SFB 35 - Transmembrane Transporters in Health and Disease

6th SFB-Symposium 2013

Scientific Sessions and Postersession
September, 23th - 25th 2013
Great Lecture Hall, Bernhard Gottlieb University Clinic of Dentistry
Sensengasse 2a, 1090 Vienna

Konstantinos Beis
Randy Blakely
Peter Chiba
Susan PC Cole
Lynette C. Daws
Raimund Dutzler
Keith Henry

Jonathan Javitch
Karl Kuchler
Oliver Kudlacek
Claus Juul Loland
Poul Nissen
Michael Robinson
Gary Rudnick

John Schuetz
Erin Schuetz
Gerhard Schütz
Maria Sibilia
Steffen Sinning
Harald Sitte
Emad Tajkhorshid

Robert Tampé
Michael Trauner
Roxanne Vaughan
Da-Neng Wang
Klaus Wanner

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# 6th SFB35 Symposium 2013, Vienna

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### Poster Presentations

- **Monday, September 23**: EVEN poster numbers
- **Tuesday, September 24**: ODD poster numbers
- **Wednesday, September 25**: free poster viewing
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Scientific Programme

Monday, September 23

11:00  Registration

13:00  Opening Remarks, Welcome Address

13:10  **Plenary Lecture 1:**
       *(Chair: Haley Melikian - UMASS Medical School, USA)*

13:15  Plenary Speaker: **Randy Blakely** (Vanderbilt Kennedy Center, USA)
       *Glutamate Transporter Modulated Dopamine Homeostasis: Discovery of a Missing Link*

14:00  **Session 1:**
       *(Chair: Kazumitsu Ueda - Kyoto University, Japan)*

14:05  Speaker 1: **John Schuetz** (St. Jude Children's Research Hospital, USA)
       *A porphyrin transporter is required for optimal survival of N-MYC mediated myeloid leukemia*

14:30  Speaker 2: **Susan PC Cole** (Queens University, Canada)
       *Misfolding Mutants of the MRP1 (ABCC1) Transporter: Useful tools in determining domain-domain interactions*

14:55  Speaker 3: **Konstantinos Beis** (Imperial College London, UK)
       *Structure and function of an antibacterial peptide ABC-exporter*

15:20  Special talk: **Niels Chr. Danbolt**
       *Electronic notebook systems - A call for action*

15:30  **Coffee/Tea break**

16:10  **Session 2:**
       *(Chair: Anders Kristensen – University of Copenhagen, Denmark)*

16:15  Speaker 1: **Emad Tajkhorshid** (University of Illinois, USA)
       *Atomic Resolution Description of Structural Transition Pathways in Membrane Transporters with Advanced Molecular Simulation Technologies*

16:40  Speaker 2: **Gerhard Schütz** (Technical University of Vienna, Austria)
       *Subunit stoichiometry of membrane proteins determined by singe molecule analysis*
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17:05 Speaker 3: **Harald Sitte** (Medical University of Vienna, Austria)

*Amphetamine actions at the serotonin transporter rely on the availability of phosphatidylinositol-4,5-bisphosphate*

17:30 **Evening Poster Session - EVEN poster numbers (2 h)**

*Refreshments/Snacks/Buffet*

*Practical Demonstrations*

**Tuesday, September 24**

08:30 **Registration**

08:55 **Plenary lecture 2:**

*(Chair: Michael Freissmuth, Medical University Vienna, Austria)*

09:00 Plenary Speaker: **Raimund Dutzler** (University of Zurich, Switzerland)

*Activation and inhibition of prokaryotic pentameric ligand-gated ion channels*

09:45 **Coffee/Tea break**

10:15 **Session 3:**

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

10:20 Speaker 1: **Karl Kuchler** (Medical University of Vienna, Austria)

*The Molecular Basis of ABC Transporter-Mediated Drug Resistance from Yeast to Man – From Structural Modeling to Transport Mechanisms?*

10:45 Speaker 2: **Peter Chiba** (Medical University of Vienna, Austria)

*The dual core architecture of ABC transporters: possible implications for function and folding*

11:10 Speaker 3: **Erin Schuetz** (St. Jude Children's Research Hospital, USA)

*Drug Transporters at the Blood-Arachnoid-CSF Barrier*

11:35 **SOLVO Lecture**

*R&D and pharmaceutical applications in the transporter field*

11:45 **Plenary lecture 3:**

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

11:50 Plenary Speaker: **Da-Neng Wang** (NYU Langone Medical Center, USA)

*Structure and mechanism of a sodium-dependent carboxylate transporter - Implications in fatty acid synthesis and obesity*
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12:35 Lunch / Poster Session - ODD poster numbers

15:15 Session 4:
(Chair: Daniela Pollak – Medical University Vienna, Austria)

15:20 Speaker 1: Michael Robinson (University of Pennsylvania, USA)
Glutamate transporters, mitochondria, and astrocytes: Biology reveals itself

15:45 Speaker 2: Maria Sibilia (Medical University of Vienna, Austria)
The EGF-receptor-Transporter connection: exploring the unexpected

16:10 Speaker 3: Klaus Wanner (LMU Munich, Germany)
MS Binding Assays and their application to the development of new GABA uptake inhibitors

16:35 Coffee/Tea break

17:05 Session 5:
(Chair: Ana Carneiro – Vanderbilt University Medical Center, USA)

17:10 Speaker 1: Roxanne Vaughan (University of North Dakota, USA)
Regulation of Dopamine Transporter Phosphorylation and Function by the Peptidyl Prolyl Cis-Trans Isomerase Pin1

17:35 Speaker 2: Keith Henry (University of North Dakota, USA)
Unraveling Ion Coupling in the Human Serotonin Transporter: the different roles of Na

18:00 Speaker 3: Lynette C. Daws (University of Texas, USA)
Organic cation transporters and the plasma membrane monoamine transporter: Uncovering novel targets to treat depression

18:30 Guided Tour: Vienna City Center

20:00 Reception Townhall of Vienna / Dinner
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**Wednesday, September 25**

08:30  Registration

08:55  **Plenary lecture 4:**

   *(Chair: Baruch Kanner – BioJerusalem, Israel)*

09:00  Plenary Speaker: **Poul Nissen** (Aarhus University, Denmark)

   *New insight of the mechanism of P-type ATPase ionpumps and neurotransmitter: sodium symporters*

09:45  **Coffee/Tea break**

10:15  **Session 6:**

   *(Chair: Walter Sandtner – Medical University Vienna, Austria)*

10:20  Speaker 1: **Claus Juul Loland** (University of Copenhagen, Denmark)

   *Identification and molecular properties of the allosteric binding site in the serotonin transporter*

10:45  Speaker 2: **Steffen Sinning** (Aarhus University, Denmark)

   *Cation binding to the Na2 site of the serotonin transporter drives the conformational changes necessary for transport*

11:10  Speaker 3: **Gary Rudnick** (Yale University, USA)

   *LeuT: we have to talk about sodium*

11:35  **Leica Keynote Lecture: Boris Zarda**

   *Super-Resolution Microscopy: New Horizons for Life Scientists*

11:55  **Lunch / Poster Session - free poster viewing**

14:25  **Poster Bullets**

   *(Chair: Heinz Bönisch – University of Bonn, Germany)*

14:30  3 Short Talks of Selected Poster Presenters (12’ + 3’)

15:15  **Coffee/Tea break**
**6th SFB35 Symposium 2013, Vienna**

15:45 **Session 7:**
*(Chair: Thomas Stockner – Medical University Vienna, Austria)*

15:50 Speaker 1: **Oliver Kudlacek** (Medical University Vienna, Austria)
*Fasciola hepatica, a parasite with many transporters*

16:15 Speaker 2: **Robert Tampé** (Goethe-University Frankfurt, Germany)
*Transport Machineries in Adaptive Immunity and Viral Escape Strategies*

16:40 Speaker 3: **Michael Trauner** (Medical University Vienna, Austria)
*Molecular regulation of hepatobiliary ABC transporters in cholestatic liver diseases*

17:05 **Plenary Lecture 5:**
*(Chair: Ulrik Gether – University of Copenhagen, Denmark)*

17:10 Plenary Speaker: **Jonathan Javich** (Columbia University NY, USA)
*Substrate-modulated gating dynamics in a Na+-coupled neurotransmitter transporter homologue*

17:55 **Closing Remarks, End of meeting**
*Harald Sitte* (Medical University of Vienna, Austria)

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.
Plenary Lecture 1

PL-01 Glutamate Transporter Modulated Dopamine Homeostasis: Discovery of a Missing Link

Randy Blakely
Vanderbilt University, USA

Glial cells are well known to modulate the excitability of neurons in the CNS through their expression of glutamate transporters, expressed both synaptically and extrasynaptically. Owing to the fact that improper expression of these transporters can actually lead to neuronal death, glia have developed multiple, but as yet ill-defined, control mechanisms to induce or suppress glutamate transporter expression and activity. In a genetic screen for the induction of hyperdopaminergic states in the model system C. elegans, we discovered an as yet unstudied gene, which we term swip-10, that when mutated, leads to glutamate-dependent hyperexcitation of dopamine neurons. The expression of swip-10 in glia and the ability of mutants of glutamate transporters to reproduce, and of glutamate receptors to block, hyperdomapinergia, supports a role for swip-10 as a regulator of extracellular glutamate homeostasis. The presence of a close mammalian ortholog is now being examined for a similar role at mammalian glutamatergic synapses, with the latest findings presented in my lecture.
PL-02 Activation and inhibition of prokaryotic pentameric ligand-gated ion channels

Raimund Dutzler
University of Zurich, Switzerland

The pentameric ligand-gated ion channels (pLGICs) are ionotropic neurotransmitter receptors that mediate electrical signaling at chemical synapses. The X-ray structures of two prokaryotic homologues have provided first insight into the detailed architecture of the family at high resolution. The structure of GLIC, a proton-activated channel from the cyanobacterium Gloebacter violaceous shows an open conformation of the pore. The channel conducts cations with similar properties as the nicotinic acetylcholine receptor (nAChR). The transmembrane pore is funnel-shaped with a wide hydrophobic entrance at the extracellular side that narrows to a hydrophilic intracellular opening. In this region conserved residues coordinate ions which have lost a large part of their hydration shell. The structure of ELIC, a pLGIC from the plant pathogen Erwinia chrysanthemi, shows a non-conducting conformation that was obtained in the absence of ligands. In its structure the extracellular half of the pore is occluded by bulky hydrophobic residues that likely prevent ion conduction. ELIC is activated by a set of primary amines that include the neurotransmitter GABA. The protein forms cation selective channels with large conductance that slowly desensitize in the presence of ligands. Both proteins were used to study distinct mechanisms of channel inhibition. Ion conduction in GLIC is inhibited by the same set of open channel blockers that also act on nAChRs. In ELIC acetylcholine acts as competitive inhibitor, which binds to the agonist site and stabilizes the closed conformation of the channel. Divalent cations, in contrast, are allosteric modulators, which bind to a site distant from the agonist binding site where they interfere with gating. The strong structural similarity to their eukaryotic counterparts make ELIC and GLIC important model systems for the pLGIC family that will ultimately allow a detailed comprehension of mechanistic properties that are still poorly understood.

