregnancy due to so far limited pharmacokinetic and safety data. The aim of this study was to evaluate whether lamivudine, emtricitabine and etravirine can interact with placental ABCB1 and ABCG2 transporters and if their administration could lead to ABC transporter-mediated drug-drug interactions with other antiretrovirals confirmed as ABC transporter substrates, such as abacavir and tenofovir disoproxil fumarate (TDF). Employing *in vitro* accumulation and transport assays in MDCKII-ABCB1, MDCKII-ABCG2 and control MDCKII-parent cells we revealed that neither lamivudine nor emtricitabine are substrates of human ABCB1 and ABCG2. Consistently, the transplacental pharmacokinetics of these drugs did not show to be affected by Abcb1/Abcg2 using *in situ* dually perfused rat placenta (reaching the ratio between fetal-to-maternal and maternal-to-fetal clearances ~ 1). Interestingly, high inhibitory potency of etravirine to ABCG2, but not to ABCB1 was observed in Hoechst 33342 accumulation assay. The drug was able to completely diminish the active transport of abacavir (300 nM) and TDF (33 nM) across MDCKII-ABCG2 monolayers when applied at concentration 0.1 μ M and 1 μ M, respectively. In addition, etravirine significantly increased TDF concentrations in fetal perfusates employing *in situ* experiments. No such interaction was observed in etravirine-abacavir combination on the rat placenta, which might be explained by lower affinity of abacavir to ABCB2 when compared to TDF and by predominant ABCB1-mediated transport of abacavir across the placenta. To conclude, lamivudine and emtricitabine do not bear the risk of pharmacokinetic drug-drug interactions (DDI) on placental ABC transporters, while ABCG2-mediated DDI between etravirine and TDF could lead to affected transplacental transfer of TDF. Further studies are needed to evaluate the significance of this DDI in clinical settings.

PP-09 Role of nucleoside transporters in transplacental permeation of nucleoside-derived antivirals.

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Adequate therapeutic concentrations of antivirals in the fetal circulation are required for effective prevention of mother-to-child transmission of HIV, HBV and/or HCV. As many antivirals are structurally derived from nucleosides it can be hypothesized that their cellular uptake might be mediated by nucleoside transporters. In our study we aimed to investigate whether equilibrative nucleoside transporters (ENTs; *SLC29A*) and/or concentrative nucleoside transporters (CNTs, *SLC28A*) play any role in transplacental permeation of anti-HIV drugs (abacavir, zidovudine, and emtricitabine) or anti-HCV drug (ribavirin). For this purpose, we employed *in vitro* (accumulation in placental choriocarcinoma derived BeWo cells), *ex vivo* (accumulation in fresh villous fragments and microvillous membrane vesicles isolated from human placenta), and *in situ* (dual perfusion of rat term placenta) experimental approaches. We found that nucleoside transporters significantly affect ribavirin and to a lesser extent abacavir transplacental pharmacokinetics. On the other hand, transplacental transfers of zidovudine and emtricitabine were completely independent of nucleoside transporters. Moreover, using PCR analysis we demonstrated that expression of some placental nucleoside transporters is gestation stage-dependent (in particular CNT2; *SLC28A2*) and can be

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modulated by epigenetic or differentiation affecting agents. Therefore, we suggest that fetal exposure to and efficacy of ribavirin and abacavir might be affected by drug-drug interactions on nucleoside transporters as well as by inter-/intraindividual differences in placental expression of these transporters. In conclusion our findings further broaden current knowledge on mechanisms involved in antiviral transfer from maternal to fetal circulation and might contribute to more evidence-based selection of antiviral medication for prevention of ante-/perinatal infection of the developing fetus.

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PP-10 Ligand-based *in silico* classification model and *in vitro* validation identifies two new BCRP inhibitors

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The breast cancer resistance protein (ABCG2, BCRP) is a member of the ABC (ATP binding cassette) transporter superfamily. It is a transmembrane efflux transporter and is expressed in the physiological barriers of the human body like liver, kidney, gastrointestinal tract, blood-placenta, blood-testis and blood-brain barrier, where it plays a role in protecting the tissues from various, chemically unrelated toxins. Based on its expression profile and its broad range of substrate specificity, BCRP can influence the pharmacokinetics of therapeutic drugs. Therefore, the identification of substrates and inhibitors of BCRP in the early drug discovery phase can help to avoid or predict drug-drug interactions.

In order to predict BCRP inhibition, a ligand-based *in silico* classification model was built using a 978-compound BCRP inhibition dataset previously collected and curated¹. Afterwards, the small molecules subset of DrugBank was screened to find potentially new inhibitors. Ten structurally diverse compounds were selected from the highest ranked substances and were tested *in vitro*. Using steady-state cellular mitoxantrone accumulation assay in BCRP expressing PLB985 cells, we identified roflumilast and cisapride as new BCRP inhibitors with an IC₅₀ value of $0.9 \pm 0.2 \mu\text{M}$ and $0.4 \pm 0.1 \mu\text{M}$, respectively.²

This interdisciplinary study showed that the classification model may be an efficient tool for pre-filtering compound datasets for perpetrators of BCRP-mediated drug-drug interactions, and therefore, can reduce the necessary amount of *in vitro* tests.²

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PP-11 Nanoscopic organization of monoamine transporters in living cells

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Monoamine transporters (e.g. Dopamine transporter (DAT), Serotonin transporter (SERT)) in the plasma membrane of presynaptic nerve terminals and glial cells aid in the removal of specific monoamine neurotransmitters from the extracellular space of the synapse, thereby terminating their actions. It has been suggested that the functional unit of these transporters is monomer. However, there exists some evidence to suggest that DAT form higher oligomeric structures on the membrane while previous work from our group have characterized the oligomerization behavior of SERT in cell lines. We propose to employ different single molecule methods to obtaining information about the oligomeric state, interaction kinetics, mobility, nanoscopic organization and activity of DAT in cell lines and SERT in cultures of primary neurons.

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PP-12 Ligand-based discovery of NHA2 modulators

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NHA2 is a sodium/hydrogen exchanger that is expressed in β -cells of Langerhans islets. Previous epidemiological data in humans suggest a possible role of NHA2 in the pathogenesis of diabetes mellitus, arterial hypertension and osteoporosis^[1]. No pharmacological data has been reported so far for this protein. To identify small molecule modulators of this transporter, we have used ligand based virtual screening^[2] to select 180 compounds that were evaluated with a functional complementation assay in salt-sensitive yeast, leading to a novel series of inhibitors. The best compound (CJE236) has an IC₅₀ of 44 nM and inhibits insulin secretion dose-dependently in NHA2 wild-type, but not NHA2 knock-out primary islets^[3]. In an effort to further explore NHA2 pharmacology, we have performed additional screening on a fragment library inspired from the chemical universe database GDB-17^[4], and identified four new series of inhibitors with a μ M range activity.

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PP-13 Physiology of an essential basic and neutral amino acid transporter family from apicomplexan parasites

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Apicomplexan parasites are obligate intracellular parasites underlying many infectious diseases, including malaria, toxoplasmosis, and cryptosporidiosis. Throughout evolution they have become increasingly dependent on the scavenging of essential nutrients such as amino acids from host cells due to the loss of anabolic pathways. As a result, apicomplexan membrane transporters have taken on a vital role in parasite survival and virulence. Recently, we have characterised a new family of apicomplexan transporters. Designated as the AAAT (Apicomplexan Amino Acid Transporter) family, the two most well characterised transporters, AAAT1 and AAAT6-1, are predominately basic amino acid antiporters.

AAAT1 is an almost obligatory exchanger with exclusive specificity for L-arginine. In contrast, AAAT6-1 demonstrates more L-arginine uniport and a broader specificity, exchanging various neutral amino acids and ornithine in addition to L-arginine. AAAT1 has a high affinity for L-arginine but is insensitive to L-lysine, while AAAT6-1 is a lower affinity L-arginine transporter, but is competitively inhibited by L-lysine with high affinity. AAAT1 and AAAT6-1 harbour a puzzling response to the changes of the membrane potential: the L-arginine-induced inward currents increase when the membrane potential is depolarised. Moreover, addition of arginine causes an initial depolarisation spike, which disappears when oocytes are preloaded with substrate. We hypothesise these observations may underlie an unusual voltage-gating mechanism not often observed for membrane transporters. As both AAAT1 and AAAT6-1 are essential for parasite survival and, in the case of AAAT1, essential for virulence of *Plasmodium berghei*, this novel apicomplexan transporter family present themselves as excellent candidates for pharmacological targeting.

PP-14 Strategies for bacterial over-expression of the canonical and variant hOCTN2 transport proteins

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The OCTN subfamily includes three members involved in carnitine and organic cation transport, namely OCTN1, 2 and 3. The OCTN2 transport protein is known as Na⁺ dependent carnitine

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transporter, but no information is available on its structure. The expression of a splice variant of this protein has been also reported [1]. This variant is 24 aa longer with respect to the canonical OCTN2 and is retained in the endoplasmic reticulum. For this protein neither transport function nor structure are known. With the aim to obtain OCTN2 and its variant in large scale for both structural and functional characterization heterologous over-expression has been performed. Starting from MegaMan Human Transcriptome Library the cDNAs coding for both OCTN2 and OCTN2vt variant have been amplified by PCR using primers specific for the desired variants. After amplification, the canonical OCTN2 cDNA has been cloned into several plasmids and several *E. coli* strains have been used to express the corresponding protein. Only after modification of the second codon of the OCTN2 gene from CGG (Arg) to AAA (Lys) reliable expression of the protein has been obtained[2]. Starting from this observation the cDNA sequence has been optimized according to *E. coli* codon usage. Strong over-expression of 6His-OCTN2 protein has been obtained in Lemo21(DE3) cells transformed with the optimized gene after 6 hours of induction at 28°C. The OCTN2vt splice variant, containing an insertion of 72 nucleotides has been cloned into pET-21a(+) expression vector adding a C-terminal 6His tag. To promote the expression of OCTN2vt protein, as in case of the canonical OCTN2 protein, the second codon has been mutated. Then, Rosetta(DE3) competent cells have been transformed with the OCTN2vt-pET21 construct. The protein synthesis has been induced for 4 hours at 28°C. In these conditions, the OCTN2vt-6His protein and 6His-OCTN2 have been over-expressed in the insoluble fraction of induced cell lysate in an aggregate form. An on column refolding strategy has been adopted. The 6His tagged proteins have been denatured with Urea and Sarkosyl. The denaturing agents have been substituted by dodecyl maltoside on IMAC and the proteins purified by imidazole gradient. Yields of 0.5 mg/L and 5 mg/L cell culture were obtained for hOCTN2vt and hOCTN2, respectively. This is the starting point for reconstitution studies using proteoliposomes and for crystallization trials.

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PP-15 Effect of disease-causing mutations on the ATPase activity of the purified and reconstituted human ABCB6 protein

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Effect of disease-causing mutations on the ATPase activity of the purified and reconstituted human ABCB6 protein

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Mutations of the ABCB6 gene cause Dyschromatosis Universalis Hereditaria (DUH), which is characterized by hypo- and hyper-pigmented macules of irregular size and shape. The causal link of ABCB6 function and the DUH phenotype is currently unknown, and the transported substrate remains to be identified. To assess the effect of DUH mutations on ABCB6 function, wild-type and mutant ABCB6 variants were expressed using the Sf9 insect expression system. His-tagged proteins were efficiently extracted, purified and reconstituted in various lipids. ABCB6 shows significant ATPase activity ($K_m=0.059$ mM, $V_{max}=225$ nmol/mg/min), which is abolished by mutagenesis of the conserved lysine to methionine in the Walker A motif. Analysis of disease-causing variations identified mutations resulting in the loss of basal ATPase activity, suggesting a

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causal link between ABCB6 function and the DUH phenotype. Our results provide the basis for future studies on the structure/function of ABCB6.

PP-16 Transport mechanism of divalent metal ion transporters (SLC11 family)

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Iron is an essential trace element in humans playing a vital role in a variety of physiological processes. Dysregulation of its homeostasis can lead to severe diseases such as anemia and iron overload disorders. Dietary uptake of its non-heme form is mediated in the form of ferrous iron (Fe^{2+}) by the intestinal H^+ -coupled divalent metal ion transporter DMT1 (SLC11A2). In addition, a splice variant of intestinal DMT1 plays a key role in the uptake of iron from acidified endosomes into the cytosol of red cell precursors, and loss-of-function mutations in the SLC11A2 gene lead to microcytic anemia, as first discovered in the *mk* mouse and Belgrade rat strains and later in several human patients. While X-ray diffraction studies have revealed that DMT1 and its bacterial homologue, ScaDMT, belong to the LeuT-fold transporter superfamily, several details of its transport mechanism remain elusive. Open questions include how the translocation of the Fe^{2+} ion across the membrane is coupled to the cotransport of protons (H^+), and which residues are responsible for establishing the coupling mechanism. Starting from *in silico* pK_a estimations of titratable amino acid side-chains, we have identified several candidate residues that may play a role in proton binding during the transport cycle. Our molecular dynamics simulations have highlighted that one of these residues (E127 in ScaDMT, E193 in hDMT1) is responsible for H^+ -dependent stabilization of intracellular regions, as well as regulation of opening motions on the intracellular side of the transporter. Functional studies revealed that E193 mutants of hDMT1 indeed have altered sensitivity toward pH changes, with reduced divalent metal ion uptake, suggesting a role for this residue in H^+ -coupled Fe^{2+} transport. In support of our findings, we propose a transport mechanism where E193 acts as a proton sensor that modulates the transport of Fe^{2+} across the membrane.