References
PL-03  Structure and mechanism of a bacterial sodium-dependent dicarboxylate transporter — Implications in fatty acid synthesis and obesity

Romina Mancusso, Da-Neng Wang
New York University School of Medicine, USA

In liver and adipose cells, cytosolic citrate is a major precursor for the synthesis of fatty acids, triacylglycerols, cholesterol and low-density lipoprotein. The cytosolic citrate concentration partially depends on direct import across the plasma membrane via the Na⁺-dependent citrate transporter (NaCT), a member of the divalent anion/Na⁺ symporter family (1, 2). Mutations of the homologous transporter gene in flies (INDY, I’m not dead yet) result in reduced fat storage through calorie restriction (3). NaCT-knockout mice are both slimmer and protected from obesity and insulin resistance (4). We have determined the 3.2 Å crystal structure of an INDY homolog from Vibrio cholerae (5). The protein consists of two halves that are related by an inverted twofold symmetry. One citrate molecule and one sodium ion are bound per protein, and their binding sites are formed by highly-conserved amino acid sequence motifs. Comparison of the structures of the two symmetrical halves of the transporter suggests conformational changes that propel substrate translocation.

References:
PL-04  New insight of the mechanism of P-type ATPase ionpumps and neurotransmitter:sodium symporters

Poul Nissen
Aarhus University, Denmark

P-type ATPases like Na+,K+-ATPase establish and maintain electrochemical gradients for ions and lipids across membranes. Similarly, secondary transporters such as the neurotransmitter:sodium symporters (NSS) use the energy stored in these gradients for uphill transport.

We have studied several proteins of the P-type ATPase and NSS families using for example X-ray crystallography, biochemistry and electrophysiology. Recently, we have obtained new insight of how ion pumps and secondary transporters continue from occluded states to subsequent, open states.
PL-05  Substrate-modulated gating dynamics in a Na⁺-coupled neurotransmitter transporter homologue

Jonathan A. Javitch
Columbia University, USA

Neurotransmitter/Na⁺ symporters (NSS) terminate neuronal signaling by recapturing neurotransmitter released into the synapse in a co-transport (symport) mechanism driven by the Na⁺ electrochemical gradient. NSS for dopamine, noradrenaline and serotonin are targeted by the psychostimulants cocaine and amphetamine, as well as by antidepressants. The crystal structure of LeuT, a prokaryotic NSS homologue, revealed an occluded conformation in which a leucine (Leu) and two Na⁺ are bound deep within the protein. This structure has been the basis for extensive structural and computational exploration of the functional mechanisms of proteins with a LeuT-like fold. We have used single-molecule fluorescence resonance energy transfer imaging to reveal reversible transitions to an inward-open LeuT conformation, which involve the movement of transmembrane helix TM1a away from the transmembrane helical bundle. We have investigated how substrate binding is coupled to structural transitions in LeuT during Na⁺-coupled transport. Substrate binding from the extracellular side of LeuT facilitates intracellular gate opening and substrate release at the intracellular face of the protein. In the presence of alanine, a substrate that is transported ~10-fold faster than leucine, we observed alanine-induced dynamics in the intracellular gate region of LeuT that directly correlate with transport efficiency. Collectively, our data reveal functionally relevant and previously hidden aspects of the NSS transport mechanism that emphasize the functional importance of a second substrate (S2) binding site within the extracellular vestibule. Substrate binding in this S2 site appears to act cooperatively with the primary substrate (S1) binding site to control intracellular gating more than 30 Å away, in a manner that allows the Na⁺ gradient to power the transport mechanism.
Optimal survival of N-MYC myeloid leukemias require a porphyrin exporter

Yu Fukuda1, Yao Wang1, Shinjiro Nagai1, Bruce Fanshawe1, Ayten Kandilci2, Brian Sorrentino3, Geoff Neale4, Yiping Fan4, Gerard Grosveld2, John D. Schuetz1

1 St Jude Children's Research Hospital, USA
2 Dept. of Genetics and Tumor Cell Biology, St. Jude Children’s Research Hospital, Memphis, TN, USA
3 Dept. of Hematology, St. Jude Children’s Research Hospital, Memphis TN, USA
4 Hartwell Center for Bioinformatics and Biotechnology, St. Jude Children’s Research Hospital, Memphis, TN, USA

Identifying pathways facilitating the survival of certain leukemias may reveal unique vulnerabilities. Here we determined that pediatric AML with a unique N-MYC gene signature strongly upregulated genes in the porphyrin biosynthetic pathway. However, the significance and relative importance of porphyrin biosynthesis and homeostasis in myeloid leukemias is unknown. We show N-MYC strongly activates de novo porphyrin biosynthesis. Using a murine model of N-MYC myeloid leukemia we demonstrated that absence of the porphyrin exporter, Abcg2, impaired N-MYC leukemogenesis and blocked self-renewal of N-MYC expressing hematopoietic progenitors. Consistent with this, in N-MYC hematopoietic progenitors, Abcg2 absence in vivo strongly reduced the number of leukemic stem and myeloid progenitors. Inhibition of porphyrin biosynthesis or reintroduction of ABCG2 in N-MYC expressing hematopoietic progenitors mostly restored self-renewal. In human myeloid leukemias expressing ABCG2, inhibition of ABCG2 produced porphyrin elevation that was accompanied by mitochondrial ROS as well as loss of both mitochondrial membrane potential and viability. In total, these results reveal a critical dependence of N-MYC myeloid leukemias upon the porphyrin exporter, Abcg2, in mice and suggest that modulating porphyrin levels in patients with these AMLs may be a promising approach to improve therapeutic efficacy.
Misfolding mutants of MRP1 (ABCC1): Useful tools for investigating domain-domain interactions of the transporter

Susan P.C. COLE
Queen's University, Canada

The 190 kDa MRP1 is encoded by the ABCC1 gene found on chromosome 16p13.1 [1]. Human MRP1 transports many anticancer drugs (e.g. doxorubicin), often in a GSH-dependent manner, as well as many other therapeutic agents (eg atorvastatin) [2]. MRP1 also effluxes organic anions including metabolites conjugated to GSH, glucuronate and sulphate. Its most important physiological substrates are the pro-inflammatory cysteinyi leukotriene C4 (LTC4) and the antioxidant tripeptide GSH and its prooxidant dimer, GSSG. MRP1 belongs to a subset of ABC proteins with 2 nucleotide binding domains (NBDs) and 3 membrane spanning domains (MSDs) comprised of 17 transmembrane (TM) α-helices. While its substrate specificity appears mostly determined by the TMs, we have focused on the cytoplasmic loops (CL) that bring the NBDs into proximity to the MSDs. The 60 amino acid long CL5 has a high density of charged amino acids [3]. Ala substitution of Arg501, Glu507, or Arg532 markedly reduced transport activity, due to reduced substrate affinity. In contrast, Ala substitution of Lys513, Lys516, Glu521 and Glu535 caused misfolding resulting in greatly reduced MRP1 levels at the cell surface. Three of these residues lie at CL5/NBD2 interface indicating that its integrity is needed for proper folding, assembly and membrane trafficking of MRP1, an idea supported by the observation that mutation of His1364 and Arg1367 in NBD2 at the CL5 interface also reduced MRP1 levels [3]. 4-PBA treatment of cells expressing the misfolding mutants allowed E521A and E535A to traffic to the plasma membrane; however, the mutants showed mechanistically distinct transport deficiencies associated with different structural conformations [4]. K513A levels could be improved by exposure to glycerol, DMSO, PEG as well as 4-PBA, but transport activity was only fully restored by 4-PBA or DMSO [5]. NBD2 in the transport-compromised PEG or glycerol-rescued K513A proteins had adopted a more open conformation than NBD2, a change accompanied by differences in ATP binding/hydrolysis. In contrast to K513A, K516A levels were not significantly improved by chemical chaperones but in more permissive insect cells, were comparable to wild-type MRP1. However, K516A transport in insect cell membranes was reduced by >80% due to reduced substrate affinity and a more open MSD2 conformation. Overall, our studies show that CL5 serves multiple functions far greater than simply connecting TM9 to TM10. Our data indicate that through its charged amino acids, CL5 participates in interactions with NBD1/NBD2 as well as MSD2, which are variously important for MRP1 transport function as well as for the proper folding and assembly of the transporter into its fully transport-competent structure.

Structure and function of an antibacterial peptide ABC-exporter

Konstantinos Beis
Imperial College London, United Kingdom

Bacteria under nutrient starvation are capable of producing antimicrobial peptides for survival, such as bacteriocins and microcins, by targeting closely related species. They have dedicated ABC transporters to export the toxins out of the producing cells, which in some cases are related to immunity of the cells to their own toxins. We have determined the crystal structure of the lasso peptide microcin J25 ABC exporter McjD at 2.7 Å resolution. McjD is the first structure of a peptide ABC transporter. McjD adopts a new conformation, nucleotide-bound outward occluded, and does not display any intertwining of the transmembrane helices, contrary to other ABC exporters. Our functional and biochemical data show specificity towards MccJ25, but not to other lasso peptides or to linear microcins. ABC exporters need to alternate from an inward to an outward conformation in order to transport their substrates from the cytoplasm to the periplasm in an ATP-dependent manner with subunit intertwining. However, the current model does not explain in detail how the transition from an inward facing to an outward facing conformation occurs. Comparisons with the inward open MsbA and outward open Sav1866 structures show that McjD has structural similarities with both states. The McjD structure provides a good model of the transition from an outward- to an inward-facing state. We also propose that the initial step of the transition is ATP-independent.
Atomic Resolution Description of Structural Transition Pathways in Membrane Transporters with Advanced Molecular Simulation Technologies

Mahmoud Moradi, Giray Enkavi, Po-Chao Wen, Jing Li, Zhijian Huang, Saher Shaikh, Emad Tajkhorshid

University of Illinois at Urbana-Champaign, USA

Membrane transporters are the principal mediators of active exchange of materials across the cellular membrane. These complex molecular machines constitute highly sophisticated, fine-tuned molecular pumps that efficiently couple various sources of energy in the cell to vectorial transport of a wide range of molecules across the membrane, often against the electrochemical gradient. Substrate binding and translocation along the transport pathway in membrane transporters are closely coupled to numerous, largely unknown protein conformational changes of varying magnitude and nature that are induced by and/or coordinated with the energy-providing mechanisms. A detailed description of the mechanism of membrane transporters, therefore, relies on high-resolution methodologies offering simultaneous spatial and temporal resolutions that can describe the dynamics of the process at an atomic level. In this talk, some of the recent methodological advances in our lab and latest results from employing molecular dynamics simulations performed on a number of membrane transporters and the molecular events involved in their function revealed by these simulations will be presented. In particular, we have been able to find the most optimal pathways for large-scale structural transitions of several key membrane transporters and quantify the energetics associated with these transitions using advance free energy calculations strongly support the unprecedented quality of the characterized pathways. Membrane transporters are the principal mediators of active exchange of materials across the cellular membrane. These complex molecular machines constitute highly sophisticated, fine-tuned molecular pumps that efficiently couple various sources of energy in the cell to vectorial transport of a wide range of molecules across the membrane, often against the electrochemical gradient. Substrate binding and translocation along the transport pathway in membrane transporters are closely coupled to numerous, largely unknown protein conformational changes of varying magnitude and nature that are induced by and/or coordinated with the energy-providing mechanisms. A detailed description of the mechanism of membrane transporters, therefore, relies on high-resolution methodologies offering simultaneous spatial and temporal resolutions that can describe the dynamics of the process at an atomic level. In this talk, some of the recent methodological advances in our lab and latest results from employing molecular dynamics simulations performed on a number of membrane transporters and the molecular events involved in their function revealed by these simulations will be presented. In particular, we have been able to find the most optimal pathways for large-scale structural transitions of several key membrane transporters and quantify the energetics associated with these transitions using advance free energy calculations strongly support the unprecedented quality of the characterized pathways.
Subunit stoichiometry of membrane proteins determined by single molecule analysis

Gerhard J. Schütz

Vienna University of Technology, Austria

During their random motion, biomolecules experience a manifold of interactions that transiently conjoin their paths. It is extremely difficult to measure such binding events directly in the context of a living cell: interactions may be short lived, they may affect only a minority fraction of molecules, or they may not lead to a macroscopically observable effect. We describe here a new single molecule imaging method that allows for detecting and quantifying associations of mobile molecules. By “thinning out clusters while conserving the stoichiometry of labeling” (TOCCSL) we can virtually dilute the probe directly in the cell, without affecting the fluorescence labeling of single clusters. Essentially, an analysis region is created within the cell by photobleaching; this region is devoid of active probe. Brownian diffusion or other transport processes lead to reentry of active probe into the analysis region. At the onset of the recovery process, single spots can be resolved as well separated, diffraction-limited signals. State of the art single molecule microscopy then allows for characterizing the spots in terms of their composition and mobility (1-4). Particularly, we used the new method to characterize the oligomerization state of the serotonin transporter (SERT). We found a variety of coexisting oligomerization states up to pentamers; those oligomers were found to be stable over several minutes in the live cell plasma membrane.

Supported by the FWF, grant F3519-B20


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

Harald H Sitte
Medical University Vienna, Österreich

Neuronal functions require phosphatidylinositol-4,5-bisphosphate (PIP$_2$) to regulate ion channels and other proteins. Monoamine transporters control neurotransmission by removing monoamines from the extracellular space. They also display channel properties, but their regulation by PIP$_2$ has so far not been reported. The psychostimulant amphetamine acts on monoamine transporters to stimulate transporter-mediated currents and efflux and thereby increases the levels of extracellular monoamines. Direct or receptor-mediated activation of phospholipase-C (PLC) reduced the levels of PIP$_2$ in the plasma membrane and amphetamine-evoked currents through recombinant serotonin transporters (SERT); application of a PIP$_2$-scavenging peptide mimicked this effect. PLC activation also diminished amphetamine-induced reverse transport without altering serotonin uptake; PLC-mediated inhibition of efflux was not observed in cells expressing GABA transporters.

Furthermore, we used homology modelling and docking to examine the binding site on the SERT. A subsequent mutagenesis study supported the notion that PIP$_2$ directly binds to the transporters. These data demonstrate the PIP$_2$-dependence of current and reverse transport, but not inward transport, in monoamine transporters.

The Molecular Basis of ABC Transporter-Mediated Drug Resistance from Yeast to Man – From Structural Modeling to Transport Mechanisms?

Karl Kuchler¹, Narakorn Khunweeraphong¹, Anna Cseke¹, Martin Valachovic¹, Thomas Stockner²

¹ Medical University of Vienna, Austria
² Institute of Pharmacology, Medical University of 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.

A subset of yeast efflux pumps plays a major role in the so-called pleiotropic drug resistance (PDR) phenomenon, where overexpressed ABC transporters such as Pdr5 confer resistance to a vast variety of xenobiotics. This phenomenon resembles multidrug resistance in cancer cells conferred by membrane proteins such as P-glycoprotein, BCRP/ABCG2 or MRP, and it poses serious difficulties when occurring in microbial pathogens. Our key hypothesis is that the principle drug transport mechanisms may be conserved among fungal PDRs and mammalian ABCGs. We have thus pursued both a detailed structure-function and comparative structural modeling analysis of a yeast PDR transporter and its mammalian ABCG2 orthologue with the aim to discover common mechanistic aspects of drug substrate transport. In my lecture, I will provide a comprehensive review about the role of fungal drug resistance ABC transporters, as well as elaborate on the usefulness of comparative structural modeling strategies to decipher mechanisms of xenobiotic transport in medically relevant mammalian ABC transporters.

Our work is supported by a grant from the Austrian Science Foundation (SFB035-04).
The dual core architecture of ABC transporters: possible implications for function and folding

Peter Chiba1, Yaprak Dönmez2, Elena Rudashevskaya1, Narakorn Khunweeraphong1, Harald Sitte3, Gerhard Ecker4, Thomas Stockner5

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ATP binding cassette (ABC) transporters, which are found in all extant phyla, have been causally related to the etiology of at least 25 human diseases. Moreover, pharmacokinetics of systemically administered drugs can be influenced by multispecific members of the ABC-transporter family. The minimal functional unit of ABC transporters comprises of four domains, two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs), which in humans are arranged in tandem. All human ABC transporters are exporters. We demonstrate that the rotational C2-symmetry of the proteins brings forth a dual core architecture forming two (potentially overlapping) substrate binding sites. These are identical in half transporters and similar, nevertheless biochemically discernible in full transporters. In the latter group of transporters, for which ABCB1 (P-glycoprotein) serves as a paradigm, different solutes have dissimilar binding probability ratios (Parveen et al 2011). We pioneered a photolabeling approach, which in combination with mass spectrometry and MD-refined structural modeling allowed experimental proof of a dual core architecture of ABCB1. Binding probability of protonable substrates to either of two symmetry related binding sites can be modified by introducing symmetrically positioned selector-residues with a substrate-equivalent charge state. This experimental strategy enabled us to study the importance of active site residues in each binding mode without interference from the other. The presence of two composite substrate binding sites at the TMD-interface and two composite nucleotide binding sites at the NBD-interface might be important for function, as a pairwise dedication between them might exist. Folding deficiency has been identified as a frequent cause for ABC transporter malfunction in inherited diseases, not only in cystic fibrosis, but also among others in intrahepatic cholestasis and gout. The importance of the dual core concept for rescue of folding deficient variants by pharmacological chaperones will be discussed.

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Drug Transporters at the Blood-Arachnoid-CSF Barrier

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The subarachnoid space, where CSF flows over the brain and spinal cord, is lined on one side by arachnoid barrier (AB) cells that form part of the blood-cerebrospinal fluid (CSF) barrier. However, despite the fact that drugs are administered into the CSF, and CSF drug concentrations are used as a surrogate for brain drug concentration following systemic drug administration, the tight junction AB cells have never been examined for whether they express drug transporters that would influence CSF and CNS drug disposition. Hence, we characterized drug transporter expression and function in mouse and human AB cells. Immunohistochemical analysis showed P-glycoprotein (Pgp) and BCRP in mouse and human AB cells, but not other meningeal tissue. Microarray analysis of mouse and human arachnoidal tissue revealed expression of many drug transporters. Immortalized AB cells express functional Pgp on the apical membrane and BCRP on both membranes. Consistent with Pgp on the AB apical membrane (dura facing) and BCRP on the basal membrane (CSF facing), Pgp substrates were removed faster from the CSF space of Pgp WT vs. KO mice while BCRP substrates were removed faster from the CSF of BCRP KO vs. WT mice following intrathecal injection. Thus, AB drug transporters contribute to the CSF drug permeation barrier. However, we realized that studies using mice with global deletion of drug transporters are inadequate to understand how AB drug transporters (independent of drug transporters at the blood brain barrier (BBB) endothelial cells or choroid plexus (CP) epithelial cells) influence CSF drug disposition which is important to understand since CSF drug concentration is frequently used as a surrogate for the free drug concentration in the brain, and because the expression and/or pumping direction of drug transporters at the three sites sometimes oppose one another. Hence we have been developing mice with conditional deletion of Pgp and BCRP at the three brain barrier sites. These studies have implications for better understanding of the influence of drug transporters along the blood-cerebrospinal route, in particular, to CSF and CNS drug disposition.
Glutamate transporters, mitochondria, and astrocytes: Biology reveals itself

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Glutamate is the predominant excitatory neurotransmitter in the mammalian brain. After release, a family of sodium-dependent transporters both shape excitatory signaling and prevent excessive activation of glutamate receptors that can lead to neuronal death. Unlike most classical neurotransmitters, the bulk of glutamate clearance is into astrocytes. In the forebrain, this activity is mediated by GLT-1 (also called EAAT2). Decreases in GLT-1 expression and impaired glutamate transport activity have been associated with both acute and chronic neurodegenerative diseases. In this presentation, I will briefly describe two stories.

First, I will describe a project that we have been pursuing in collaboration with Dr. Jeff Rothstein. We have been defining the mechanisms that control transcriptional regulation of GLT-1. Using both in vivo and in vitro approaches, we made the surprising observation that different subtypes of astrocytes engage different transcriptional mechanisms to control GLT-1 expression.

In the second hour of my presentation, I will describe an analysis of proteins that were found to interact with GLT-1 in brain tissue using co-immunoprecipitation and mass spectrometry. We found that mitochondrial proteins/mitochondrial proteins overlap with GLT-1 more than would occur by chance. As is true for neurons, we find that the fraction of mobile mitochondria is regulated by neuronal activity in organotypic cultures prepared from neonatal hippocampus; dampening activity increases the fraction of mobile mitochondria. Furthermore, we have developed evidence that neuronal activity dampens mobility of mitochondria by a mechanism that depends on transport activity. We have also found that oxygen glucose deprivation causes a delayed loss of mitochondria within the astrocytic processes.
The EGF-receptor-Transporter connection: exploring the unexpected

Maria Sibilia
Medical University of Vienna, Austria
Abstract not available
MS Binding Assays and their application to the development of new GABA uptake inhibitors.