PP-17 A GRIN1/GRIN2A antagonist increases cell accumulation and cytotoxic efficacy of sorafenib in mouse hepatocellular carcinoma cells

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The most common types of solid tumors like hepatocellular carcinoma (HCC) inevitably acquire resistance against anticancer therapy. The increased expression of ABC transporters in cancer cells is a well-established cause of chemoresistance. Our hypothesis is that the most efficient strategy to reverse the ABC transporter mediated chemoresistance is to modulate the transporter expression instead of inhibiting the transporter function. GRIN1/GRIN2A is a heterodimeric subtype of *N*-methyl-*D*-aspartate receptor that is highly expressed on the cell membrane of cancer cells and takes part in the up-regulation of ABC transporter expression. We have developed selective GRIN1/GRIN2A antagonist (LBG-10119) with the aim of down-regulating ABC transporter expression and make cancer cells more susceptible for anticancer drugs. In addition, the aim was to develop antagonist, which is not able to cross the blood-brain barrier and thus avoid the risk of CNS adverse effects, common for GRIN1/GRIN2A antagonists.

The blood-brain barrier permeation of LBG-10119 was determined with the *in situ* mouse brain perfusion technique at 100 μM . The *in vivo* pharmacokinetics in mice were studied using 10 mg/kg bolus *i.p.* injection. The effect of LBG-10119 on cell accumulation of efflux transporter probes digoxin and fluorescein as well as anticancer drug sorafenib was studied in mouse HCC cells. In addition, the potentiating effect on sorafenib's cytotoxic efficacy was studied in HCC cell using resazurin based cytotoxicity assay.

LBG-10119 was not detected in the brain after the *in situ* brain perfusion, and *in vivo* pharmacokinetic analysis showed that the compound reached $\text{AUC}_{0-240 \text{ min}}$ of 3020 $\mu\text{M} \times \text{min}$ and 575 $\text{nmol/g} \times \text{min}$ in plasma and liver, respectively. Incubation of HCC cells for 72 h with 10 μM LBG-10119 increased the accumulation of the efflux probes fluorescein and digoxin resulting in 2-fold and 1.5-fold increase in the cell accumulation compared to control. In addition, 10 μM LBG-10119

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increased the cell accumulation of sorafenib significantly from 11.9 nmol/mg to 18.3 nmol/mg. No LBG-10119 was detected in the cells. Sorafenib reduced the HCC cell viability to 84 %, 78 % and 73 % with 1 μ M, 2.5 μ M and 5 μ M concentrations, respectively. The addition of 100 μ M LBG-10119 in the growth medium increased the cytotoxic efficacy of sorafenib leading to 33 %, 30 % and 35 % of remaining viability. LBG-10119 at 100 μ M did not have cytotoxic efficacy itself.

In conclusion, the GRIN1/GRIN2A antagonist LBG-10119 increases the cell accumulation of efflux probes and sorafenib. Importantly, LBG-10119 potentiates the cytotoxic efficacy of sorafenib in mouse HCC cells. As LBG-10119 was not detected in the cells, it is likely that the compound is not an efflux inhibitor, but the increased cell accumulation of efflux substrates is due to down-regulation of transporter expression. This will be further investigated by the use of mass spectrometry based quantitative transporter proteomics.

PP-18 Elucidation of the conformational changes of ABCG2 in permeabilized cells

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ABCG2 (BCRP) is an ABC transporter that is able to extrude a large variety of chemotherapeutic drugs from cells and it is often involved in the multidrug resistance of cancer cells. ABCG2 is a “half-transporter” consisting of a single nucleotide binding domain (NBD) and a single transmembrane domain (TMD). The functional transporter is formed by homo-dimerization of two identical “half-transporter” molecules. Based on crystal structures of several ABC transporters ABCG2 is believed to alternate between nucleotide-free inward and nucleotide-bound outward facing conformations. Previous studies revealed that the reactivity of ABCG2 with the 5D3 anti-ABCG2 mAb is dependent on the conformational changes of the transporter.

In the present study, we show that after washing out the ATP from cells permeabilized by bacterial toxins the ABCG2 molecules become trapped in a 5D3-reactive conformation. Addition of ATP at conditions either allowing (presence of ATP/Mg²⁺ at 37 °C) or preventing ATP hydrolysis (absence of Mg²⁺ or low temperature) switches ABCG2 into a 5D3-dim state in a nucleotide concentration dependent manner, suggesting that nucleotide hydrolysis is not required for this conformation change. Consistent with the formation of the ABCG2-ADP-V_i complex, addition of vanadate (V_i) increased the apparent affinity of nucleotide binding under hydrolysis conditions. However, when ATP hydrolysis was prevented, the apparent affinity was not influenced at all by V_i, confirming that ATP hydrolysis and the subsequent release of the γ -phosphate is a prerequisite for the formation of the ABCG2-ADP-V_i complex. We also found that ADP also brings about a switch of ABCG2 to the 5D3-dim conformation, although it binds to ABCG2 with lower affinity compared to ATP. Taken together, our results suggest that the conformational changes between the 5D3-reactive and non-reactive conformations are caused by nucleotide binding and dissociation.

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PP-19 Regulation of serotonin transporter activity in animal models of peripheral inflammation – relevance to inflammation-induced depression

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Depression is a complex disorder precipitated in susceptible individuals by various stress factors. Inflammation is thought to be one such stressor, and chronic inflammatory diseases are often associated with depression. Molecular mechanisms underlying inflammation-induced depression are poorly understood. One target for immune system modulation of neuronal function is the

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serotonin transporter (SERT), a key regulator of serotonergic neurotransmission and a primary antidepressant target. Employing the widely used lipopolysaccharide (LPS) model of sickness and depression-like behaviour, we show that SERT activity is upregulated in the frontal cortex of rats 24h after LPS injection. The increase in SERT activity is not caused by enhanced mRNA or protein expression but due to posttranslational modification of the transporter. To overcome limitations of the rather acute LPS model, we have recently established a more clinically relevant model of chronic inflammatory diseases, namely the collagen-induced arthritis (CIA) mouse model. In the CIA model SERT activity is elevated in the hippocampus but not in other brain regions. Similar to the LPS model, the upregulation of SERT activity in CIA mice occurs at a post translational level due to an increase in the V_{max} for 5-HT uptake and is trafficking independent. Moreover, CIA induced elevation of SERT activity is temporally correlated with symptoms of depression-like behaviour (anhedonia) in these mice, suggesting that this model is suitable to study molecular mechanisms underlying depression triggered by chronic peripheral inflammation. Together with previous studies by others our results suggest that the effects of inflammation on SERT regulation in the CNS varies in a temporal and spatial manner. These differences are likely to reflect the shift from acute sickness behaviour toward true depression-like behaviour under chronic inflammatory conditions. Thus, these animal models allow us to characterise the neurochemical and behavioural consequences of acute and chronic peripheral immune system activation, as well as *in vivo* mechanisms of serotonin transporter regulation relevant to human disease.

PP-20 Generation and characterization of a GlyT1b/c *knock-in* mouse

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The amino acid glycine acts as a neurotransmitter at many inhibitory synapses predominantly in the caudal regions of the central nervous system, where it binds to strychnine-sensitive Glycine Receptors. Additionally, glycine has been shown to act as an essential coagonist of N-Methyl-D-Aspartate Receptors. This dual function of glycine requires a strict regulation of the extracellular glycine concentrations, which is accomplished by high affinity glycine transporters (GlyTs). While GlyT2 is expressed by glycinergic neurons, GlyT1 expression is found throughout the CNS, by glial as well as neuronal cells.

Up to now three GlyT1 subtypes have been identified, GlyT1a, GlyT1b, and GlyT1c, which are encoded by a single gene. Whereas GlyT1b and GlyT1c share the same promoter, GlyT1a expression is controlled by a different promoter element. It was suggested that the expression pattern of the individual GlyT1 subtypes might correlate to the individual cell types expressing GlyT1. Here GlyT1a was suggested to be preferentially expressed by glial cells, GlyT1b by neurons, and GlyT1c in extra-neuronal tissues. Analysis of cell-type specific *knock-out* mice has suggested different functions of GlyT1 dependent on cell type, but detailed information about the function of the different isoforms are still missing.

In this study, the *in vivo* functions of GlyT1b/c were analysed by a genetic approach. To determine the function and expression patterns of GlyT1b, a GlyT1b/c reporter *knock-in* (GlyT1b KI) mouse line was generated. GlyT1b/c expression was abolished by insertion of a Luciferase-FP635 (LucR) double reporter downstream of the endogenous GlyT1b/c start codon. Accordingly, this mouse line should carry a complete GlyT1b/c deficiency, and simultaneously label the GlyT1b/c expressing cells.

The GlyT1b/c KI mice were born at the expected Mendelian ratio and survived to adulthood, although they showed reduced size and weight compared to wildtype littermates. They show a hypoactive phenotype, with intermittent strong hypotonic episodes.

Analysis of the reporter expression revealed highest GlyT1b/c promoter activity in caudal regions of the brain, but also in thalamus, cortex and hippocampus. In line with highest expression of GlyT1b in brain stem and spinal cord the GlyT1 specific uptake activity was strongly reduced in membrane preparations from these brain regions. Analysis of developmental changes in the reporter expression suggests that GlyT1b expression is strongly regulated with lowest expression around birth.

Taken together our data demonstrate that GlyT1b/c contributes to the control of glycine dependent (inhibitory and excitatory) neurotransmission also in the mature nervous system. Our data suggest that GlyT1b/c in addition to its function in the regulation of motor control also contributes to the

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control of anxiety. Further studies will have to analyse the electrophysiological effects of GlyT1b deficiency on excitatory and inhibitory synapses.

PP-21 Secrets of ABCG2/BCRP revealed by the new ABCG fold

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ABCG2 is an ATP Binding Cassette transporter protein, containing a nucleotide binding domain and a transmembrane region. It functions as a homodimer multidrug resistance (MDR) transporter in the plasma membrane, extruding a wide range of molecules from the cell. ABCG2 affects the pharmacokinetics of various drugs, and protects the stem cells and cancer (stem) cells from xenobiotics and chemotherapeutic agents. This transporter is present in key pharmacological barriers and drug metabolizing organs. However, ABCG2 is also involved in the transport of endobiotics, e.g. porphyrines and uric acid. Its Q141K variant exhibits decreased functional expression leading to increased drug accumulation and decreased urate secretion. To overcome MDR and correct ABCG2 related pathological phenomena, learning its structural and dynamic properties is a major objective in the field. Still, there has been no reliable molecular model available for this protein, as the published structures of other ABC transporters could not be properly fitted to the ABCG2 topology and experimental data. The recently published high resolution structure of a close homologue, the ABCG5/ABCG8 heterodimer, revealed a new ABC transporter fold, unique for ABCG proteins. We generated a structural model of the ABCG2 homodimer based on this fold and confirmed its validity using experimental data. We also performed molecular dynamics simulations and in silico docking to understand the effect of mutations including R482G and Q141K and the mechanism of substrate recognition. The ABCG2 model we present may have significant impact on understanding drug distribution and toxicity, as well as drug development against cancer chemotherapy resistance or gout.

PP-22 The external gate of the human and Drosophila serotonin transporters requires a basic/acidic amino acid pair for 3,4 methylenedioxymethamphetamine (MDMA) translocation and the induction of substrate efflux

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The substituted amphetamine, 3,4-methylenedioxy-methamphetamine (MDMA, ecstasy), is a widely used drug of abuse that induces non-exocytotic release of serotonin, dopamine, and norepinephrine through their cognate transporters as well as blocking the reuptake of neurotransmitter by the same transporters. The resulting dramatic increase in the synaptic levels of these neurotransmitters leads to psychotropic, stimulant, and entactogenic effects. The mechanism by which amphetamines drive reverse transport of the monoamines remains largely enigmatic, however, promising outcomes for the therapeutic utility of MDMA for post-traumatic stress disorder and the long-time use of the dopaminergic and noradrenergic-directed amphetamines in treatment of attention-deficit hyperactivity disorder and narcolepsy increases the importance of understanding this phenomenon. Previously, we identified functional differences between the human and Drosophila melanogaster serotonin transporters (hSERT and dSERT, respectively) revealing that MDMA is an effective substrate for hSERT but not dSERT even though serotonin is a potent substrate for both transporters. Chimeric dSERT/hSERT transporters revealed that the molecular components necessary for recognition of MDMA as a substrate was linked to regions of the protein flanking transmembrane domains (TM) V through IX. Here, we performed species-scanning mutagenesis of hSERT, dSERT and C. elegans SERT (ceSERT) along with biochemical and electrophysiological analysis and identified a single amino acid in TM10 (Glu394, hSERT; Asn484,

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dSERT, Asp517, ceSERT) that is primarily responsible for the differences in MDMA recognition. Our findings reveal that an acidic residue is necessary at this position for MDMA recognition as a substrate and serotonin releaser.

PP-23 Whole exome sequencing of SLC transporters in chemotherapy outcome of breast carcinoma patients

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Background and Aims: Breast cancer is the second most common cancer in women worldwide. Solute carrier (SLC) transporters can confer to multi-drug resistance via decreased influx of anticancer drugs. The major goal of our study was to investigate associations of the expression and genetic variability of SLC transporters with prognosis and response of breast carcinoma patients.