Wanner Klaus T., Sindelar Miriam, Schmitt Sebastian

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MS binding assays, a novel technique for the characterization of ligand-target interactions, are closely related to radioligand binding assays, but avoid all drawbacks that result from radioactivity associated with radiometric assays. Most importantly no labelling of the ligands to be monitored by MS is required.\[1\] As will be demonstrated for the GABA transporter mGAT1 (SLC6a1), a target representing the most important subtype of the γ-aminobutyric acid transporters, MS Binding Assays can be used in saturation experiments, competition experiments, or kinetic studies. In addition, competitive MS binding assays can also be employed for drug screening. Even libraries generated by Dynamic Combinatorial Chemistry (DCC) may be screened, provided appropriate measures are taken to render them pseudostatic. The latter approach will be exemplified for mGAT1 as well. Importantly, in this case libraries are generated in the presence of the protein target and under conditions that are compatible with the screening process. That way, they resulting incubation mixtures can be directly used for hit detection by MS making the whole screening process highly efficient.\[2\]\[3\]

Furthermore, a new GABA uptake assay for hGAT1 based on MS will be presented.


Regulation of Dopamine Transporter Phosphorylation and Function by the Peptidyl Prolyl Cis-Trans Isomerase PIN1

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The rat dopamine transporter is phosphorylated in the membrane proximal region of the N-terminus on Thr53, a residue that is followed by proline, making it specific for proline-directed kinases such as ERK. In previous studies we found that Thr53 mutations affected the kinetics of transport and amphetamine-stimulated efflux, suggesting a role for Thr53 phosphorylation in these processes. Phosphorylation of proline-directed residues strongly impacts protein conformation by regulating cis-trans isomerization of the phosphoacceptor-proline peptide bond. Cis-isomerization of these bonds in turn slows the rate of phosphoacceptor dephosphorylation by preventing actions of phosphatases. The peptidyl prolyl cis-trans isomerase, PIN1, specifically isomerizes phospho-Ser/Thr-Pro motifs and catalyzes the return of the bond to the trans configuration, allowing dephosphorylation to occur. To test for such activities on DAT we have used the PIN1-specific inhibitor juglone. In both rat striatal synaptosomes and heterologous expression systems juglone treatment rapidly increased the phosphorylation level of Thr53, suggesting that PIN1 acts on DAT and regulates dephosphorylation of this site. In addition, juglone strongly stimulated [3H]DA efflux from rat striatal synaptosomes, supporting a mechanistic link between efflux and Thr53 phosphorylation. These results identify a novel mechanism for regulation of DA efflux and suggest PIN1 as an enzyme that functions in post-phosphorylation control of DAT function.
Unraveling Ion Coupling in the Human Serotonin Transporter: the different roles of Na

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Neurotransmitter transporters of the SLC6 family of proteins, including the human serotonin transporter (hSERT), utilize Na\textsuperscript{+}, Cl\textsuperscript{−} and K\textsuperscript{+} gradients to induce conformational changes necessary for substrate translocation. Dysregulation of ion movement through monoamine transporters has been shown to impact neuronal firing potentials and could play a role in pathophysologies such as depression and anxiety. Despite multiple crystal structures of prokaryotic SLC transporters indicating the location of both (or one) conserved Na\textsuperscript{+} binding sites (termed Na1 and Na2), much remains uncertain in regard to the movements and contributions of these cation-binding sites in the transport process. In this study, we utilize the unique properties of a mutation of hSERT at a single, highly conserved asparagine on TM1 (N101) to provide several lines of evidence demonstrating mechanistically-distinct roles for Na1 and Na2. Mutations at N101 alter the cation dependency of the transporter, allowing Ca\textsuperscript{2+} (but not other cations) to functionally replace Na\textsuperscript{+} for driving transport and promoting 5-HT-dependent conformational changes. Furthermore, in two-electrode voltage clamp studies in \textit{Xenopus} oocytes, both Ca\textsuperscript{2+} and Na\textsuperscript{+} illicit 5-HT-induced currents in the N101 mutants and reveal that, although Ca\textsuperscript{2+} promotes substrate-induced current, it does not appear to be the charge carrier during 5-HT transport. These findings, in addition to functional evaluation of Na1 and Na2 site mutants reveal separate roles for Na1 and Na2 and provide insight into initiation of the translocation process, as well as a mechanism whereby the reported SERT stoichiometry can be obtained despite the presence of two putative Na\textsuperscript{+} binding sites.
Depression and related disorders cause much suffering and lost productivity worldwide, compounded by the fact that a least half of patients are not effectively treated by currently available medications. The most commonly prescribed antidepressant drugs belong to the selective serotonin (5-HT) reuptake inhibitor (SSRI) class and act by blocking the serotonin transporter (SERT), the high-affinity “uptake 1” transporter for 5-HT. Increases in extracellular 5-HT that follow are thought to be critical for initiation of downstream events needed for therapeutic effects. A potential explanation for their limited therapeutic efficacy is the recently characterized presence of organic cation transporters (OCT1, OCT2 and OCT3) and plasma membrane monoamine transporter (PMAT) in brain. These “uptake 2” transporters have low-affinity but high-capacity to take up biogenic amines and thereby, may limit the ability of SSRIs to increase extracellular 5-HT to therapeutically relevant levels. Consistent with the idea that “uptake 2” transporters limit antidepressant-like activity of SSRIs, we found that in adult mice, blockade of these transporters with decynium-22 (D22) enhanced the 5-HT uptake inhibiting effect and antidepressant-like activity of the SSRI, fluvoxamine. Similarly, when SERT expression was genetically reduced, D22 inhibited 5-HT clearance and produced antidepressant-like effects. The potency of D22’s antidepressant-like activity was reduced in OCT3 knockout mice, suggesting that in addition to OCT3, other D22-sensitive transporters contribute to its antidepressant-like effects.

Depression is often co-morbid with alcoholism and increased risk for both disorders has been associated with low expressing/functioning gene variants of SERT. Ethanol is known to increase extracellular 5-HT; however, the mechanism(s) through which ethanol induces this increase remains unclear. We found that in mice with a constitutive reduction or loss of SERT, ethanol robustly inhibited 5-HT clearance. We also found that SERT heterozygous and knockout mice have increased levels of mRNA and protein for OCT3 compared with wild-type mice, raising the possibility that ethanol inhibits 5-HT uptake via OCT3. Our preliminary data support this notion. Moreover, like D22, we found that ethanol also enhances the ability of SSRIs to inhibit 5-HT clearance. These findings point to OCT3, and not SERT, as a site where ethanol inhibits 5-HT uptake.

Together these data show that “uptake 2” transporters, putatively OCT3, play a prominent role in 5-HT uptake when SERT function is either genetically or pharmacologically compromised. Together these data suggest that OCT3 may be a valid target for the development of new antidepressants with improved therapeutic efficacy.
Identification and molecular properties of the allosteric binding site in the serotonin transporter

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The serotonin transporter (SERT) plays a key role in regulating serotonin (5-HT) homeostasis and is the major pharmacological target for antidepressants (ADs) such as S-citalopram (S-CIT) in the treatment of depression and anxiety disorders. In addition to the high-affinity binding site for ADs overlapping with the substrate binding site (S1), data have supported the existence in SERT of a low-affinity allosteric binding site for inhibitors such as S-CIT and clomipramine. Binding of S-CIT to the allosteric site modulates ligand binding properties of the S1 site. In this presentation I will show experimental evidence supported by molecular modelling suggesting that the allosteric binding site is localized to the extracellular vestibule of SERT. In addition, I will describe our efforts in the development of ligands possessing high-affinity and high selectivity towards the allosteric binding site. We hypothesize that such an allosteric inhibitor could possess a new clinical potential compared to the current ADs either as separate entities or as an adjuvant therapy improving the clinical efficacy of existing drugs targeting S1.
S6-02  Cation binding to the Na2 site of the serotonin transporter drives the conformational changes necessary for transport

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The serotonin transporter contains two sodium binding sites, Na1 and Na2. It remains unclear what the distinct role of the individual sites are with respect to substrate binding, conformational changes and substrate translocation. By combining mutagenesis with functional studies and electrophysiology we show that Na2 is the primary determinant of functional sodium affinity in serotonin uptake, i.e. Na2 is the cation binding site primarily driving the translocation of substrate from the extracellular to the intracellular side. We show that the cation selectivity of hSERT can be manipulated by mutagenesis of Na2 and that the utilization of an intracellular K+ or H+ to drive the conformational change from inward-facing to outward-facing is achieved through binding to the Na2 site. By mutation of Na2 we are able to alter the cation selectivity of the translocation process.
Serotonin transporter (SERT) couples the reuptake of serotonin to the influx of sodium and chloride and the efflux of potassium. The ability of transporters like SERT to catalyze coupled transport ultimately requires that conformational changes are triggered by binding events. To understand how binding site occupation influences conformational change, we have studied the bacterial SERT homologue LeuT, which transports Na⁺ together with an amino acid substrate. Several publications have shown that Na⁺ influences the conformation of LeuT, stabilizing outward-open conformations. Because LeuT binds two Na⁺ ions, we examined the possibility that occupation of the Na1 and Na2 sites have different effects on substrate binding and conformational change. We began with the hypothesis that Na1, which directly coordinates the substrate carboxyl group, is responsible for the Na⁺-dependence of substrate binding and that Na2, which is sandwiched between the stationary scaffold and the mobile 4-helix bundle of LeuT, is responsible for stabilizing outward-open LeuT conformations. Mutations designed to mildly disrupt the Na1 and Na2 sites provided some support for this hypothesis, but also revealed some surprising aspects of the Na⁺ sites. Several of these results were replicated using mutants of SERT, suggesting that they may provide insight into the role of Na⁺ in all transporters in this family.
Fasciola hepatica, a parasite with many transporters

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The liver fluke Fasciola hepatica infests farm animals and people to cause the eponymous liver fluke disease (fascioliasis). This parasitic disease occurs all over the world; there is no effective vaccine; for the past several decades the drug of choice has been the anthelmintic drug triclabendazole. Predictably, fluke populations resistant to triclabendazole have emerged.

Several mechanisms have been proposed to account for this resistance. ABC transporters of Fasciola hepatica may be involved, because inhibitors of mammalian ABC transporters render resistant fluke strains susceptible to triclabendazole. Sequence data bases indicate that the Fasciola hepatica genome harbours a large number of transporters that fall into distinct of ABC transporter subfamilies. Their biochemical properties, in particular their substrate specificity, are not known. This is also true for their tissue- and life cycle-dependent expression. Because Fasciola hepatica has to switch hosts and to cope with very different environments, it is likely to rely on many different transporters. Accordingly, transporters may not only account for emerging drug resistance but also constitute interesting candidate drug targets presented as Achilles heels by the individual stages during the life cycle of the worm. We therefore initiated a survey of Fasciola hepatica transporters by cloning several transporters using samples of fresh flukes from local slaughter houses. One of these transporters had highest homology to ABC transporters of the B-subfamily. When heterologously expressed in HEK293 cells, this nematode transporter supported efflux of rhodamine123, which was blunted by typical blockers of P-glycoprotein (ABCB1). Most importantly, rhodamine via the Fasciola hepatica transporter – but not via human ABCB1 - was inhibited by triclabendazole.

These experiments provide a proof-of-principle by showing that (i) an ABC transporter can account for triclabendazole resistance and (ii) that the nematode orthologue may be sufficiently diverse to allow for selective targeting. In the past, huge efforts were made to develop ABC transporter blockers to overcome drug resistance in cancer therapy in humans. This approach has been questioned and recently abandoned because blockage of human ABC transporters is associated with severe adverse events (e.g., due to penetration of toxic compounds into the brain). Proteins of the nematode Fasciola hepatica may be targeted to overcome resistance to anthelmintic drugs without incurring risks of major adverse effects on people or farm animals.
Transport Machineries in Adaptive Immunity and Viral Immune Evasion

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Goethe-University Frankfurt, Germany

The recognition and elimination of virally or malignantly transformed cells is the pivotal task for the adaptive immune system. For immune surveillance, a snapshot of the cellular proteome is displayed on major histocompatibility (MHC) class I molecules as immunodominant epitopes for recognition by cytotoxic T-cells. The knowledge about the track from the equivocal protein to the presentation of peptides has greatly expanded, leading to an astonishingly elaborated understanding of the MHC I peptide loading pathway. This seminar will report on this complex process, which rests on ABC transporters, chaperones, and ER quality control. The contribution of the individual proteins and subcomplexes as well as the architecture and dynamics of the peptide-loading complex will be discussed, including mechanisms of viral immune evasion.
Molecular regulation of hepatobiliary ABC transporters in cholestatic liver diseases

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Cholestatic liver diseases are characterized by impaired heptobiliary excretory function leading to accumulation of bile salts (BS), hepatocellular damage, inflammation, fibrosis/cirrhosis, and cancer. While BS retention results in hepatocellular damage, continued/disproportionate BS efflux via the canalicular bile salt export pump (ABCB11) in relation to the canalicular phospholipid export pump (ABCB4) may critically determine downstream bile duct injury in cholestatic diseases ('toxic bile concept'). Nuclear receptors such as the farnesoid X receptor (FXR) are central regulators of BS metabolism and transport. Deletion of FXR in the ABCB4 knockout (ABCB4\textsuperscript{-/-}) mouse model of sclerosing cholangitis improved bile duct injury but aggravated hepatocellular damage as reflected by increased levels of serum ALT, ALP and BS in comparison to single ABCB4\textsuperscript{-/-}. This may be explained by reduced bile flow, biliary BS-output and expression of ABCB11. However, hepatic and intestinal BS-homeostasis was lost resulting in elevated CYP7A1 (BS synthesis) which was not compensated by pronounced induction of alternative BS detoxification (CYP2B10, CYP3A11) and basolateral BS efflux pumps (ABCC3 and 4). These findings support the toxic bile concept and central role of FXR as key regulator of ABC transporters.

Breast cancer-related protein (ABCG2) and multidrug resistance protein (ABCB1) may function as compensatory canalicular efflux system for BS (and carcinogens) in cholestasis, but could also contribute to cell injury and carcinogenesis in the biliary tract. Hepatic expression of ABCB1a and b (but not G2) was increased in ABCB4\textsuperscript{-/-} and bile duct-ligated mice. FXR ligands induced ABCB1a and b in vivo and in vitro, suggesting a central regulatory role for BS. To identify novel pharmacological strategies for regulation of ABC transporter expression, we screened a panel of nuclear receptor agonists and antagonists in vitro. In addition to FXR and PPAR, thyroid hormone receptor (TR) ligands targeting the major hepatic isoform TR\textsubscript{81} were identified as novel stimulators of ABCB1a, C3 and 4, but not other ABC efflux pumps (including G2). Moreover, norUDCA potently induced ABCB1a, C3 and 4, but not other ABC efflux pumps (including G2). Moreover, norUDCA significantly decreased the area under the liver time-activity curves (AUC\textsubscript{18F}) of \textsuperscript{18}F-ciprofloxacin in both WT and ABCB4\textsuperscript{-/-} mice, most likely reflecting induction of ABC transporter expression. This results indicate, PET tracer studies may help to functionally visualize and assess the adaptive ABC transporter response in cholestasis and under therapeutic conditions.
Poster Presentations

Monday, September 23: EVEN poster numbers

Tuesday, September 24: ODD poster numbers

Wednesday, September 25: free poster viewing
**PP-01** Tailoring activity thresholds according to bioassays for use in classification models of P-Glycoprotein inhibitors

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**Background:** P-glycoprotein (Multidrug resistance protein 1, ABCB1) inhibitors are promising agents for the use in cancer chemotherapy in order to overcome multidrug resistance. In addition, they are involved in bioavailability and safety of drugs. Thus, reliable in silico procedures to predict P-glycoprotein inhibitors are of great interest.

**Objective:** Examining whether a classification dataset taking the individual values of a standard compound (e.g., verapamil) measured in every assay as separation criterion, is performing better than by taking a generalized threshold.

**Methods:** Compound bioactivity data for P-glycoprotein inhibitors was retrieved from the open access database ChEMBL version 15 (410 compounds). 2D descriptors were calculated using the MOE software. Attribute selection and model building was done in WEKA 3.7.8 by using different classifier algorithms. Validation was done by 10-fold cross validation as well as by prediction of an external test set, consisting of 310 in-house tested compounds (all measured in the same assay set-up).

**Results and Discussion:** The best internal models (10-fold cross-validation) were achieved with Random Forest Decision Tree for the models built upon both thresholds. Models built under the pre-condition of taking an assay-tailored verapamil threshold in order to separate inhibitors from non-inhibitors outperform the models with a generalised threshold when it comes to the prediction of an external test set, due to a more balanced predictive power regarding inhibitors and non-inhibitors (sensitivity and specificity) in the external test set.

The research leading to these results has received support from the Austrian Science Fund (FWF), grants F03502 and W1232, as well as from the Innovative Medicines Initiative Joint Undertaking under grant agreements no. 115002 (eTOX) and 115191 (Open PHACTS), resources of which are composed of financial contribution from the European Unions Seventh Framework Programme (FP7/2007-2013) and EFPIA companies’ in-kind contribution.

**PP-02** Unfolding the structure of LeuT\textsubscript{Aa} employing Luminescence Resonance Energy Transfer

**Azmat Sohail**, Oliver Kudlacek, Markus Daerr, Peggy Stolt-Bergner, Gerhard F. Ecker, Michael Freissmuth, Klaus Wanner, Thomas Stockner, Walter Sandtner, Harald H Sitte

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3. Research Institute of Molecular Pathology, Campus Vienna Biocenter, Austria
4. Department of Medicinal Chemistry, University of Vienna, Austria

**Background:** Neurotransmitter sodium symporters (NSS) are located in the brain and retrieve neurotransmitters from the synaptic cleft to end synaptic transmission. Solute carrier class six proteins (SLC6) are of great pharmacological importance in terms of their localization and function. The crystal structures obtained from a bacterial homolog, the leucine transporter LeuT\textsubscript{Aa}, in open to outward, occluded and open to inward conformations are present in frozen state with high resolution (Krishnamurthy et al., 2012). Due to its close kinship with SLC6 proteins, LeuT\textsubscript{Aa} serves as a paradigm for these transporters.

**Methods:** In order to address the dynamicity of the substrate transport cycle in LeuT\textsubscript{Aa}, we use the Lanthanide encoded resonance energy transfer (LRET) technique. This method is a spin-off of the fluorescence resonance energy transfer method according to Förster employing the introduction of the genetically encoded lanthanide binding tags (LBT) as donor elements. Exogenous cysteine residues labelled with cysteine specific fluorophores are used as acceptor elements. This technique is an alternative to address the movement of helices, with great resolution and has been employed successfully to examine potassium channels (Sandtner et al., 2007).

**Results:** We screened for the functional LBT\textsubscript{mutants} using the scintillation proximity assay. The LeuT-A335-LBT-G336 mutant displayed function in terms of its binding activity. Within this background, we generated cysteine mutants. To date, we have successfully measured the intramolecular distances in different LBT\textsubscript{LeuT}_{Cys} mutants. Furthermore, we observed intramolecular distance changes from these purified proteins in detergent micelles.

**Conclusion:** Our LRET measurements will help us to understand the transport cycle and help to complete the missing steps in substrate transport cycle of LeuT\textsubscript{Aa}. Currently, we focus on the reconstitution of purified LeuT\textsubscript{Aa} into liposomes and have our LRET measurements in a reconstituted system that allows to use more physiological ionic gradients.

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PP-03  Age dependent reversal of COMT influence on resting state connectivity in regions relevant for executive and cognitive functioning.

Bernhard M Meyer 1, Julia Huemer 2, Ulrich Rabl 1, Lucie Bartova 1, Ana Popovic 1, Helmut Haslacher 3, Thomas Perkmann 5, the IMAGEN Consortium 4, Harald Esterbauer 5, Ewald Moser 6, Siegfried Kasper 1, Harald H Sitte 5, Gunter Schumann 4, Lukas Pezawas 1

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Introduction: The Valine158Methionine (Val158Met) polymorphism of the COMT gene results in lower enzymatic activity and higher dopamine availability in Met carriers. Dopamine effects on behavior follow an inverted U-shaped dose-response curve, with both deficient and excessive amounts of dopamine activity predicting poor cognitive task performance. In humans, results overall suggest a decrease in dopamine levels from puberty to adulthood. These age-dependent changes could affect the position of the Val158Met genotypes on an inverted U-shaped curve and lead to a differential effect of the Val158Met polymorphism during development. While numerous fMRI studies investigated the genotype effect in the context of task-dependent cognitive functioning, recent studies suggest an alteration within the default mode network (DMN) and the executive control network (ECN) at rest. We therefore tested the hypothesis of an age dependent reversal of COMT influence on resting state functional connectivity in regions relevant for executive and cognitive functioning.

Methods: Data of 106 healthy young adults included in an imaging genetics protocol in Vienna, Austria (FWF project nr. F3514-B11) were gender and COMT matched to 106 randomly-drawn 14 year-olds included in the IMAGEN project. All participants were instructed to relax, stay awake and lie still, while keeping their eyes closed. After preprocessing, we selected a spherical seed from resting state literature within the DMN and ECN intersection (anterior medial prefrontal cortex - amPFC xyz 1, 54, 21) to calculate the mean time series for functional connectivity analysis. Based on these single subject fisher z transformed seed-to-voxel maps, we calculated the interaction of COMT Val158Met genotype and both age groups with respect to the covariate gender. We further investigated whether these effects are also relevant for connections of a ‘true network’ measurement, namely partial correlations. We used the familywise approach 3dClustSim for multiple comparison correction at a voxel-wise threshold of p<0.001.

Results: We observed a significant (p<0.05 corrected) age-dependent reversal of COMT Val158Met effects on resting state connectivities between the amPFC and the left vlPFC, left dorsolateral prefrontal cortex (dlPFC), left and right parahippocampal gyrus (PHG). These interaction effects were robust even when focusing on ‘true network’ measurements. Adult Val homozygous had a dose-dependent increased and adolescent Val homozygous a decreased connectivity compared to Met homozygous for all reported regions.