Material and methods: Expression profile of twenty-one human SLC transporters was evaluated in 33 post-treatment tumor tissue samples from patients treated by neoadjuvant chemotherapy regimens based on 5-fluorouracil, anthracycline and/or taxanes and in 50 tumor tissue samples taken from patients prior to any treatment. Paired non-tumor control tissues were available from a subset of the patients. SLCO1A2/OATP1A2 and SLC19A1/FOLT protein levels were detected by western blot in tumors (n = 42). Whole exome sequencing of SLC46A1 and SLCO1A2 was performed by SeqCap EZ choice (Roche) on 454 GS Junior (Roche) platform in a subset of 36 neo-adjuvantly treated patients.

Results: Significant associations of intratumoral transcript levels of *SLC46A1* with the expression of hormonal receptors were found in both sets. Higher *SLCO1A2* transcript levels were significantly associated with the response (partial or complete) of neo-adjuvantly treated patients. *SLC19A1* associated with shorter progression-free survival (PFS) of neo-adjuvantly treated patients.

Numerous variants were found in coding regions and neighboring intron segments of *SLCO1A2* (157 variants) and *SLC46A1* (105 variants). Three single amino acid (AA) substitutions were predicted as damaging *in silico*. E172D AA change in rs11568563 and F265L in a novel SNP (chr12:21453399 A>G) of *SLCO1A2* are located in Major Facilitator Superfamily domain, the large and diverse group of secondary importers. Fourteen genetic alterations were considered as likely affecting binding of transcription factors (Regulome DB) in *SLC46A1*.

Conclusion: *SLC46A1* and *SLC19A1* represent candidate genes potentially modifying progression and prognosis of breast cancer patients. *SLCO1A2* represents potential predictive marker and target for the design of a new therapy in patients with breast carcinoma. Validation of these putative biomarkers in independent follow-up studies and study of underlying mechanisms are now in our focus.

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PP-24 Insights into the molecular basis for substrate binding and specificity of the wild-type L-arginine/agmatine antiporter AdiC

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Pathogenic enterobacteria need to survive the extreme acidity of the stomach in order to successfully colonize the human gut. Enteric bacteria circumvent the gastric acid barrier by

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activating extreme acid-resistance responses such as the arginine-dependent acid resistance system. In this response, L-arginine is decarboxylated to agmatine thereby consuming one proton from the cytoplasm. In *Escherichia coli*, the L-arginine/agmatine antiporter AdiC facilitates the export of agmatine in exchange of L-arginine, thus providing substrates for further removal of protons from the cytoplasm and balancing the intracellular pH. We have solved the crystal structures of wild-type AdiC in the presence and absence of the substrate agmatine at 2.6 Å and 2.2 Å resolution, respectively. The high-resolution structures made possible the identification of crucial water molecules in the substrate-binding sites, unveiling their functional roles for agmatine release and structure stabilization, which was further corroborated by molecular dynamics simulations. Structural analysis combined with site-directed mutagenesis and the scintillation proximity radioligand binding assay improved our understanding of substrate binding and specificity of the wild-type L-arginine/agmatine antiporter AdiC. Finally, we present a potential mechanism for conformational changes of the AdiC transport cycle involved in the release of Agm into the periplasmic space of *E. coli*.

PP-25 The human LAT1 (SLC7A5) transporter: function and relationships with its ancillary companion CD98 (SLC3A2).

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LAT1 (SLC7A5) is a neutral amino acid transporter characterized by heterodimeric structure with the glycoprotein CD98 (SLC3A2). The interaction occurs via a disulfide between conserved Cys-164 of LAT1 and Cys-109 of CD98. The LAT1/CD98 heterodimer is localized in plasma membranes and its function in mediating amino acid transport has been firstly assessed in cell systems [1]. Very recently, a lysosomal localization of LAT1 has been also described, which is important for mTORC1 activation [2]. The terrific interest in LAT1 function and structure derives from its over-expression in many tumors suggesting this protein as a novel pharmacological target [3]. In spite of the huge number of papers on this transporter published in the last decade, very few information is available on its structure/function relationships. Using complementary experimental models such as intact cells systems, proteoliposomes and recombinant over-expressed human LAT1 (hLAT1), we have defined several issues concerning LAT1 structure/function relationships. Histidine is a major substrate, while other amino acids are transported with preferential binding on the external or the internal side of the transporter. hLAT1/CD98 heterodimer has been extracted from SiHa cells, reconstituted in proteoliposomes and transport has been studied as BCH sensitive [³H]His(ex)/His(in) antiport. The S-S reducing agent DTE stimulated transport activity indicating that covalent interaction (disulfide) of Cys-164 of hLAT1 with Cys-109 of hCD98 is not required for transport function. Using the recombinant hLAT1 alone, transport activity overlapping that measured with intact cells or with reconstituted native protein was detected, while no amino acid transport could be measured in hCD98 reconstituted proteoliposomes. In conclusion, it has been demonstrated that hLAT1 is the transport competent unit of the heterodimer, while CD98 has no role in substrate recognition and transport. Residues critical for function have been identified by bioinformatics, on the basis of the homology model of hLAT1 obtained using as template the structure of Arginine/Agmatine transporter (AdiC) from *E. coli*.

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PP-26 Ligand binding site comparison of human, mouse and rat P-glycoprotein transporter

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P-glycoprotein (P-gp) is an ABC transporter of the multidrug resistance (MDR) sub-family, extensively expressed in the intestinal epithelium, hepatocytes and the blood brain barrier among other tissues. The transporter protein is involved in ATP-dependent efflux of xenobiotic compounds with broad substrate and inhibitor specificity. It plays a major role in multidrug resistance and influences the bioavailability of drug candidates.

In early stages of drug development, the pharmacokinetic and toxicity profiles of a drug candidate are determined in animal models (usually mouse and rat) before it is tested in human beings. Therefore, besides developing predictive *in silico* models for the identification of inhibitors for human P-gp, it is beneficial to establish predictive models for mouse and rat to reduce the number of compounds to be tested further stages. However, sufficient data is not available to build predictive models for rat and mouse P-gp.

The aim of this study is to compare the binding site interaction profiles of human, rat and mouse P-gp to assess the usability of *in vitro* human activity data in development of *in vivo* prediction models for rat and mouse.

Since no human and rat X-ray crystal structures are available, homology models were built based on the corrected mouse P-gp structure (PDB code: 4M1M) using MODELLER 9.13[1]. Due to lack of availability of rat P-gp inhibition data in public bioactivity databases (ChEMBL, BindingDB etc.), exhaustive literature search was performed in PubMed that fetched a total of 18 rat P-gp inhibitors. Subsequently, GLIDE docking was used to dock known P-gp inhibitors into the human, mouse and rat structures, followed by analysis of ligand interactions with protein binding site residues.

The generated human and rat homology models show a high stereochemical quality with normalized DOPE scores of -0.741, -0.804, and Qmean values of 0.630, 0.632 respectively. The sequence identity of the human, rat and mouse transmembrane domains were greater than 60%. Docking results further showed a significant overlap between the binding site interacting residues in the three species, whereby the residues Phe, Ile, Tyr in transmembrane helix (TMH) 5, Phe, Leu and Ile in TMH 6, Met, Tyr in TMH 11 and Phe, Met, Ala, Gln in TMH 12 are involved.

This indicates the possibility of similar binding mode of the human, mouse and rat P-gp inhibitors, thus pointing towards the usability of *in vitro* human P-gp data for *in vivo* rat and mouse prediction models.

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PP-27 Oligomerization of human dopamine transporter (hDAT)

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Monoamine transporters of the SCL6 family are found on presynaptic neurons and terminate the neurotransmission by depleting the presynaptic cleft by reuptake of neurotransmitters. The group monoamine transporter includes the dopamine transporters (DAT), the serotonin transporters (SERT) and the norepinephrine transporter (NET) which couple substrate transport with ion gradients of sodium and chloride. They play a vital role in the normal functioning of the central nervous system. Dysfunction of these transporters lead to depression, Parkinson disease etc. These transporters are known to function as oligomers in the cell membrane and studies reported that different oligomeric sizes are present. However the binding orientations and the residues

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critical for the oligomerization are poorly understood. In this study we extensively scrutinized the oligomeric forms of the human dopamine transporter (hDAT) using molecular dynamics simulations and applying the MARTINI coarse grained force field. Two monomers of hDAT were inserted into the POPC membrane in random orientations and the assembly of the monomers were analyzed over 2 μ s time. The convergence of the simulations are shown by the interaction energy plot and the orientations identified by density plot. This study indicates that hDAT forms dimers through four distinct orientations. These results allowed us to propose a testable hypothesis of residues located within the oligomer interface.

PP-28 Biophysical characterization of Human Sideroflexin 5

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Mitochondria are a hub for cellular metabolism, with transport of substrates across the impermeable inner mitochondrial membrane essential for many physiological processes. Proposed transport proteins are the Sideroflexin (SFXN) family that have been implicated in mitochondrial disease with neurological, motor and hematological manifestations, among others. Using cellular models, the molecular function of this protein family remains unclear. Presented here is the isolation of human SFXN5 protein using recombinant protein expression in *Saccharomyces cerevisiae* and downstream biophysical analysis. For developing the protein towards structural studies, natively bound lipid species have been elucidated by thin-layer chromatography and a thermal stability assay based on protein thiol chemistry (CPM assay) to determine the affect of supplementation of lipid on protein stability. The CPM assay is also used to determine protein stability in a range of detergents and pH, to push the protein into conditions amenable to crystallography. Identification of the SFXN family function will aid in basic and translational mitochondrial research, and structural detail of the protein will lead to elucidation of the protein mechanism.

PP-29 How does the N-terminus of the human serotonin transporter regulate the conformational cycle of the transporter?

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The monoamine transporter for serotonin (SERT) mediates the re-uptake of serotonin, thus terminating neurotransmission. SERT is a sodium/chloride-dependent symporter: substrate and co-transported ions bind the outward facing state of SERT, which subsequently undergoes a series of conformational changes to reach the inward facing state. After release of the cargo, SERT completes the cycle by returning to the outward facing state. Alternatively, SERT can operate in the exchange-mode: substrate and ions are bound by the inward-facing state, which then returns to the outward facing state without completing the cycle. This mode is triggered by amphetamines (amph): SERT seesaws through half-cycles, where amphetamines are transported inward to drive serotonin efflux. The switch between forward and reverse mode is accounted for the alternate access model; the structural correlates are the conformational changes in the hydrophobic core of SERT. In addition, regulatory input (e.g., N-terminal phosphorylation) and the oligomeric state of SERT modify the extent of amph-induced release, supporting the N-terminal lever hypothesis: efflux relies on an oligomeric arrangement, where the N-terminus of one amph-bound SERT moiety triggers the counter-transport of substrate by the adjacent moiety. The two models are not mutually exclusive. The aim of the current study was to explore how the hypothetical lever action of the N-terminus affects the transition of SERT through the conformational cycle. Limited tryptic digestion showed that the conformation of the N-terminus of SERT differed in the empty inward- and outward-facing and the amph-bound state: in the absence of Na⁺ (i.e. in the inward-facing state), the N-terminus of SERT was rapidly cleaved, but the presence of *para*-chloroamphetamine protected the N-terminus against trypsin. This showed that the conformational state of the hydrophobic core was relayed to the N-terminus. It also predicts a segment in the N-terminus that binds the hydrophobic core of a SERT moiety. Accordingly, we generated truncations by removing the first 22, 32 or 42 amino acids of the N-terminus (SERT- Δ N22, -32 or -42 constructs). All constructs were expressed at the cell surface, bound inhibitors and transported substrate in the

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forward mode with affinities and turnover numbers comparable to the WT. In contrast, efflux was greatly reduced in cells expressing $\Delta N32$ or $\Delta N42$ (but not $\Delta N22$). The individual steps of the transport cycle were assessed by measuring currents associated with substrate binding (*i.e.*, capacitive gating currents) and transport in the forward mode (*i.e.*, steady-state currents) using whole cell patch clamp. The recovery rate of these currents was significantly lower for $\Delta N32$ than for WT-like $\Delta N22$, showing that $\Delta N32$ is less likely to operate in the reverse mode. These findings are consistent with the lever hypothesis and allow for a unified model, where the role of the N-terminus is to impose a switch in the kinetic cycle.

PP-30 Molecular structure and function analysis of human ABCG2 based on crystal structure of human ABCG5/G8

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Multidrug resistant (MDR) in cancer is a crucial problem for tumor treatment. Unregulated expression and malfunction of ABCG2 (Breast Cancer Resistance Protein, BCRP) was identified as a major cause of MDR in human. The understanding of the structure-function relationship and of the transport mechanism of ABCG2 would be a valuable advantage for improving clinical therapy and developing novel drugs for cancer medication.