Conclusions: The results suggest that COMT’s influence on functional connectivity at rest is not static during development and might have important implications for developmental psychopathology.

References:  
PP-04 Capturing large conformational changes of NSS-transporters by MD simulations.

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The human serotonin transporter (hSERT) is the principal medical target for antidepressants.[i] The results obtained from molecular dynamics simulations of hSERT with bound serotonin and of LeuT with substrate (Ala or Leu) will be presented revealing some characteristics of the transport mechanism linked to the large-scale conformational change from an outward-open structure to the inward-open form, including sequence of release of co-transported sodium ion(s) and substrate.[ii] Differences detected in the protein dynamics when binding either the substrate or an inhibitor (competitive or non-competitive) will be presented,[iii] and finally the necessity of a second substrate in the so-called S2-site will be discussed based on unbiased MD simulations with two substrates. The results hint to a very delicate network of interactions that gate the dynamics of the protein.


PP-05 Searching for specific and clinically relevant BCRP (ABCG2) probe

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Background: Breast Cancer Resistance Protein (BCRP/ABCG2) belongs to the G subfamily of the ATP Binding Cassette (ABC) transporter proteins. BCRP localized at the apiical membrane of epithelial cells of barrier tissues, handling a broad variety of endogenous and exogenous compounds. BCRP plays a role in drug absorption, disposition and excretion and regulates clinically relevant drug-drug interactions. Testing for BCRP is recommended by the regulatory agencies (FDA/EMA), although there is no widely accepted BCRP probe for the various in vivo and in vitro assays.

Aim: To find a clinically relevant BCRP probe that is specific for BCRP, accepted by the regulatory agencies, applicable for various in vivo and in vitro assays, and commercially available.

Methods: Five candidates (chlorothiazide, rosuvastatin, sulfasalazine, teriflunomide and topotecan) were selected based on literature data and in-house experience. First, all five compounds were tested in vesiculare transport assay using BCRP, MDR1, MRP2, and MRP4 overexpressing Sf9 or Hi5 membranes. Four of these compounds were then measured in vectorial transport assay on Caco-2 cells and BCRP overexpressing MDCKII cells.

Results: Chlorothiazide, sulfasalazine and teriflunomide were selective for BCRP in the vesicular transport experiments. In the monolayer system both chlorothiazide and teriflunomide were specific for BCRP.

Conclusion: Based on our results and literature data, chlorothiazide and teriflunomide meet all the required criteria for being a clinically relevant BCRP probe. However, further clinical data, for chlorothiazide in particular, are needed to establish these compounds as BCRP probes.
Elucidating chaos: Uncovering the mechanistically-distinct roles of the two Na⁺ binding sites in the human serotonin transporter

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Neurotransmitter transporters of the SLC6 family of proteins, including the human serotonin transporter (hSERT), utilize Na⁺, Cl⁻ and K⁺ gradients to induce conformational changes necessary for substrate translocation. Dysregulation of ion movement through monoamine transporters has been shown to impact neuronal firing potentials and could play a role in pathophysiologies, such as depression and anxiety. Despite multiple crystal structures of prokaryotic SLC transporters indicating the location of both (or one) conserved Na⁺ binding sites (termed Na1 and Na2), much remains uncertain in regard to the movements and contributions of these cation binding sites in the transport process. In our work, we utilize the unique properties of a mutation of hSERT at a single, highly conserved asparagine on TM1 (N101) to provide several lines of evidence demonstrating mechanistically-distinct roles for Na1 and Na2. Mutation at N101 alters the cation dependency of the transporter, allowing Ca²⁺ (but not other cations) to functionally replace Na⁺ for driving transport and promoting 5-HT-dependent conformational changes. Furthermore, two-electrode voltage clamp studies in Xenopus oocytes reveal that, while Ca²⁺ promotes substrate-induced current, it is not co-transported with 5-HT. These findings, in addition to functional evaluation of Na1 and Na2 site mutants, reveal separate roles for Na1 and Na2 and provide insight into initiation of the translocation process, as well as a mechanism whereby the reported SERT stoichiometry (1 Na⁺ in : 1 Cl⁻ in : 1 5-HT in : 1 K⁺ out) can be obtained, despite the presence of two putative Na⁺ binding sites.

Dissection of the intramolecular communication pathways within the multidrug resistance transporter P-glycoprotein

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In 1966 Jardetzky (Nature 211, 969-70) proposed that membrane pumps function by exposing their ligand-binding pocket alternately on different sides of the membrane. For primary active pumps like ATP-Binding-Cassette (ABC) transporters, coupling of the ligand-binding sites of the transmembrane domains (TMDs) to the ATP catalytic cycle of the nucleotide-binding domains (NBDs) is fundamental to the transport mechanism but is poorly understood at the molecular level. Structure data suggest signals are transduced through intracellular loops of the TMDs which slot into a groove on the NBDs. At the base of this groove is the Q-loop. We report the first evidence of the crucial role played by the Q-loops in the coupling mechanism of the drug efflux pump ABCB1. We mutated the eponymous glutamine in one, or both, NBD Q-loops and measured the impact on ABCB1 conformation and function. Two lines of evidence prove that the double mutant is trapped in the inward-open state which retains affinity for drug but cannot couple to the ATP cycle. Our data also describe remarkable redundancy within the mechanism of ABCB1 which requires only one of its two Q-loops to function. This allowed us to elucidate transduction pathways from twin drug-binding cavities to the Q-loops using point mutations to favour one cavity over the other. The data show the Q-loop is the central flexion point where the aspect of the drug-binding cavities is coupled to the ATP catalytic cycle. This conduit is fundamental to the molecular mechanism and is conserved in both ABC exporters and importers.
PP-08  Insights in the implication of the human OCTN1 (SLC22A4) transporter in pathophysiology.

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The OCTN1 (SLC22A4) transporter belongs to the Organic Cation Transporter Novel sub-family, found only in vertebrates. Despite the delay in defining the physiological role of OCTN1 in intact cell systems, its involvement in human pathophysiology was assessed from epidemiological and genetic evidences. A variant of OCTN1 (L503F) has been linked to the Inflammatory Bowel Diseases (IBDs) and different types of cancer. To gain further insights in the structure, the physiological role and the molecular bases of pathology links, we over-expressed the human (h)OCTN1 in E.coli. After purification the protein was reconstituted in proteoliposomes for transport assay. It was revealed that hOCTN1 transports acetylcholine besides TEA (the most acknowledged non-physiological substrate), carnitine and ergothioneine. The transporter mediates also efflux of acetylcholine from proteoliposomes, regulated by Na⁺ and K⁺. These data, corroborated by the finding of an identical orientation of the transporter in proteoliposomes and in cell membranes, indicated a role for OCTN1 in exporting acetylcholine from cells. This is consistent with the existence of the non neuronal cholinergic system recently described by several research groups in the same tissues where OCTN1 is expressed. In non neuronal districts acetylcholine released from cells, regulates inflammatory processes and many other functions. Noteworthy, the acetylcholine efflux mediated by hOCTN1 was impaired in the L503F variant. This may be the molecular link between this variant and one of the IBDs, the Crohn’s disease, explaining the typical chronic inflammation. hOCTN1 was found to be sensitive to SH reagents used for targeting Cys residues. We have then investigated the effects of HgCl₂ and methylmercury, the most diffused mercury pollutants, which are known to react with Cys thiol groups. Both the reagents strongly inhibited the transport function suggesting an involvement of hOCTN1 in mercury toxicity. The inhibition by mercurials was reversed by SH reducing agents, confirming the interaction with Cys residues. The molecular mechanism of the interaction was investigated using site directed mutagenesis, constructing single mutants in which each of the seven Cys of OCTN1 was substituted with Ala. The effect of the mercury compounds on the [³H]acetylcholine transport mediated by the mutants in proteoliposomes was tested. Only C50A and C136A mutants displayed a reduced inhibition by mercurials, demonstrating that the two Cys are critical for the interaction and inhibition by mercurials and, hence, also for transport mechanism. An homology structural model of hOCTN1 has been built using as template the glycerol 3-phosphate transporter. The model highlighted that the two Cys are exposed towards the extraliposomal (extracellular) side thus being available to the reagents. These data suggest a further involvement of hOCTN1 in human pathophysiology as a first level target of mercury and other SH reactive xenobiotics.

PP-09  Serotonin transporter function in blood platelets is associated with neural activity in the default-mode network.

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The serotonin transporter (5-HTT) is abundantly expressed in humans by the serotonin transporter gene SLC6A4 and removes serotonin (5-HT) from extracellular space. A blood-brain relationship between platelet and neuronal 5-HT uptake is presumed, but it is unknown today, if kinetic parameters of platelet 5-HT uptake can serve as estimator of neural activity in humans.

In this functional magnetic resonance imaging (fMRI) study we show an inverse relationship between an in vitro parameter of 5-HT uptake, platelet maximal 5-HT uptake velocity (Vmax), and BOLD signaling specifically in core regions of the default-mode network (DMN). Moreover, a positive correlation in a highly complementary cortical network encompassing primarily sensory-motor areas was detected, while no associations between platelet Vmax and subcortical BOLD signaling were found. Notably, no impact of SLC6A4 variants on the correlative relationships between platelet Vmax and BOLD signaling were detected, while our data suggest a genetic effect of SLC6A4 variants on platelet Vmax. This pilot study in healthy subjects thereby provides novel evidence of a correlative relationship between platelet 5-HTT uptake kinetics and neural activation in cortical networks. Particularly, our data suggest that a blood marker of 5-HTT uptake (platelet Vmax) is capable to predict BOLD signaling in a distributed cortical network that spatially corresponds to the DMN.
PP-10  Exploring candidate proteins involved in methylmercury (MeHg) transfer at the human materno-fetal barrier: Expression of the multidrug resistance-associated protein 2 (MRP2/ABCC2)

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Background: The organic mercury compound MeHg is a prevalent environmental toxicant, which is formed in aquatic systems and biomagnified along aquatic food chains. Consequently, humans are exposed to MeHg by consumption of contaminated fish and seafood. Ingested MeHg actively traverses the placenta, accumulates in fetal tissues and behaves as a neurodevelopmental toxicant. As deduced from other organs and species, multiple proteins may be involved in the complex MeHg toxicokinetics at the human placental barrier, which is composed of two cellular layers, the epithelial syncytiotrophoblast (STB) and fetal endothelial cells (FEC). However, still very little is known on the human placental mechanisms involved in mercury toxicokinetics.

Aim: One candidate protein for cellular efflux of MeHg is the multidrug resistance-associated protein 2 (MRP2/ABCC2), which is involved in renal and hepatic export of mercuric ions in rats and zebrafish, and export of mercapturates from human placental fetal organs in pregnant rats following exposure to methylmercury. In the human placenta, expression of MRP2 mRNA has been unequivocally demonstrated. However, protein expression, with predominant localization at the apical membrane of STB, remains contradictory and was investigated in this study.