In our study, we applied both theoretical and experimental approach to predict and generated the topology structure of human ABCG2. Sequence alignments and biophysical properties analysis of all mammalian ABCGs and yeast PDRs revealed several prominent structural elements like the presence of Elbow helix at the beginning of TM1 and the re-entry helix immediately following TM5. Recently, the crystal structure of the heterodimeric human sterol transporter ABCG5/G8 has been resolved (Lee J.Y., *et al.*, Nature 2016). We found many of our predictions in structural features of an ABCG2 model developed using the ABCG5/G8 structure as template. To validate predictions from our analysis we evaluated mutation of the intra- and extracellular loops. Thereby, we identified charged residues that are located close to the transmembrane interface and in the extracellular loops. These residues were selected to determine, if they are involved in charge-charge interaction, important for folding or simply exposed to water. Importantly, we are interested, if these residues are involved in regulating conformational changes during substrate translocation. The importance of charged residues were examined by using neutralizing and charge-reversal mutants. Mutant were expressed in mammalian cells and examined for protein expression, protein stability, membrane localization and the efflux of the ABCG2 substrate mitoxantrone. Specificity was evaluated by the presence of the ABCG2-specific inhibitor Ko143. Our results showed that negative charged residues which are located in the first intracellular loop of the TMD have high impact on protein expression as well as function. In contrast, the positive charged residues of the intracellular loop 1 have a low effect on transport function. The results validate or structural model based on ABCG5/G8 which therefore can serve structure-function relationship analysis and can be used to elucidate the transport mechanism of ABCG2.

PP-31 Oxysterol-related transporters in breast cancer patients: gene expression study

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Oxysterols are oxidative derivatives of cholesterol that naturally occur in human body and play important roles in physiological and pathological processes. This study is focused on the role of oxysterols in hormonal therapy of estrogen receptor-expressing (ER+) breast cancer patients. Current studies revealed that 27-hydroxycholesterol binds to ER molecule and modulates its activity. Oxysterols also bind anti-estrogen binding sites and thus may interfere with hormonal

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therapy drugs, e.g., tamoxifen. In this study, we focused on oxysterol-related transporters that can influence the oxysterol level in cell.

Tumor tissue samples from a total of 193 breast cancer patients were collected and included in this study. In the first part of the study, gene expression of 14 ABC and four SLCO genes was quantified and compared between 17 ER+ and 12 ER- breast tumors. Genes with significantly different expression were analyzed in the next phase of study, where the gene expression level was measured in 164 ER+ breast cancer samples and compared with clinical data of patients.

Six ABC genes with different expression between ER+ and ER- tumors, namely *ABCA2*, *ABCA9*, *ABCA10*, *ABCC1*, *ABCG1*, and *ABCG2* – and solute carrier transporter gene *SLCO1A2* were included in the second part of the study. *ABCA2*, *ABCA9*, *ABCA10* and *ABCG2* significantly associated with tumor stage, grade, and Ki67 expression. *SLCO1A2* gene expression was below the limit of quantification in most samples.

In conclusion, four oxysterol-transporting ABC transporter genes seem candidates for future studies. The most interesting candidate is *ABCG2* gene whose expression associated with disease-free survival of patients. Analysis of protein level and genetic variability of these candidate genes will be the next step of our study.

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PP-32 Two-Class Classification of Drug-Induced Cholestasis by Integrating Hepatic Transporters' Predictions

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Cholestasis, together with hepatocellular and mixed type, is one of the three types of drug-induced liver injury (DILI), one of the major challenges in drug development.[1] In particular, cholestatic and mixed hepatocellular and cholestatic type are the two most severe manifestations of DILI and yield almost half of the recorded cases of DILI.[2] There is growing evidence indicating the important role of hepatic transporters for the development of cholestasis. More specifically, there is references in literature implicating BSEP, MDR3, BCRP, MRP2, P-gp, NTCP, OATPs, MRP3 and MRP4.[3] In this study we used k-nearest neighbors as a base-classifier, in order to perform 2-class classification for cholestasis, using a set of 93 2D physicochemical descriptors and predictions of hepatic transporters' inhibition. Among the transporters predictions used are BSEP, BCRP, P-gp, OATP1B1 and OATP1B3. Moreover, we investigated the suggested by literature implication of hepatic transporters in cholestasis. For this, we compared whether the inclusion of the transporters' inhibition predictions contributes to a significant increase in model's performance in comparison to the plain use of the 93 2D physicochemical descriptors. Our findings showed that including the hepatic transporters predictions for modeling cholestasis significantly improves the model's performance. Interestingly, our findings seem to implicate not only BSEP –which is considered the most associated with cholestasis transporter- but suggest a synergistic effect from the contribution of all hepatic transporters. The final optimal model is validated via both 10-fold cross validation and external validation. It performs quite satisfactory resulting in 0.688 ± 0.011 for accuracy and 0.727 ± 0.014 for AUC for 10-fold cross-validation (mean $\pm$ standard deviation from 50 iterations).

Acknowledgements

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PP-34 Complementary in vitro tools to investigate renal drug transport

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Renal excretion is an important pathway for the elimination of endogenous and xenobiotic substances. A wide range of efflux and uptake transporters are expressed in the renal epithelial cells to regulate the excretion and the reabsorption of various kinds of organic anions, cations, peptides and nucleosides. The primary proximal tubule cell (PTC) monolayer assay, established by Dr Colin Brown (Newcastle University, UK) is a unique in vitro approach for investigating renal drug handling, identifying drug-transporter interactions, potential nephrotoxicity and transporter mediated drug-drug interactions. Unlike other primary renal cell models, this monolayer assay maintains the full complement and expression level of endogenous renal transporters, resulting in a more physiologically relevant, and therefore predictive, model of drug handling in the clinical setting. While the PTC model serves as a holistic approach to study renal handling of drug molecules and endogenous substrates, double transfected monolayer assays could complete the results gained in PTC model with functional characteristics, such as substrate specificity and transport mechanisms involved in the renal elimination. Certain double transfected monolayer assays have been developed to investigate the underlying mechanisms of renal elimination of both organic anion (MDCKII-OAT1/BCRP and MDCKII-OAT3/BCRP) and cation (MDCKII-OCT2/MATE1 and MDCKII-OCT2/MATE2-K) compounds. Transport of selected drugs and physiologically relevant endogenous substrates such as metformin, methotrexate, PAH, urate, TEA and E3S has been investigated with both PTC and appropriate double transfectant monolayers. The results highlight the complementary nature of the two models.

PP-35 Vortioxetine binding to select serotonergic G-protein coupled receptors

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Major depressive disorder is commonly linked to imbalances in the serotonergic nervous system, and it has traditionally been treated by inhibiting the serotonin transporter (SERT), thus decreasing the rate of serotonin uptake and prolonging signaling times¹. The traditional treatments show a high degree of side effects and a high number of non-responding patients, and drug development has thus recently been focused on relieving more of these side effects by targeting several serotonergic proteins at once. An example of such a recent antidepressant is vortioxetine² which targets SERT, the serotonergic cys-loop ion channel receptors, and a subset of the serotonergic G-protein coupled receptors (GPCRs). Vortioxetine binding to SERT has been established,³ but how vortioxetine binds to the receptors remains unclear. We set out to determine how vortioxetine binds to the serotonergic GPCRs 5HT_{1A}, 5HT_{1B}, and 5HT₇. These receptors were chosen because vortioxetine acts differently to these three targets; being a full agonist at 5HT_{1A}, a partial agonist at 5HT_{1B}, and an antagonist at 5HT₇. We applied an induced fit docking protocol using both crystal structures and homology models in combination with molecular dynamics simulations and free energy calculations to assess the most likely binding mode of vortioxetine on each GPCR. Our results suggest that vortioxetine occupies a similar binding pocket in all three receptors, but with significant variations that aid in explaining the different effects of vortioxetine when binding to the different GPCRs.

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PP-36 A framework for allosteric activation of secondary active transport

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We recently showed that Zn^{2+} is an allosteric activator of substrate transport by the human dopamine transporter (DAT). The ability to stimulate a secondary active transporter is a desired ligand quality (i.e. in the treatment of diseases caused by loss of function mutants). However, to date no drug is available that was described to work this way. In this study, we decided to further explore this subject by taking advantage of DAT and the fact that it is endowed with an endogenous binding site for Zn^{2+} . This site can accommodate the divalent cation Ni^{2+} , which we identified as a stimulator of substrate uptake. This extended our repertoire of testable modulators and helped us pinpoint novel aspects in the stimulation of transport by such ligands. These accelerate the transport rate by displaying differing affinities for conformational intermediates adopted in the transport cycle. In this study we show that activators of substrate transport do not only differ in their affinities for the outward and inward facing conformations (as previously described) but also in their affinities for the apo and substrate bound conformations. Moreover, we demonstrate that preferred binding of the apo conformations can render their stimulatory action less sensitive to inhibition by high intracellular Na^+ concentrations. We show that augmented stability against a rise in the intracellular Na^+ concentration is achieved by conversion of a higher affinity/lower capacity into a lower affinity/higher capacity transporter.

PP-37 A prediction method for P-glycoprotein-mediated drug-drug interactions at the human blood-brain barrier based on results from (R)-[^{11}C] verapamil PET studies

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P-glycoprotein (P-gp) is expressed at the blood-brain barrier (BBB) and plays a key role in restricting brain penetration of several drugs. Concomitant administration of drugs which are transported by P-gp may lead to P-gp-mediated drug-drug interactions (DDIs) at the BBB causing central side effects. Despite the widespread availability of *in vitro* information on the interaction of drugs with P-gp, methods to predict the *in vivo* DDI potential of drugs are rare. The purpose of this study was to establish a prediction method for brain DDIs based on previous results from positron emission tomography (PET) studies in rats and humans with the P-gp substrate (R)-[^{11}C] verapamil (VPM) ¹⁻³.

At first, we established physiologically-based models for brain distribution of VPM in rats and humans. To predict human brain concentration-time profiles of VPM using this model, we had to determine the efflux clearance of VPM from brain via P-gp ($\text{CL}_{\text{efflux}}$). We first performed a sensitivity analysis using rat PET data to estimate $\text{CL}_{\text{efflux}}$ of VPM from rat brain. The estimated value of rat $\text{CL}_{\text{efflux}}$ was 4 mL/min/kg, which was similar to cerebral blood flow in rats. Next, assuming that human $\text{CL}_{\text{efflux}}$ equals human cerebral blood flow, we tried to predict human brain concentration-time profiles of VPM using the human model. The predicted values of brain area under the concentration-time curve (AUC) and maximum concentration in brain (C_{max}) of VPM were 125 kBq*min/mL and 5.2 kBq/mL, respectively, which were similar to observed values calculated from human PET data (observed brain AUC and C_{max} were 142 kBq*min/mL and 3.0 kBq/mL, respectively). Finally, we predicted the effect of the P-gp inhibitor tariquidar on VPM brain penetration in humans based on the *in vitro* K_i value of tariquidar for inhibition of VPM transport via P-gp. It was predicted that AUC and C_{max} of VPM will be 2.1- and 1.5-fold increased by tariquidar co-administration, respectively, whereas observed brain AUC and C_{max} increases of VPM by tariquidar co-administration were 2.3-fold and 2.2-fold, respectively.

Our results suggest that the established model can predict the brain concentration-time profile and DDI potential of P-gp substrates in humans.

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PP-38 Pharmacological Characterization of Fluorinated Phenmetrazines

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Background: Legal highs, commonly referred to as research chemicals or bath salts, comprise a large body of psychoactive compounds which are not covered by legal regulations. Generally, the chemical structures of these drugs are based on parental compounds, which interact with transporters or receptors for neurotransmitters in the nervous system. However, these compounds elude the legal regulations by chemical modifications. Since the first wave of legal highs, which comprised the bath salts mephedrone and 3,4-methylenedioxyparovalerone (MDPV, "zombie drug"), new psychoactive substances (NSPs) enter the illegal drug market at an alarming rate. Recently, a fluorinated version of the amphetamine-like drug phenmetrazine (PM, tradename Preludin) has become a thoroughly discussed psychostimulant in online drug forums. As, to our knowledge, no information is available on fluorinated phenmetrazines, we sought to test 2-,3- and 4-fluorophenmetrazine (2-FPM, 3-FPM, 4-FPM) for their psychoactive properties at monoamine transporters (MATs) for dopamine (DAT), serotonin (SERT) and norepinephrine (NET).

Methods: We used radiotracer flux experiments to determine the effects of the various FPMs on uptake and efflux mediated by MATs in heterologous expression systems. We further used *ex vivo* and *in vivo* experiments to validate the obtained from *in vitro* experiments. Both, efflux experiments carried out in synaptosomes prepared from whole rat brain and studies on forward locomotion in freely moving mice were applied.

Results: The tested phenmetrazine-derivatives were found to competitively inhibit uptake mediated by DAT, NET and SERT with affinities in the low micromolar range. Moreover, efflux studies revealed that 2-,3- and 4-FPM are capable of inducing efflux via all three MATs in a monensin and dose-dependent manner. Most importantly, systemic administration of 2-,3- and 4-FPM resulted in dose-dependent elevations of basal locomotion.

Discussion: The present study includes the first pharmacological characterization of a novel family of NSPs. The obtained datasets show that the fluorinated phenmetrazines exert their psychostimulant effects in an amphetamine-like fashion. As such, the presence of 2-,3- or 4-FPM in the nervous system results in efflux of the monoamines dopamine, serotonin and noradrenaline via their cognate high-affinity transporters. Knowledge about the molecular mechanism of action of drugs of abuse is a prerequisite to estimate the potential neurotoxicity associated with the abuse thereof. Release of model substrates evoked by 2-,3- and 4-FPMs from synaptosomes allows to make the educated guess, that abuse of the tested FPMs may bears the risk of enhanced neurotoxicity.

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PP-39 Cell-specific functions of the murine thyroid hormone transporter Mct8 (Slc16a2) within the hypothalamus-pituitary- thyroid axis

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The monocarboxylate transporter 8 (MCT8; SLC16a2) represents a specific thyroid hormone (TH) transporter that facilitates uptake and efflux of the prohormone T4 and the receptor active TH T3. Patients with inactivating mutations in the MCT8 gene suffer from a severe psychomotor retardation conceivably due to a deficient TH transport into the brain [Allan-Herndon-Dudley Syndrome (AHDS)]. Furthermore, AHDS patients exhibit abnormal serum TH levels with high T3 and low T4 levels as well as a central resistance to TH pointing to a compromised function of the hypothalamus-pituitary- thyroid axis in the absence of MCT8. The latter hypothesis was substantiated by studies in Mct8 ko mice that fully replicate the patients' endocrine abnormalities and exhibit elevated hypothalamic-thyrotropin releasing hormone (TRH) and pituitary-thyroid stimulating Hormone (TSH) expression. However, the exact pathogenic mechanisms by which these abnormal parameters are produced are still poorly defined.

Here, we report on the generation and analysis of Mct8 mutant animals that lack Mct8 either in forebrain neurons (FN), endothelial cells of the blood-brain barrier (BBB) or in tanycytes, thus in specialized glia cells located around the third ventricle. For this purpose, conditional Mct8 fl mice were crossed with transgenic mice expressing cre recombinase controlled by the promoter region of Camk2a (for FN-deletion), Slco1c1 (for BBB deletion) and Nestin (for tanycyte deletion), respectively. Successful inactivation of Mct8 in the respective cell type was confirmed by immunohistochemistry and in situ hybridization analysis.

Analysis of serum TH levels in Mct8 mutant mice did not reveal any significant changes in circulating T3 or T4 levels. Moreover, mice lacking Mct8 specifically in forebrain neurons did not show any changes in hypothalamic TRH expression indicating that Mct8 is not critical for the transport of T3 into T3-sensing hypothalamic TRH- neurons. However, absence of Mct8 at the BBB strongly induced hypothalamic TRH mRNA levels to a similar extent as in the global Mct8 ko mice. This finding underscores the importance of Mct8 at the BBB for proper T3 transport and, consequently, accurate T3 sensing in hypothalamic neurons. Finally, inactivation of Mct8 in tanycytes did not affect hypothalamic TRH expression, but resulted in increased pituitary TSH expression. Ongoing experiments are expected to reveal to which extent a lack of proper TH sensing in Mct8 deficient tanycyte contributes to the elevated TSH levels found in Mct8 ko mice as well as in MCT8 patients.

PP-40 Investigating the intracellular hydrophobic lid of the human dopamine transporter *in silico* and *in vitro*

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Dopamine is a neurotransmitter involved in addiction, learning and memory. Dopamine is translocated by the human dopamine transporter [hDAT] through coupling to the transmembrane sodium gradient. Zinc is thought to regulate dopamine, as it has been found to specifically bind to and inhibit substrate uptake at physiological concentrations (10- 30 μ M). The mutations that disrupt the intracellular hydrophobic lid (Y335A and T432F) have been previously found to drastically reduce uptake activity of substrate by hDAT. This project investigates the effect of zinc inhibition on hDAT, with the help of a mutagenesis approach, both computationally and experimentally.

Transport activity of hDAT with mutations present in the intracellular hydrophobic lid can be rescued to near wild-type levels through the addition of zinc, in a concentration-dependent manner. In our computational approach, we found that a transient water file forms reaching from the intracellular milieu to sodium bound in the Na2 site.

The recovery of uptake activity in the presence of zinc will be fully investigated as to the conformational effects of these mutations and the effect of zinc on the normal transport cycle. The transient nature of the sodium 2 site water file may possibly explain the sodium leak of the Y335A mutant but a more thorough investigation is required. Further, molecular dynamics simulations of

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hDAT will be conducted in the presence of zinc for mutant and wild-type. A full series of simulations will also be conducted on the Y335A_T432F mutant.

PP-42 Understanding the role of apical pore tyrosine residues in substrate gating of human P-glycoprotein.

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ABCB1 is an ATP-binding cassette multidrug transporter that actively expels chemically diverse potentially toxic compounds from cells. The precise molecular mechanism underlying transport is not fully understood. Substrate translocation is thought to occur according to the concept of alternating access (Jardetzky 1966). The X-ray structure of a eukaryotic homolog of human ABCB1 from *Cyanidioschizon merolae* (Kodan et al. 2014), which is a homodimer, addressed the aspect of substrate gating and identified a tyrosine residue in the apex of the central cavity (Y358) as being part of the extracellular gate. We mutated analogous tyrosine residues in both the N- and C-terminal halves of human ABCB1 (Y310 and Y953) to alanine and characterized these mutants in both a steady state and a zero-trans efflux protocol. Our group previously showed that rhodamine 123, as well as other paradigmatic substrates, are able to bind to the transporter in two modes, which are related to each other by 180° rotational symmetry. Introduction of positively charged arginine residues in helix 2 (Q132R) and 8 (Q773R) deselects one of these two binding modes for rh123. The Y310A and Y953A mutations were combined with these mode selector residues. All single mutants showed reduced transport activity and higher steady state loading than wild type. One of them, mutant Y953A/Q773R showed loading, which was identical with that of negative controls (cells containing a nonfunctional transporter harboring the E556Q mutation). Nevertheless, the rate for zero-trans efflux was several-fold higher than the rate observed in the absence of a functional transporter. Therefore, this mutant can still transport rhodamine 123 along a concentration gradient, but lacks active transport characteristics. Similar behavior is found for mutant Y310A/Q725L/Q132R, but the effect can only be made visible, when combining it with the Q725L mutation (change of a polar to a hydrophobic residue (Leucine in both CmABCB1 (L135) and in the aligning position of the other half of human P-gp (L65)) while the other two mutants (Y310A/Q725L/Q773R; Y953A/Q132R) remained active transporters. Experiments identify symmetric tyrosine residues that are involved in formation of the extracellular gate of human ABCB1. Therefore rotational symmetry of the transporter is reflected in function, not only with respect to ATP and substrate binding, but also for the ultimate step of cargo translocation, which involves opening and closing of the extra cytoplasmic gates.

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PP-43 Swapping the selectivity of ring-substituted methcathinones for human dopamine and serotonin transporter

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Dopamine and serotonin transporters (DAT and SERT, respectively) are members of the solute carrier 6 (SLC6) transporter family. Both are associated with a number of human disorders and

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therefore, they are important targets for clinically relevant therapeutics. In addition, DAT and SERT are directly involved in the action of and for psychostimulant drugs, including cathinones and their methylated derivatives. Methcathinone (MCAT) and congeners are amphetamine-type drugs, which are characterized by high abuse potential. Evaluation of a large set of analogs showed that adding a bulky chemical group to the 4-position of the phenyl ring can change the selectivity between DAT and SERT in a synaptosomal release assay [1]. In particular, the serine in position 149 (S149) of the human DAT and the alanine in position 169 (A169) in the human SERT were proposed to act as key amino acids in the selectivity of these compounds [1, 2]. However, no functional studies are present in the literature to corroborate this hypothesis. Sequence alignments indicate that S149 in hDAT is homologous to A169 in hSERT. Therefore, we decided to generate mutations to exchange both amino acids and test whether this change swapped transporter selectivity among the following 4-MCATs: MCAT, 4-OCH₃, 4-Br MCAT, 4-CF₃ MCAT. A robust selectivity profile is maintained between the rat and the human transporters: for all the compounds tested, the selectivity conforms with previous data obtained from a synaptosomal release assay [1]. The only exception was seen with 4-OCH₃-MCAT suggesting that possible species differences can be present. Surprisingly, no difference was found between the wild-type and mutant transporters for all the 4-MCATs tested, both in hDAT and hSERT suggesting that the S149 in hDAT and A169 in hSERT are, on their own, not sufficient to determine selectivity of 4-MCAT analogs and that other residues, or more likely a combination of residues, are involved. Our conclusion is supported by pharmacoinformatics which suggests alternative mutations that can be combined with the mutations tested so far. Our study may provide important information for the synthesis of new therapeutic and possibly more selective compounds.

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PP-44 Inhibition of P-glycoprotein by curcumin and its synthetic derivatives

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Curcumin has shown promise as a chemopreventive agent against a variety of human cancers. However its functionality is limited and to overcome some of its limitations synthetic derivatives of curcumin have been designed. P-glycoprotein (P-gp) a plasma membrane bound ATP-binding cassette (ABC) transporter is known for enabling cancer cells to evade response to treatment through the efflux of chemotherapeutic agents.

This study assesses the impact of curcumin and the synthetic derivatives of curcumin on P-gp activity in parental drug sensitive CCRF-CEM and multidrug resistant CEM/ADR5000 cell lines. Our findings show that some of the synthetic derivatives are more cytotoxic than curcumin and also that they are able to inhibit the efflux activity of P-gp and slow for uptake of doxorubicin in the CEM/ADR5000 cell line in the doxorubicin uptake assay. Molecular docking studies also showed us the binding pocket within the P-gp where curcumin and its synthetic derivatives bind and this was on the modulatory site of the P-gp. ATPase assay further confirmed that curcumin and its synthetic derivatives did not cause basal- or verapamil-stimulated ATP activity.

curcumin and its synthetic derivatives could be potential lead agents against P-gp drug efflux activity which is causing multidrug resistance in cancer cells and this could be an addition in the methods being put forth to fight cancer

PP-45 New fluorescent methods for the functional investigation of drug transporters

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In the past few years, the role of OATPs (Organic Anion Transporting Polypeptides) in drug absorption, disposition, drug clearance and drug-drug interactions (DDI) has been intensely investigated. ATP Binding Cassette proteins (ABCs) have already been in the spotlight of drug developers for a long time. Members of both of these transporter families are expressed in tissue barriers and organs, like the intestine, liver, blood-brain barrier, placenta and kidney. It has also

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been shown that they have partially overlapping substrate specificity, which implies that they might cooperate in the translocation and homeostasis of their substrates. If these pathways are interrupted by DDIs, it might lead to adverse reactions or even toxicity. Therefore, predicting potential interactions with OATP- and ABC- drug transporters is of great scientific interest.

The major impediment to large scale screen drug-transporter interaction tests is the limited availability of suitable test substrates. Fluorescent compounds are more convenient, safe and cost-efficient than isotope-based functional assays. Therefore, fluorescent OATP or ABC substrates are good candidates for large scale screens considering issues of sensitivity and costs.

Here we describe new fluorescent substrates for OATPs, 1B1, 1B3 and 2B1, as well as for ABCs, ABCC2 and ABCG2. We have confirmed the transport of these fluorescent molecules in intact cells or membrane vesicles with elevated OATP or ABC expression. Moreover, by using stable cell lines we have developed a new fluorescent substrate based assay for functional test of OATPs (1B3, 2B1) which is suitable for screening of novel OATP-interacting molecules.

PP-46 Identification and improvement of modulators of Divalent Metal Ion Transporter 1 (DMT1)

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DMT1 is a proton coupled iron transporter which plays an essential role in iron homeostasis, and whose deregulation is linked to hemochromatosis. Our aim is to develop small molecule inhibitor of DMT1 which can be used further for physiological studies or future developments in therapeutic applications. Starting with inhibitors previously reported as DMT1 inhibitors, we performed ligand-based virtual screening (LBVS) in the ZINC database using our recently reported shape and pharmacophore similarity algorithm xLOS,^[1] and purchased over 200 pharmacophore analogs, which led to the identification of 2 hit compounds.^[2] One of this compound proved to act as a non-competitive inhibitor of DMT1 and was therefore investigated further. While analogs could not be identified from commercial catalogs, the compound was readily prepared by condensation of the parent hydrazine with acetylacetone, which enabled the synthesis of a series of analogs from which an optimized inhibitor reaching the activity of the parent literature compound was identified. This new inhibitor has a significantly smaller size and therefore a better ligand efficiency compared to references .

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PP-47 Vesicular glutamate transporters use the glutamate binding site to transport inorganic phosphate

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Vesicular glutamate transporters (VGLUTs) accumulate the neurotransmitter glutamate in synaptic vesicles. Transport depends on a V-ATPase dependent electrochemical proton gradient ($\Delta\mu\text{H}^+$) and is proposed to be regulated through two anion and one cation binding site located on VGLUT, allowing the transporter to adjust to the changing ionic conditions during vesicle filling. Intriguingly, VGLUTs, when inserted into the plasma membrane, are functioning as sodium phosphate cotransporters, with transport occurring in the direction opposite to glutamate transport. How these two transport activities in opposing directions can be accommodated by the same molecule and whether there is interference between these activities remains unresolved.

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Using a reconstitution approach, we used an exogenous proton pump to drive VGLUT-mediated transport either in liposomes containing purified VGLUT1 or in synaptic vesicles fused with proton-pump-containing liposomes.

Our data show that phosphate binds to the glutamate binding site and competes with the transport of glutamate and chloride. Moreover, VGLUT actively transports phosphate in a $\Delta\mu\text{H}^+$ dependent manner. In addition, we observed a Na^+ -dependent phosphate transport activity in VGLUT under conditions mimicking the plasma membrane orientation, which was inhibited by glutamate and chloride.

We conclude that VGLUT transports phosphate through the glutamate binding site but with a lower affinity. Furthermore, when VGLUT is localized on the plasma membrane, phosphate transport is strictly coupled to Na^+ , thus utilizing the inwardly directed Na^+ gradient as a driving force for uptake.