Methods: Tissue collected from term healthy human placentas was either immediately frozen or HOPE-fixed and paraffin-embedded. The expression of MRP2 protein was analyzed using a polyclonal rabbit-anti-human MRP2 antibody (R260) from Cell Signaling Technology. For western blotting experiments, total protein lysates, crude membrane fractions or enriched apical STB fractions were prepared. Localization of protein was studied by immunofluorescence microscopy (IFM) on 2-4 µm tissue sections. CaCo 2 cells were used as positive control.

Results: While expression of a 180-200 kDa protein was clearly shown in CaCo 2 cells by western blotting and IFM, we could not demonstrate expression of MRP2 in human term placenta.

Conclusion and outlook: The currently available results show that MRP2, one major candidate for human placental MeHg efflux, is absent from the apical membrane of STB derived from healthy term placentas. As we can not exclude up-regulation of MRP2 protein in STB following exposure to MeHg, isolated trophoblast cells as well as CaCo2 cells will be exposed to MeHg in vitro and analysed for MRP2 expression. The hypothesized role of MRP2 in MeHg efflux will be investigated by siRNA-mediated ABCC2 knockdown in an appropriate alternative cell model (e.g., BeWo cells). Upported by LifeScience2010, NFB

PP-11  Human haploid cell genetics to study solute carrier specificity

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The identity and integrity of even the smallest biological systems is preserved by carefully regulating the exchange with the environment. The exchange between biosystems and their environment occurs mainly through small chemical molecules. This is true at the level of a single cell or a human being. The largest group of proteins transporting molecules across membranes are members of the solute carrier protein (SLCs) family; almost 400 members exist in humans. Although most of these transporters have unknown physiological function, several have gained interest within the scientific community, following the observation that drugs may "hitchhike" on these carriers that normally act on endogenous substrates. We would like to pursue the hypothesis that it is possible to chart many new routes and thus describe the main molecular interface between chemistry and biology in molecular detail.

The identification of orphan transporters by means of next-generation sequencing. By comparing wild type vs SLC knockout and characterized. In order to monitor drug/metabolite entry into cells, we use transcriptional readouts of a collection of cell lines in which each human gene that is not essential in KBM7 cells is knocked out individually depending on a certain SLC transporter. Furthermore, we are pursuing a metabolomics approach to identify the endogenous ligands of orphan transporters by means of mass spectrometry. Based on our results, we will attempt to derive a "pharmacophore" chemoinformatic model for each SLC cargo, which will in turn be able to predict further SLC substrates. Our genetics/mass spectrometry/cheminformatic approach will potentially allow us to chart many new routes and thus describe the main molecular interface between chemistry and biology in molecular detail.
PP-12  Intrahepatic changes in bile acid composition protects BSEP (ABCB11) KO mice from hepatic inflammation in methionine choline-deficient diet induced NASH

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Background: Bile acids (BAs) activate G-coupled and nuclear receptors controlling hepatic glucose and lipid metabolism as well as inflammation. Non-alcoholic-steatohepatitis (NASH) is characterized by increased hepatic lipid storage and inflammation. BAs are transported into bile via the bile salt export pump (BSEP; ABCB11). BSEP transgenic mice display less fat accumulation but severe inflammation. We therefore hypothesized that absence of BSEP may sensitize to hepatic steatosis while at the same time reducing inflammation in a mouse model of NASH.

Methods: Wildtype (WT) and BSEP knockout (KO) mice were fed a methionine-choline-deficient (MCD) diet for 5 weeks to induce NASH. Liver RNA profile analysis was performed by RT-PCR. Serum biochemistry, hepatic TG, BA content/composition as well as liver histology were assessed.

Results: MCD feeding induced hepatic TG accumulation (1.8-fold) in WT mice and to a lower extent in BSEP KO mice. In line, mRNA expression of de novo lipogenesis and fatty acid transport markers was repressed to a higher extent in MCD fed BSEP KO mice. In contrast to WT animals, BSEP KO were protected from hepatic inflammation (reflected by Tnfa, F4/80, Mcp1 and iNOS expression) induced by MCD feeding. mRNA expression of the BA-importer Ntcp was down-regulated by 60% in WT mice upon MCD but to a greater extent in BSEP KO mice. Conversely, expression of Mrp4 and Ostb was increased (10-fold; 2.8-fold) only in BSEP KO mice. Measurement of hepatic bile acid composition uncovered a distinct increase in hepatic CDCA (3-fold) and CA (3-fold) concentration (both BAs are known FXR agonists) in BSEP KO MCD mice compared to WT controls, whereas amounts of hepatic UDCA and b-muricholic acid were decreased.

Conclusion: Absence of BSEP protects from hepatic inflammation and reduces fatty acid storage by modulating hepatic bile acid transport and intrahepatic bile acid composition. These effects are most likely mediated via FXR induction. Thus, pharmacological modulation of bile acid transport/metabolism could constitute a new therapeutic option for modulating inflammation during progression from fatty liver to NASH.

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PP-13  The cocaine analog RTI 82 adducts to Phe319 in the rat dopamine transporter and is accommodated by the S1 binding site.

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The dopamine transporter (DAT) is a member of the SLC6A family of secondary-active Na+/Cl−-dependent neurotransmitter transporters and functions to terminate dopaminergic signaling through reuptake of dopamine (DA) from the synapse. The psychotropic drug cocaine binds to DAT inhibiting DA uptake resulting in increased synaptic DA levels. Currently, there are data supporting the existence of two separate cocaine-binding sites on DAT (the centrally located S1 and the more extracellularly positioned S2). Furthermore, cocaine antagonism has been argued to occur by both competitive (binding to S1) and non-competitive (binding to S2) mechanisms, which leaves many unanswered questions concerning the molecular determinants for cocaine-DAT interaction.

In order to better understand cocaine blockade of DAT, we utilized a photoactivatable cocaine analog, RTI 82, to determine ligand/transporter contact sites. These studies included use of LeuT-based comparative models to perform small molecule docking of RTI 82 as well as biochemical peptide mapping analyses of crosslinked RTI 82/DAT complexes. Our findings revealed that the photoactive phenyl azido moiety of RTI 82 adducts to Phe319 in rDAT and that the cocaine-like core of RTI 82 was bound in the S1 site. Molecular dynamic simulations of the docked models suggested a pi-pi stacking interaction between the phenyl azido moiety of RTI 82 and the phenyl side chain of Phe319. To investigate this interaction, we used site-directed mutagenesis to introduce mutations at Phe319 and analyzed their impact through competitive uptake and binding assays. Mutation of Phe319 to Tyr, Trp or Met resulted in DA-transport competent proteins whereas substitution to Cys or Asp yielded non-functional transporters. These findings suggest that hydrophobicity is required at position 319 for DAT function. Furthermore, we observed that the Tyr, Trp, or Met substitutions did not significantly alter RTI 82 potency (<3-fold change). The absence of potency changes with the Met mutant argues that the interaction between Phe319 and RTI 82 may be due to van der Waal forces rather than pi-pi stacking of the aromatic rings.

Additionally, using the substituted cysteine accessibility method (SCAM) to target residues in S1 and S2, we assessed the ability of RTI-82 to protect from MTSET attack. In these studies, RTI 82 could not protect the S2 residues Asp475 and Ala479 from MTSET inactivation suggesting that RTI 82 is positioned in S1. To further support these results, we are evaluating RTI 82-mediated protection of S1 residues Asp79, Ser421, and Ile483 and the S2 residues Trp84 and Ile159 from MTSET inactivation. This work was funded by the National Institute On Drug Abuse of the National Institutes of Health under Award Number R01DA027845 to LKH and RAV.

PP-14  Single molecule force spectroscopy on oligomeric receptors using branched crosslinkers

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Conventional single molecule force spectroscopy allows for measurement of the unbinding force between a ligand molecule and its corresponding receptor. A linear heterobifunctional poly(ethylene glycol) (PEG) linker is used to couple the ligand to the atomic force microscope (AFM) tip while the receptor is immobilized chemically or electrostatically on the sample surface. Common functionalization procedures starting with amino-functionalization of the tip via (3-aminopropyl)triethoxysilane (APTES) result in a statistical population of 0 – 2 ligands per tip apex. This approach restricts the use of force microscopy to the investigation of single ligand-receptor pairs. For extension of this technique to the analysis of dimeric or oligomeric receptors, a new generation of crosslinkers was synthesized. The hitherto existing linear crosslinker was modified to comprise a dendron-like structure carrying two or four terminal functional groups for ligand attachment.

In case of the bivalent linker, lysine was chosen as branching element. The carboxyl was elongated with a β-amino group. In a multistep reaction, a 2*2 “fork” with four terminal amino groups was synthesized. In order to avoid forks with <4 prongs, it was necessary to ensure 100% efficiency of amide bond formation. This was achieved by in-situ activation of the carboxyls with 1-
In conclusion, the synthesis of bi- and tetravalent crosslinkers is based on a modular system of linear heterobifunctional PEGs, of trifunctional branching units, and a wide variety of terminal coupling functions. This allows for adaptation to virtually any kind of receptor-ligand system, and for fine adjustment of ligand-ligand distance by use of proper prong-lengths in the forked linker.

ABC transporters constitute a ubiquitous superfamily of membrane pumps containing an evolutionary conserved ATP-binding cassette domain. They are responsible for energy-driven efflux of a great variety of substrates including amino acids, ions, sugars, as well as synthetic and naturally occurring xenobiotics and toxins. A subset of yeast pumps plays a major role in the so-called pleiotropic drug resistance (PDR) phenomenon, where overexpressed ABC transporters such as \textit{S. cerevisiae} Pdr5 confer resistance to a vast variety of xenobiotics and drugs. This phenomenon resembles multidrug resistance in cancer cells conferred by membrane proteins such as P-glycoprotein, BCRP or MRP, and poses serious difficulties when occurring in microbial pathogens, including fungal pathogens such as \textit{Candida spp}.

In a follow-up work of the original Pdr5 structure-function analysis\textsuperscript{1}, we took a closer look at ten Pdr5 mutant strains where only one non-synonymous mutation was found in the coding region. Equal expression was confirmed by Western blotting of total cell extracts. We next tested the Pd5 variants on plates containing different classical Pdr5 drug substrates such as rhodamines and azoles to identify those mutations that lead to a change in substrate specificity rather than a complete loss of activity. This resulted in a set of four mutants, namely H248Y (two independent mutants), L914F and G1441D. While the first two are localized close to conserved motifs responsible for ATP hydrolysis, the latter is located in the large external loop between helices 11 and 12. We further characterized these mutants using liquid growth assays and ATPAse activity measurements to better understand their role in Pdr5 function. In addition, we used molecular structural modeling to localize and place the topology of the mutations in a possible functional conformation of the Pdr5 drug exporter.