PP-48 Brain uptake and pharmacokinetics of targeted ketoprofen prodrugs in mice

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PURPOSE: Blood-brain barrier (BBB) is the main obstacle for the delivery of drugs to the central nervous system. The utilization of L-type amino acid transporter 1 (LAT1) highly expressed in the BBB holds promise for the targeted drug delivery into the brain. The aim of the study was to evaluate the BBB-uptake mechanism and systemic pharmacokinetics of novel prodrugs of ketoprofen designed to utilize LAT1.

METHODS: In the present study, five amino acid prodrugs of ketoprofen were synthesized and their affinities for LAT1 were assessed *in vitro* in LAT1-overexpressing human breast cancer cells (MCF-7). The prodrugs' ability to cross the BBB was studied using *in situ* mouse brain perfusion technique. In addition, the pharmacokinetic profile (plasma, brain and liver) of the prodrugs, ketoprofen and its release from the prodrugs were determined *in vivo* in mice after 25 $\mu\text{mol/kg}$ i.p. bolus injection. The concentrations of the compounds were determined by the developed liquid chromatography-mass spectrometry method after simple and rapid sample preparation.

RESULTS: The prodrugs inhibited the uptake of LAT1 substrate [^{14}C]-L-leucine in MCF-7 cells by 24-93%. All of the compounds were able to cross the BBB with the K_{in} values ranging from 0.60×10^{-5} to 4.48×10^{-5} mL/s/g. *In vivo* experiments revealed that all prodrugs reached the brain after i.p. injection. Importantly, two prodrugs released ketoprofen in the brain. Thus, the brain to plasma (K_p) – values of the released ketoprofen were 2- and 4-fold higher compared to the K_p -value after ketoprofen dosing. Furthermore, $\text{AUC}_{\text{liver}}$ values of ketoprofen released from the prodrugs were significantly lower compared to $\text{AUC}_{\text{liver}}$ of ketoprofen itself.

CONCLUSIONS: All the designed prodrugs were able to bind to LAT1 *in vitro* and cross the mice BBB. Two of studied prodrugs efficiently delivered ketoprofen into the brain. Compared to ketoprofen dosing, the peripheral exposure to released ketoprofen from the prodrugs was significantly lower. Therefore, the development of novel prodrugs designed to utilize LAT1 can offer the means for targeted delivery of neuroprotective agents, such as ketoprofen, into the brain, simultaneously avoiding peripheral adverse effects.

PP-49 A potential role of the putative mitochondrial transporter ABCB6 in vacuolar detoxification

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Given the controversy regarding the cellular localization and function of ABCB6, our goal was to investigate the ABCB6 in yeast. Based on evolutionary trees built from alignment of amino acid sequences the ABCB6 may be considered as a homologue of the heavy metal tolerance factor 1 (HMT1) of *Schizosaccharomyces pombe*. Half ABC transporters of the HMT subfamily are required for heavy metal tolerance in Fission yeast. It has been discovered that SpHMT1 transports cadmium into the vacuolar compartment which is functionally analogous to the mammalian

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lysosome. Our goal was to investigate the function of the ABCB6 in yeast model system: localization of ABCB6 was analyzed by fluorescent microscopy; the strains expressing ABCB6 was assayed in cytotoxicity experiments. Our results will be presented in the context of the prediction that ABCB6 is a functional orthologue of HMT1, pumping transition metal complexes into the interior of acidic vesicles.

PP-50 Quo vadis ABCB6? – Characterizing intracellular trafficking of ABCB6

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Characterizing intracellular trafficking and determining the correct subcellular localization of ATP-binding cassette (ABC) transporters might provide an important basis and a good starting point for further studies to reveal the roles of these transporters in cellular physiology and in pathologic conditions. In earlier studies ABCB6 was classified as a mitochondrial ABC transporter, responsible for the mitochondrial uptake of porphyrins. Recent studies have shown that endogenous ABCB6 is localized to the endo/lysosomal compartment of cells [1] and to the plasma membrane of mature erythrocytes (Langereis blood group antigen), whereas mitochondrial localization of ABCB6 was not confirmed. Mutations in ABCB6 have been associated with various pathological conditions such as ocular coloboma, dominant familial pseudohyperkalemia and dyschromatosis universalis hereditaria (DUH), but the exact pathophysiological role of ABCB6 in these conditions has not yet been revealed. In our current research we are focussing on characterizing the intracellular transport and trafficking pathways of endogenous ABCB6 and of disease causing mutants of the transporter, using immunofluorescence techniques and live-cell imaging. For the live-cell approach we will modify and use the RUSH system [2]. The RUSH system is based on the reversible interaction of a hook protein, which is fused to core streptavidin, with a reporter protein of interest fused to streptavidin-binding peptide. The hook protein (a mutant of the invariant chain of human MHC II) cannot leave the endoplasmic reticulum (ER), therefore all of the protein of interest should be trapped in the ER. Biotin addition causes a synchronous release of the reporter from the hook [2]. In an ongoing project we are also creating a fluorescent endogenous ABCB6 fusion protein with the CRISPR/Cas system, which will be a valuable tool for our live-cell imaging experiments. These techniques have the potential to become powerful tools to analyse consequences of mutations and polymorphic variations on the intracellular trafficking and protein targeting in pathological conditions.

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PP-51 Tuning the ion selectivity of glutamate transporter-associated uncoupled conductances

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Extracellular glutamate concentrations are tightly regulated by the excitatory amino acid transporters (EAATs). In addition to their primary role of clearing glutamate, the EAATs also possess an uncoupled chloride conductance. We have mapped the permeation pathway of this chloride conductance to the interface of the transport and scaffold domain and predict an arginine residue at the most restricted part of this pathway determines anion selectivity of this conductance. In this study, we mutate this arginine residue to a histidine in the human glutamate transporter EAAT1 and investigate the effect of the protonation state of this histidine on anion selectivity and transporter function. Our results demonstrate that a positive charge at this residue is crucial in determining anion selectivity of the uncoupled conductance of EAAT1. In addition, as the nature of

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this residue also influences the turnover rate of EAAT1, we reveal an intrinsic link between the elevator movement of the transport domain and activation of the chloride channel.

PP-52 The molecular mechanism for overcoming the rate-limiting step in monoamine neurotransmitter transport

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The monoamine transporter family consists of dopamine (DAT), norepinephrine (NET) and serotonin transporters (SERT) that mediate the reuptake of the monoamine neurotransmitters after their release during neurotransmission. These transporters play prominent roles in psychiatric disorders and are targets for drugs of abuse such as cocaine, amphetamine and ecstasy as well as anxiolytics and antidepressants.

The transporters undergo a series of concerted conformational changes in order to harness the driving force of co-transported cations to translocate the neurotransmitter across the neuronal membrane. The rate-limiting step in monoamine reuptake is the return of the empty transporter from an inward-facing to an outward-facing conformation without neurotransmitter and sodium bound. The molecular mechanism underlying this important conformational transition has not been described.

Crystal structures of the bacterial homolog, LeuT, captured in a new conformation without substrate or sodium bound shows a dramatic rotation of an absolutely conserved leucine into the substrate site. Molecular dynamics simulations combined with functional studies on SERT support that this leucine must act as an endogenous substrate mimic in the empty transporter in order for it to overcome the transition from the inward-facing to the outward-facing conformation. We also show that the local conformational changes associated with the rotation of this conserved leucine explains how cation sites are perturbed and reformed to ultimately drive vectorial transport of neurotransmitter.

PP-53 Identification of novel inhibitors of the amino acid transporter B⁰AT1 (SLC6A19), a potential target to treat type 2 diabetes

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The amino acid transporter B⁰AT1 (SLC6A19) has recently been identified as a possible target to treat type 2 diabetes and related disorders. B⁰AT1 mediates the Na⁺-dependent uptake of all neutral amino acids. For surface expression and catalytic activity, B⁰AT1 requires co-expression of collectrin (TMEM27). A CHO-based cell line was generated, stably expressing both proteins. Using this cell line, a high-throughput screening assay was developed, which uses a fluorescent dye to detect depolarisation of the cell membrane during amino acid uptake via B⁰AT1. In parallel to these functional assays, we ran a computational compound screen using AutoDock4 and a homology model of B⁰AT1 based on the high resolution structure of the highly homologous *Drosophila* dopamine transporter. The combination of these tools allowed us to identify a series of novel inhibitors of the B⁰AT1 transporter, which can serve as tool compounds to study epithelial transporters or as lead compounds for the development of drugs to block B⁰AT1. Benztropine was identified as a competitive inhibitor of the transporter showing an IC₅₀ of 20±7 μM. The compound was selective with regard to related transporters and blocked neutral amino acid uptake in inverted sections of mouse intestine.

PP-54 Antigen receptor regulation of amino acid transporter SLC7A5 couples methionine metabolism to the epigenetic control of T cell differentiation

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Methionine is required for *de novo* protein synthesis and is also the biosynthetic precursor for S-adenosylmethionine (SAM), the main methyl donor used in the DNA, RNA and histone modifications that regulate cellular transcriptional programs. This study shows that the T cell antigen receptor (TCR) controls methionine metabolism by regulating the transport of methionine across the T cell membrane and by controlling the expression of key enzymes involved in the methionine cycle. In particular, regulated methionine delivery to the cell interior is rate limiting for epigenetic histone modifications in immune activated T cells. We used high resolution mass spectrometry to identify the repertoire of amino acid transporter proteins expressed on effector CD4 and CD8 T cells. Multiple methionine transporter candidates were identified but the most abundant was the System L transporter, SLC7A5. SLC7A5 is upregulated in response to antigen receptor engagement and we show it to be the dominant methionine transporter in effector T cells. Furthermore, *Slc7a5* null CD4 T cells fail to differentiate into effector cells and cannot co-ordinate a normal program of histone methylation. The regulation of methionine uptake via SLC7A5 is thus a critical TCR controlled metabolic checkpoint for T cell differentiation.

PP-55 Structure based approaches to understand L-Amino Acid Transporter (LAT)-1 inhibition

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The Large-neutral Amino Acid Transporter 1 (LAT-1) or SLC7A5 is a sodium-independent exchanger of amino acids that preferentially transports large neutral, aromatic or branched amino acids like phenyl-alanine, leucine, isoleucine, valine, tryptophan, histidine and methionine to the cells. LAT-1 is highly expressed in the blood-brain barrier (BBB) and facilitates the transport of several prescription drugs, such as the antiparkinsonian drug L-dopa and the anticonvulsant gabapentin, across the BBB, thereby enabling their pharmacologic effects. This function at the BBB has made LAT-1 a target for drug delivery by modifying CNS-impermeable drugs such that they become LAT-1 substrates and have enhanced BBB penetration. LAT-1 is also widely expressed in different types of cancer and promotes their growth and development by providing the tumor cells with essential nutrients. Therefore, a cancer drug targeting LAT-1 can be an inhibitor that deprives the cancer cells of nutrients or a cytotoxic LAT-1 substrate with an intracellular target (e.g., a metabolic enzyme).

In this study, we present the development of a homology model of LAT-1 based on the crystal structures of homologous APC family transporters from prokaryotic organisms. Flexible molecular docking studies of Melphalan analogues of known LAT-1 activity was performed and the general binding modes of these inhibitors were investigated using common scaffold clustering method and structure based pharmacophore modeling. The binding poses of ligands were optimized using hybrid QM-MM technique to gain more insights into non covalent interactions like hydrogen bonding, halogen bonding in the binding site. Furthermore, MD simulations was employed to ascertain the stability of the predicted 3D model and that of ligands in the active site.

The 3D structure of LAT-1 was found to be of good stereochemistry as revealed by PROCHECK-NMR and QMEAN. The model was thermodynamically stable as revealed by long time MD simulations. The structure based studies demonstrated that residues Thr62, Ile63, Ile64, Gly65, Ser66, Ser143, Ser338 and Ser401 seem critical for LAT-1 inhibition as they are profoundly involved in polar interactions with ligands. Based on the results, we propose a five feature pharmacophore model consisting of two hydrophobic, one aromatic, one positive and one negative ionizable sites for LAT-1 ligands. The proposed pharmacophore model would be used to screen a large public library of compounds (ZINC, DrugBank, Chembridge and HMDB) to find potential ligands that can inhibit the LAT-1 transport mechanism.

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PP-56 Role of the canonical and non-canonical Nucleotide Binding Sites (NBSs) of the bile salt export pump in taurocholate transport

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ATP-binding cassette (ABC) transporters bind and hydrolyze ATP to energize transmembrane transport of cargo. ATP binds at two rotationally symmetric nucleotide binding sites (NBSs) at the interface of the nucleotide binding domains. About half of the human ABC proteins harbor a non-canonical NBS1. We investigated the role of this NBS in the human bile salt export pump (BSEP, ABCB11). BSEP is responsible for bile salt transport from the cytoplasm of hepatocytes into bile canaliculi. The NBD interfaces of ABCB1 and BSEP differ in only 4 residues, all of which are located in NBS1 (E502 (Q-loop), M584 (Walker B) and R1221 and E1223 (C-motif)). These residues are S, E, G and Q, respectively in the canonical site of ABCB1. We generated the M584E single and the E502S.M584E.R1221G.E1223Q quadruple mutation. Wild type and BSEP mutants were expressed in HEK293 cells and membrane vesicles were prepared (Hirano et al., 2005). The surface expression was determined by Western blotting and [³H]taurocholate uptake activity was measured using a rapid filtration technique (Gerloff et al., 1998). Substitution of non-canonical residues of NBS1 with canonical ones decreased both TC transport and ATPase activity. While the single mutant showed 90% of wt transport activity, transport in the quadruple mutant decreased to 35 percent. These results indicate that the non-canonical residues of NBS1 have evolved in coordinated manner to preserve function of the transporter. To further verify this, we mutated canonical lysine residues (which contact β and γ phosphate of ATP) of the Walker A motif of NBS1 as well NBS2 to methionine. Not unexpectedly, we found that the conserved lysine in the Walker A motif of NBS1 is as important as that of NBS2, as the mutation on either site resulted in complete loss of function. Furthermore, we mutated aspartate residues of the D-loops. The results showed that mutating aspartate to alanine in the canonical NBS2 leads to almost complete loss of transport activity. In contrast mutation of the analogous aspartate residue in NBS1 only lowered the transport rate to about 50% of wt. We also exchanged the catalytic glutamate between NBS2 and NBS1. Unexpectedly, the ensuing double mutant M584E/E1244Q showed about 15% residual transport activity as compared to wild type protein. We conclude that during evolution the non-canonical nucleotide binding site was optimized structurally and functionally to provide a platform for ATP hydrolysis in the consensus site. In addition our experiments for the first time show that NBS1 can functionally replace NBS2 in mediating taurocholate transport.

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PP-57 Gene expression of ATP binding cassette transporter ABCD4 associates with survival of stage II colorectal carcinoma patients

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Colorectal cancer presents a third most frequent malignancy in males and fourth in women. Its incidence is particularly high and increasing in Central Europe. Drug efflux performed by ATP-binding cassette transporters (ABCs) is among numerous factors contributing to tumor cell resistance in general. Recently performed gene expression profiling of all human ABCs revealed a number of important findings with implications for cancer biology. Moreover, ABCs responsible for translocation of physiological substrates, e.g., cholesterol, hormones (family A) and fatty acids (family D), etc. associated with cancer prognosis and therapy outcome. Further studies in

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independent and homogeneous patient cohorts are needed to focus functional studies and evaluate clinical utility of the previously observed associations.

In the previous study (Hlavata *et al.*, *Mutagenesis* 2012;27:187-196), we have found that intratumoral transcript levels of several ABCs significantly associated with response of colorectal cancer patients to palliative chemotherapy (stage IV patients; ABCC6, ABCC11 ABCF1, and ABCF2) and with disease-free survival of those treated by adjuvant chemotherapy (stage II and III patients; ABCA7, ABCA13, ABCB4, ABCC11, and ABCD4). Other ABCs associated with clinical characteristics of the patients (ABCA12, ABCB2/B3/B11, ABCC1/C2/C3/C7/C10, and ABCG2). The main goal of this study was to validate previous data suggesting putative prognostic roles of the above ABCs in an independent and homogeneous cohort of colorectal cancer patients. We studied patients with stage II disease because markers for therapeutic decisions are urgently needed in this group of patients.

Transcript levels of the above listed ABCs were assessed in tumor tissues from 60 stage II colorectal cancer patients by quantitative real-time PCR. Associations of their transcript levels with prognostic clinical factors as tumor size, grade, and localization, with therapy type and survival were then evaluated. Protein levels of the most interesting markers were analyzed by immunoblotting in tumors.

After comparing intratumoral transcript levels of ABCs in tumors with clinical data of patients, significantly higher level of ABCB2 in rectum and rectosigmoideum compared to colon was found ($P=0.004$). Additionally, expression of ABCD4 gene associated with the disease-free survival ($P=0.027$), namely, patients with an increased (above the median) ABCD4 expression in their tumors exhibited shorter disease-free survival than patients with lower expression. Protein expression of both genes was detected in colorectal tumors and seems biologically relevant for this cancer.

According to the present study, ABCD4 is the most interesting putative prognostic marker in patients with localized colorectal cancer. Subsequent studies will be focused on functional analysis of the mechanism behind the observed prognostic effect of ABCD4.

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PP-58 Folding correction of ABC-transporter ABCB1 by pharmacological chaperones: a mechanistic concept

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Point mutations and subsequent impaired trafficking of ATP-binding cassette (ABC) proteins are a common cause of human diseases. Dysfunction of at least 22 human ABC transporters has been linked to more than 27 disease etiologies. Available crystal structures indicate a similarity in the architecture of several members of this protein family. Their molecular architecture makes these proteins vulnerable to mutation, when critical structural elements are affected. The latter preferentially involve the two transmembrane domain (TMD)/nucleotide binding domain (NBD) interfaces (transmission interfaces), formation of which requires engagement of coupling helices of intracellular loops with NBDs. Both, formation of the active site and engagement of the coupling helices are contingent on correct positioning of ICLs 2 and 4 and thus an important prerequisite for proper folding. Aberrant folding leads to retrotranslocation from the endoplasmic reticulum to the cytoplasm and degradation by the ubiquitin-proteasome system. In recent years folding correction by specifically binding small molecules, so called pharmacological chaperones (PCs), has been advocated as a therapeutic concept. Here we show that active site compounds are capable of rescuing P-glycoprotein (P-gp) mutants $\Delta Y490$ and $\Delta Y1133$ - located at the transmission interface in pseudosymmetric position to each other - in a concentration dependent manner. We selected P-gp as a model system for studying folding because of its ability to interact with a large complement of small molecular weight compounds. The $\Delta Y490$ and $\Delta Y1133$ mutants are representative for frequent other mutations in their vicinity with respect to their position in the alpha helical domain. These include mutations in the cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7), the breast cancer related protein (ABCG2, a major contributor to gout particularly in the Japanese population), the cholesterol transporter (ABCA1) and the conjugated bilirubin transporter (ABCC2). In addition, the ability of propafenone analogs to correct folding correlates with their ability to inhibit transport of model substrates. This finding indicates that folding correction and

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transport inhibition by propafenone analogs are brought about by binding to the active site. Furthermore, we demonstrate an asymmetry in folding correction with cis-flupentixol, which reflects the asymmetric binding properties of this modulator to P-gp. Our results suggest a mechanistic model for corrector action in a model ABC transporter based on insights into the molecular architecture of these transporters.

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PP-59 Functional rescue of misfolded SLC6 transporters

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The physiological role of proteins belonging to the solute carrier 6 (SLC6) family is to transport neurotransmitters, amino acids, and osmolytes such as betaine, taurine, and creatine. Their dysfunction has been linked to different neurological and psychiatric disorders. SLC6 transporters are proteins of twelve membrane-spanning helices and cytosolic amino and carboxy termini. Mutations in the coding sequences of SLC6 transporter genes are known to cause protein misfolding. The aim of our study is to characterise and rescue misfolded/dysfunctional SLC6 transporters mutants using pharmacological chaperones. Mutations in *Drosophila melanogaster* DAT (dDAT) abolish dopamine uptake and lead to a sleepless phenotype in flies. We showed that a single-point mutation in dDAT (dDAT-G108Q) arises due to defective protein folding. The mutant exhibited no specific dopamine uptake in HEK293 or Schneider cells. Moreover, dDAT-G108Q was retained in the endoplasmic reticulum (ER), which was illustrated both by confocal microscopy and co-localisation with the ER-resident chaperone calnexin. We also found that dDAT-G108Q associates with significantly higher levels of HSP70-1A and calnexin, compared to the wild type dDAT. This interaction was markedly reduced upon treatment with noribogaine or pifithrin- μ . A combined action of the two compounds resulted in an additive effect, i.e. enhanced surface expression. The pharmacochaperoning action of noribogaine can be accounted for by its ability to stabilise the inward-facing conformation of DAT, whereas pifithrin- μ acts by inhibiting HSP70. Noribogaine and pifithrin- μ also restored sleep in vivo in dDAT-G108Q expressing flies. Interestingly, in the human creatine transporter-1 (hCRT-1), a conservative mutation of the equivalent glycine residue (hCRT-1-G132V) is linked to severe mental retardation in children. We found that this mutant has approximately 10% of the activity of the wild type hCRT-1, and confirmed that the dramatic loss of function is also due to protein misfolding and retention in the ER compartment. Evidently, this particular glycine residue plays a common, crucial role in protein folding in a variety of members of the SLC6 family. In fact, the glycine is absolutely conserved among all SLC6 transporters. It resides in the first intracellular loop, juxtamembrane to the second membrane-spanning helix, a region already known to be involved in the folding of the related serotonin transporter (1).

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Reference: (1) Koban F, El-Kasaby A, Häusler C, Stockner T, Simbrunner BM, Sitte HH, Freissmuth M, Sucic S (2015). A salt bridge linking the first intracellular loop with the C terminus facilitates the folding of the serotonin transporter. *J Biol Chem.* 290 (21): 13263-78.

PP-60 Dimerization energetics of the nucleotide binding domains of ABCB1

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P-glycoprotein also known as multidrug resistance protein (MDR1 or ABCB1) is the first member of the ATP-binding cassette superfamily that was cloned. ABCB1 can bind and export a wide range of chemically unrelated compounds, including chemotherapeutic drugs and is therefore responsible for widespread resistance of cancer cells leading to failure of treatment. The transporter shows a rotational two fold pseudo-symmetry, consisting of two transmembrane domains (TMDs) that form the substrate binding and translocation domain and of two cytoplasmic nucleotide binding domains (NBDs) that bind and hydrolyse ATP. Substrate export is energized by ATP. Several transport mechanisms including the switch, the alternating sites or the constant contact model have been proposed, which describe how catalysis, conformational changes and transport are linked, but

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many questions remained unanswered. We estimated the interaction forces between the NBDs of ABCB1 in different states of the catalytic cycle (apo or bound to ATP, ADP, ADP+Pi) to quantify the energetic dimension of nucleotide binding and hydrolysis. Therefore we calculated the potential of mean force (PMF) by pulling the NBDs apart using umbrella sampling. Alignment and orientation of the NBDs was maintained by the enforced rotation module. The PMF profile show a deep (~ -43 kJ/mol) energetic minimum at the closed dimer conformation. Interestingly, ATP and ADP showed comparable profiles, while the apo configuration exhibited a much weaker attractive force. Noticably the ADP+Pi bound configuration developed a repulsion in the closed dimer geometry. All PMFs show a secondary energy minimum at an approximately 0.5 nm NBD separation, which is caused by breaking of the water bridged hydrogen bonding network between the NBDs in the interface.

Our results therefore indicate that the NBD dimer is stabilized by the presence of bound nucleotides (ATP or ADP), while opening of the dimer is facilitated by the reaction products of ATP hydrolysis.

PP-61 Studying the catalytic cycle of P-glycoprotein using Walker B and A-loop mutants

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P-glycoprotein (Pgp) is an ABC transporter that can export a large variety of drugs from cells causing multidrug resistance. The protein contains two transmembrane domains (TMD) forming the substrate binding site(s), and two nucleotide binding domains (NBD) involved in ATP binding and hydrolysis. Based on crystal structures of several ABC transporters Pgp is believed to alternate between inward and outward facing conformations characterized by high and low substrate binding affinities, respectively. The inward facing conformation is recognized by the conformation sensitive UIC2 monoclonal antibody, while the outward facing conformation(s) do not react with the antibody. Despite accumulating structural and functional data, it is still unknown how ATP binding and hydrolysis are connected to the conformational changes of the TMDs allowing transport. Recently, we have used the UIC2-assay to characterize the transport cycle of Pgp variants containing mutations at the critical Walker A residues (Bársony O. et al. 2016).

Mutations in the conserved residues of the NBDS including the aromatic loops (Y401A/Y1044A) and the Walker B sequences (D555A/D1200 or E556Q/E12001Q) affect different steps of the catalytic cycle, all of them were found to inactivate the protein either the mutation is present at one or both NBDs supporting the hypothesis that the integrity of both NBDs is needed for transport. However, the majority of the studies reporting the inactivating effect of single catalytic site mutations have been performed using heterologous expression systems or reconstituted proteins hampering the natural membrane environment of the transporter. To avoid the above possible pitfalls we engineered mammalian cell lines expressing the mutant transporters at high level and studied them in their natural membrane environment.

We have demonstrated in calcein-AM accumulation studies and cytotoxicity assays that the single aromatic loop mutants possess residual drug efflux activity (the double mutant variant was inactive), while Walker B mutant Pgp molecules did not show any transport activity. The aromatic loop mutants preferentially reside in the UIC2-reactive conformation in intact cells similarly to the D555N/D1200N single or double mutants and they can switch to the UIC2-unavailable conformation in the presence of nucleotides and vanadate. In contrast, single and double Walker B mutants E556Q, E1201Q and QQ are preferentially in a UIC2-nonreactive state and switch to the UIC2-reactive conformation in the presence of cyclosporine A. Our results demonstrate that although all the examined single mutants can change conformation between the inward and outward facing conformations only the single A loop mutants can couple these conformational changes to substrate transport.

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Reference: Bársony et al.: Sci Rep. 2016; 6:24810.

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PP-62 A preclinical *in vivo* model for protein-conformation therapy of ABCC6-associated PXE and GACI

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A molecular pathway inhibiting ectopic calcification is initiated by ABCC6, a membrane transporter primarily expressed in liver. ABCC6 facilitates the efflux of ATP from hepatocytes, what is rapidly converted into pyrophosphate (PPi) in the liver vasculature. PPi is the major inhibitor of calcification. Mutations in ABCC6 are responsible for the currently incurable calcification disorder pseudoxanthoma elasticum (PXE) and some cases of generalized arterial calcification of infancy (GACI). Most mutations in ABCC6 are missense, many of which have preserved transport activity but are retained intracellular. We have shown earlier that the chemical chaperone 4-phenylbutyrate (4-PBA) can reorient ABCC6 mutants into the plasma membrane (1).

In a humanized mouse model transiently expressing human ABCC6 mutants in the liver, we addressed whether treatment with 4-PBA could rescue the physiological function of ABCC6 *in vivo* by restoring its potential to inhibit calcification. We used the dystrophic cardiac calcification phenotype as a marker in *Abcc6*^{-/-} mice to determine the effect of 4-PBA on mineralization.

Administration of 4-PBA to *Abcc6*-deficient mice expressing human ABCC6 mutants (identified in PXE and in GACI patients) in the livers restored the calcification inhibitory capacity of the protein to a large extent (2). We have also shown that 4-PBA triggered a two fold increase of the level of PPi in supernatants of HEK cells overexpressing mutant ABCC6.

As 75% of patients are with at least one missense allele, the potential of 4-PBA (an FDA-approved drug) seems to be a promising strategy for allele-specific therapy of ABCC6-associated calcification disorders and plasma PPi may be a useful indicator of ABCC6 activity.

(1) Pomozi et al, 2014 *J.Invest.Dermatol.* **134**: 946-953

(2) Pomozi et al, 2016 *J.Invest.Dermatol.* (under editorial review)

PP-63 Comparative analysis of putative resistance causing SNPs in ABC-transporter sequences studied in European and African *Fasciola hepatica* isolates

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The trematode *Fasciola hepatica* (liver-fluke) is a parasitic worm infecting the liver of both livestock and humans on all continents (except the Antarctic). Up to 17 million people worldwide are thought to be infested. A survey in Tyrol revealed that antibodies against *F. hepatica* were found in 73% of the cows. At present, the only effective drug is triclabendazole. However it has been used for more than 20 years and resistance of *F. hepatica* was reported already in the mid 1990s. It has been hypothesized that ABC-transporter play an important role in the mechanisms underlying the resistance which is further supported by a recent survey that examined polymorphisms in a putative ABC transporter.

RNA-seq data from four different isolates (resistant and non-resistant phenotypes) was created and all ABC-transporter transcripts were identified. Those were further analysed in order to identify mutations potentially related to the resistance phenotype. We identified a variety of alterations including SNPs and allelic variations with specific expression potentially related with the resistance. In order to validate these potential relations and to gain a broader overview of the distribution of these mutations in *F. hepatica* populations we used 96 samples from various populations in Austria and Ethiopia. For each the identified regions were extracted from the genomic DNA and sequenced on an Illumina Miseq machine. The specific composition of each analysed SNP was extracted for

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each sample and compared to the identified values. The analysis of the SNP data revealed significant potentially resistance related differences between the African and European isolates. Although these findings have to be confirmed experimentally, they provide a valuable resource for studying the resistance of *F. hepatica* to triclabendazole.

The validation and analysis in various populations of SNPs in ABC-transporter sequences in *F. hepatica* are crucial steps in the analysis of the ABC-transporter dependent development of resistance phenotypes.

PP-64 Iron oxide nanoparticles can enter inside the cells also by directly crossing plasma membranes

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Iron deficiency is one of the major global public health problem, whether caused by diseases or physiological conditions; iron supplementation or food fortification are commonly used to overcome this iron deficiency, but they present annoying side effects and are likely to cause unacceptable changes in the color and taste of foods. Nanoformulations have demonstrated interesting performances and might minimize side effects of classical formulations. It has been recently postulated that some metal NPs might cross the plasma membrane also by a non-endocytotic pathway. To verify this possibility for iron-based NPs, we recently developed a protocol based on Calcein-injected *Xenopus laevis* oocytes, that is capable to monitor the cytoplasmic changes of divalent metal ions¹.

In this work, we have first defined the relationship between Calcein quenching and intracellular iron concentration. To this aim, we have co-injected oocytes with increasing concentration of FeCl₂ with a fixed amount of Calcein. Then, we have exposed Calcein-injected oocytes to oxide (Fe₃O₄) and zerovalent (Fe⁰) iron NPs. Both species undergo dissolution, releasing ions that can be detected by Calcein quenching. Only iron oxide NPs induced a significant decrease in intracellular Calcein fluorescence.

In a following experiment, protein corona (transient modification of NP surface caused by biomolecule adsorption) was exploited to prevent plasma membranes crossing of NPs. To form this corona, we have used the negatively charged protein bovine serum albumin. We have exposed *Xenopus laevis* oocytes to the iron oxide BSA-coated NPs, that resulted unable to modify intracellular Calcein fluorescence, and therefore to modify intracellular iron concentration. This indicates that can not cross plasma membranes.

Two-electrode voltage clamp (TEVC) was used to determine possible alteration of oocytes membrane properties². A significant increase in membrane conductance was revealed in oocytes treated with Fe₃O₄ NPs. The characterization of electrophysiological membrane parameters over a period of 30 minutes showed that alterations in membrane properties appears in the first 5 minutes after treatment. After 20 minutes the difference with non-treated oocytes is strongly reduced. Moreover, iron oxide NPs with protein corona do not cause conductance increase, confirming previous data obtained with the fluorescence approach.

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PP-65 Mapping Cholesterol Binding Sites on The Human Dopamine Transporter

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The human dopamine transporter (hDAT) is essential for regulating dopaminergic neurotransmission by transporting dopamine from the synaptic cleft back into the presynaptic neuron. Dysregulation of hDAT is involved in several debilitating diseases such as Parkinson's

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disease, attention deficit hyperactive disease (ADHD) and Tourette's syndrome, among others. DAT is also the target of many illicit drugs and has been presumed to be involved in the development of addiction. It is therefore essential to further the understanding of the transporters architecture, transport mechanism and means of regulation to aid in drug development. There has been published a range of DAT structures all from the species *Drosophila Melanogaster* [1][2], which contain at least one conserved co-crystallized cholesterol molecule. It is believed that cholesterol acts in regulating hDAT activity [3], but whether its mechanism of regulation is site specific, has yet to be resolved. This question is sought to be answered within this contribution using coarse-grained molecular dynamics simulations of a hDAT homology model embedded in a mixed POPC and cholesterol membrane. Based on preliminary results it appears that there exists several cholesterol specific sites surrounding hDAT and that the conserved cholesterol site observed in DAT crystal structures is among them. Thereby concluding that this cholesterol site is not a crystallization artifact and that it also occurs on the hDAT homologue.

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PP-66 PKC- β inhibitors attenuate amphetamine and cocaine stimulated dopamine release

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Amphetamine abuse afflicts over 13 million people, and there is currently no universally accepted treatment for amphetamine addiction. Amphetamine serves as a substrate for the dopamine transporter (DAT) and reverses the transporter to cause dopamine efflux in addition to inhibiting the vesicular monoamine transporter (VMAT) to promote dopamine exocytosis. Activation of protein kinase C β enhances extracellular dopamine in the presence of amphetamine by enhancing the reverse transport of dopamine and internalizing the D2 autoreceptor. We previously demonstrated that PKC β inhibitors block amphetamine-stimulated dopamine efflux in rat striatum in vitro. In this study, we utilized in vivo microdialysis in live, behaving rats to assess the effect of the PKC β inhibitors enzastaurin and ruboxistaurin on amphetamine-stimulated increases in monoamines and their metabolites. A 30 min perfusion of the nucleus accumbens core with 1 μ M enzastaurin or 1 μ M ruboxistaurin reduced amphetamine-stimulated efflux of dopamine and its metabolite 3-methoxytyramine by approximately 50%. The inhibitors also significantly reduced extracellular levels of norepinephrine and its metabolite normetanephrine after amphetamine. The stimulation of locomotor behavior by amphetamine, measured simultaneously with the analytes, was comparably reduced by the PKC β inhibitors. Ruboxistaurin also attenuated cocaine stimulated extracellular dopamine, a process that would not be dependent upon DAT reversal. In order to see if this process was D2 autoreceptor mediated, we examined the effect of ruboxistaurin on cocaine activation when D2 receptors were blocked with raclopride. The inhibitory effect of ruboxistaurin was reduced in the presence of cocaine and raclopride, suggesting that ruboxistaurin action involved D2 autoreceptors. Using a stable isotope label retrodialysis procedure, we determined that ruboxistaurin had no effect on basal levels of dopamine, norepinephrine, glutamate, or GABA. Our results support the utility of using PKC β inhibitors to reduce the effects of amphetamine and cocaine. Funding support comes from DA11697, T32DA07268, and NIHR37 EB003320.

PP-67 Atomic force microscopy studies of dopamine transporter

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The dopamine transporter (DAT) belongs to the neurotransmitter sodium symporter family, which removes dopamine from the synaptic cleft into the presynaptic neuron. DAT is targeted not only by its substrate dopamine, but also by addictive drugs and therapeutic medicines. Cocaine binds to DAT and inhibits the uptake of dopamine, while some other drugs, e.g. 3,4-methylenedioxymethamphetamine (MDMA), enter the presynaptic neuron through DAT or the cell membrane and induce the efflux of the dopamine, thereby enhancing dopaminergic neurotransmission. The main purpose of our experiments was to investigate the kinetics and energy landscapes of binding between DAT and its ligands and substrates, e.g. the cocaine analog MFZ2-12 (2b-carbomethoxy-3b-(3',4'-dichlorophenyl)tropane) and MDMA. We used atomic force microscopy (AFM) based single molecular force spectroscopy to directly measure the intermolecular interaction on living CHO cells expressing DAT. AFM tips were functionalized with MFZ2-12 or MDMA via a flexible PEG linker. Two populations of unbinding forces were observed in the force distribution with MFZ2-12, suggesting the existence of two binding sites with different interaction strengths in DAT. After adding 50 μ M MFZ2-12 into the solution, only the high affinity binding site was significantly blocked. To identify the location of the two binding sites, DAT mutants were studied. For the mutant W84L, the stronger interaction site appeared in the force distribution even in the absence of Zn^{2+} , implying that W84L favors an outward-facing conformation. For the mutation S422A, which is located at the central S1 site, the strong interactions were significantly suppressed, confirming that the stronger unbinding events originate from the central S1 site. From force measurements with MDMA tips, we found that MDMA can bind with both DAT and the cell membrane lacking DAT. Further studies with new dopamine analogues are planned to probe conformational changes of DAT during transport.

PP-68 *CesCD*, putative ABC transporter involved in post-translational regulation of cereulide toxin synthesis?

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Cereulide is a nonribosomal peptide produced by the human pathogen *Bacillus cereus*. The toxin causes emetic symptoms upon ingestion of cereulide contaminated food. Genes for biosynthesis of cereulide are encoded on pCER270, a mega plasmid sharing its backbone with the *B. anthracis* pX01 toxin plasmid. The *ces* genes are organized as operon, including a putative hydrolase (*cesH*), a putative type II thioesterase (*cesT*), a phosphopantetheinyl transferase (*cesP*), the structural genes of the cereulide nonribosomal peptide synthetase (*cesA* and *cesB*) and the putative ABC transporter (*cesC* and *cesD*).

Despite our extensive knowledge of the transcriptional regulation of cereulide biosynthesis there is lack of evidence concerning cereulide cellular localization, transport outside the cell and the mechanism of autoimmunity of the host bacterium. The aim of our current work is to investigate the function of a putative ATP binding protein (*cesC*) and membrane binding protein (*cesD*) in these processes and the toxin's biosynthesis. Strikingly, the double deletion mutant, $\Delta cesCD$, has no cereulide phenotype despite the WT level of *cesAB* expression as well as a *cesB* protein level suggesting the involvement of the predicted transporter in post-translation regulation of cereulide biosynthesis. Furthermore, our (UP)LC-MS/MS measurements of cereulide from different cellular fractions revealed that the majority of detected cereulide is associated with the cell wall, suggesting the involvement of *cesCD* in the export of the toxin outside the cell. The ongoing transcriptional study of the transporter mutant strains as well as data on cellular localization of *cesAB* NRPS complex and cereulide itself will be presented.

PP-69 Substrate-induced conformational change in LeuT

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LeuT is a prokaryotic amino acid transporter that has been used extensively as a model for neurotransmitter transport. We recently demonstrated that the conformational change induced by Na^+ ions requires the Na2 site observed in LeuT crystal structures. We observed this

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conformational change using LeuT in *E. coli* membranes, in the absence of detergent, by a decrease in reactivity of a single cysteine (Y265C) in the cytoplasmic permeation pathway. We now show that this effect of Na^+ is observed whether K^+ or NMDG $^+$ is used as a control ion. In the presence of Na^+ , addition of alanine, a substrate, induces the reverse conformational change, opening the cytoplasmic pathway and increasing Cys-265 reactivity. Three mutations in the substrate binding site each altered the affinity of LeuT for leucine and alanine, but did not interfere with the conformational change induced by Na^+ . One of the mutations also blocked the substrate-dependent conformational change. The results suggest a mechanism by which substrate interactions with the central binding site of LeuT reverse the ability of Na^+ to stabilize outward-facing conformations of this model transporter.

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