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The alternating access model of transport were revealed, but the molecular determinants of the coupling mechanism and of the ion selectivity have not been fully elucidated yet; moreover, differences are present among the members of the family. One of the main differences is the chloride-dependence, absent in LeuT and in the members of some subfamilies (slc6a20, 19, 15 and 17). However, while in LeuT this independence is explained by the presence of a negatively charge residue in the putative chloride binding site, the members of the cited subfamilies exactly conserve the same sequence of the chloride-dependent members. The insect transporter KAAT1 sits in the middle because of its weak chloride dependence that together with the possibility to utilize K⁺ as driver ion and the ability of the driver ion to influence the substrate selectivity, unique physiological features, make this transporter a useful tool to investigate the molecular determinants of the coupling mechanism. Comparison with different NSS amino acid sequences and structural analysis point to Threonine 67 in KAAT1 as a possible determinant of the transport coupling mechanism for different reasons: the residue is not conserved among NSS members although it is in a keystone position between the two sodium binding sites of the protein; furthermore, according to the LeuTAA crystal structure, it is involved with its back cone and side chain, in leucine binding. Mutants of T67 were expressed in Xenopus oocytes and functionally characterized by uptake, electrophysiological and chemiluminescence assays. The single point mutation T67Y led to a protein correctly expressed in the oocyte membrane, with unaltered affinity for sodium and with an uptake activity comparable to that of the wild type, but chloride-independent and with a strong reduction of the transport associated current, suggesting the uncoupling of Na⁺ and amino acid fluxes. The stereoselectivity of the transport process was enhanced as proved by the poor uptake inhibition observed in the presence of the substrate D-leucine. The T67G mutant showed relatively good expression (50-60 % of wt protein - chemiluminescence assay), while its activity was drastically reduced both in uptake and in electrophysiological experiments. These experimental data, together with those reported for GAT1 showing that the reverse mutation (GAT1 Y60T) induced an increased lithium leak current and in sodium dependence, suggest that Thr67 is important in the coupling mechanism, probably participating in the conformational changes that allow the strictly coupled translocation of ion and substrate.

Human ABCG2 is a plasma membrane glycoprotein actively extruding a wide variety of compounds from the cells. ABCG2 is one of the key transporters causing multidrug resistance in cancer cells. On the other hand, this transporter has an important role in the detoxification processes in the body. Membrane cholesterol is essential for ABCG2 function, though the exact nature of this interaction is not yet known and the cholesterol sensing sites have not been identified. The cholesterol recognition amino acid consensus (CRAC) is one of the conserved motifs, shown to be involved in cholesterol binding of several proteins. Here we identified 5 potential CRAC motifs in the transmembrane region of the human ABCG2. In order to define their roles in cholesterol sensing of ABCG2, the central tyrosines of these CRACs (Y413, 459, 469, 570 and 645) were mutated and the mutants were expressed both in insect and mammalian cells for functional measurements. We found that tyrosines in CRACs of the predicted intracellular loops (459, 469 and 645) are critical for ABCG2 expression and/or function. These mutants lose their activity even in the presence of cholesterol. Interestingly, the Y469F and Y645F mutants not only retained their activity but their cholesterol sensitivity and substrate recognition was unaltered. Mutation of CRAC tyrosines located in potential transmembrane helices (413 and 570) did not diminish the functionality of the transporter, and also the cholesterol sensing of the mutants did not differ significantly from that of the wild-type protein. However, we show here that the substrate recognition of these latter mutants has been altered. As a summary we conclude that tyrosines of the potential CRAC motifs of ABCG2 are not involved in cholesterol sensing but rather in substrate recognition and proper folding of the protein. Therefore these residues can be potential targets in the design of ABCG2 inhibitory drugs.
PP-18 Characterization of functional properties and collectrin interaction of fish amino acid transporter B0AT1

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B0AT1 (SLC6A19) transporter belongs to the Na⁺ - and Cl⁻ -dependent neurotransmitters and amino acids cotransporters family SLC6. B0AT1 is a Na⁺ -dependent, but not Cl⁻ -dependent, nutrient transporter. It accepts a wide range of neutral amino acids and mediates the intestinal and kidney epithelial re-absorption of neutral amino acids. Recently the first fish ortholog, sea bass B0AT1 (Dicentrarchus labrax) was cloned and electrophysiologically characterized with TEVC (two-electrode voltage clamp) on Xenopus laevis oocytes (Margheritis et al., 2013).

The aspects of amino acid adsorption have a great relevance for the diet composition in farmed species, comparative investigations about ortholog proteins can reveal important characteristics, similarities and differences that may be useful not only for animal and human nutrition, but also to obtain details on functionality and regulation of this class of proteins at a molecular level. In this context, we have investigated the behavior of Salmo salar B0AT1, the amino acid transporter of one of the most important species in aquaculture.

The electrophysiological characterization of salmon neutral amino acid transporter, ssB0AT1 showed an unexpected feature; as the human ortholog (Singer and Camargo, 2011), it needs partner proteins like Tmem27, to be correctly expressed on Xenopus levis oocytes membrane, differently from the mouse and the sea bass transporters that have a recordable electrophysiological activity even when injected alone.

Electrophysiological measurements in Xenopus oocytes were possible only when the cRNA coding for the salmon transporter was co-injected with collectrin. Therefore the first functional recording of the ssB0AT1 was obtained in the presence of mouse collectrin.

The experiments were subsequently performed with oocytes co-injected with B0AT1 and collectrin from Salmo salar RNA and the transport current characterization was achieved. The substrate specificity, the ionic-dependence, the pH dependence and kinetic parameters were investigated.

To evaluate the possible interactions between different species, salmon collectrin was co-injected with sea bass B0AT1. Collectrin significantly enhanced the transport associated currents probably through an increase in protein expression at the oocyte surface. The effects of collectrin on the substrate affinity and on the pre-steady-state currents were analyzed suggesting that the current increment observed was due to an increase in the number of proteins expressed on the membrane rather than to an altered affinity for the substrate.

The upcoming cloning of the sea bass collectrin will allow to perform species-specific interaction experiments.
PP-19  A common structural basis for folding deficient variants of ABC-proteins reveals convergent disease etiology

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Disease causing mutations in a number of human ABC proteins (including among others ABCA1, ABCB4, ABCB11, ABCC6, ABCC7 and ABCG2,) have been linked to aberrant folding, retranslocation of proteins into the cytoplasm and subsequent proteasomal degradation (Nakagawa, 2011). ER-associated degradation (ERAD) of mutant ABC proteins results in a number of lethal or debilitating human diseases and available evidence seems to indicate misfolding and premature degradation caused by missense mutations to be a significant cause of membrane protein deficiencies.

Current focus was on compiling single amino acid mutations caused by SNPs and deletions causing transport deficiency of ABC proteins from the literature. Mutations were selected according to direct evidence for resulting in folding and/or trafficking deficiency of ABC proteins to their final cellular destination. In total 126 mutations were found in 12 members of 4 subfamilies of ABC protein (subfamilies A, B, C, and G) and localized in homology models.

The minimal functional form of ABC transporters comprises a minimum of four domains, two transmembrane domains (TMDs), which confer solute specificity and two nucleotide binding domains, which bind and hydrolyze ATP in two composite nucleotide binding sites (NBDs) thereby providing the energy for the movement of solutes across membranes. An analogous architecture can be observed in all presently available ABC exporter crystal structures. All mutations were projected onto a model of the ABCB11 protein to analyze the distribution pattern of the mutations in ABC proteins and to identify potential common characteristics.

We found that mutations cluster in regions, which are critical for either domain folding or domain interface formation, including charge interactions between intracellular loops of TMDs and NBDs. Despite their different function, including transport, ion conductivity and channel regulation and diverse resulting diseases, the distribution of the trafficking deficient mutations reveal a structurally convergent disease etiology.

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PP-20  Flotillin-1 is a novel SERT interacting protein

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Activation of PKC is known to decrease the surface expression of 5-HT due to SERT internalization (endocytosis) hereby decreasing 5-HT uptake and increasing 5-HT bioavailability. However the molecular machinery of SERT endocytosis remains largely unknown. Identification of the molecules involved in the endocytosis of SERT is critical for the investigation of the underlying mechanism. Here, we aimed to elucidate some of the key players involved by concentrating SERT in the lipid-raft fractions by sucrose gradient centrifugation and screening of SERT interacting proteins, potentially involved in SERT endocytosis. Interestingly, we found that an abundant amount of Flotillin-1 concentrating within the same fraction containing SERT enrichment. This observation lead us to hypothesize that Flotillin-1 might interact with SERT and furthermore affect its transport and physiological function. This finding was confirmed by co-immunoprecipitation experiments and will be followed up by mass spectrometrical and FRET analysis. Our results propose Flotillin-1 as novel interacting partner of SERT. Currently, the physiological relevance of the SERT-Flotillin-1 interaction are being studied both in-vitro and in-vivo.
PP-21 Real-time uptake of fluorescent ASP+ via the organic cation transporter 3

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Background: The organic cation transporter 3 (OCT3) shows a broad expression pattern in the nervous system and has been detected in neurons and glial cells. Here, OCT3 serves as an additional re-uptake system for neurotransmitters, such as dopamine, serotonin and norepinephrine, and therefore OCT3-mediated uptake has been termed “uptake 2.”

Methods: We measured OCT3-mediated uptake of the fluorescent OCT3 substrate 4-di-1-ASP (4-(-4-(dimethylamino(styryl)-N-methylpyridinium (ASP+)) in real-time in HEK293 cells stably expressing the human and the mouse isoforms of OCT3. Data obtained on OCT3-mediated uptake of fluorescent ASP+ were compared to uptake of tritiated 1-methyl-4-phenylpyridinium (MPP+). We further used mass spectrometry to assess the phosphorylation status of OCT3.

Results: We show that ASP+ is selectively taken up via OCT3, which allows for sensitive, real-time assessment of transport via OCT3. Hence, we analyzed the mode of action of several OCT3 substrates and transport inhibitors, such as the stress hormone corticosterone and decynium-22. All results obtained from ASP+uptake studies are comparable with data obtained from uptake studies with tritiated MPP+ and are in line with previously published reports. Finally, we tested if OCT-mediated uptake is sensitive to phosphorylation and found that the protein kinase C inhibitor GF109203X inhibited uptake.

Discussion: The use of ASP+ as substrate for OCT3 provides the opportunity to analyze OCT3-mediated transport with a high temporal resolution and is comparable to OCT3-mediated uptake of tritiated MPP+. Uptake inhibition by corticosterone was comparable using either ASP+ or MPP+ and similar to inhibition of protein kinase C.

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PP-22 Improving oral drug delivery: Computational studies of peptide transporter

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High oral bioavailability is a desirable property of any drug. One way to achieve this is by designing a drug or prodrug that can be transported across the gut lining by endogenous nutrient transporter, such as PepT1 (SLC15A1). Expressed primarily in the small intestine, PepT1 not only transports di- and tripeptides from protein digestions using proton electrochemical gradient, but also facilitates absorption of peptide-mimetic drugs, like many β-lactam antibiotics, into the blood. The crystal structures of bacterial peptide transporters, PepTSt [1] and PepTSo [2], provide an excellent model for the structural studies of PepT1 given their high sequence similarity and conserved key residues.

The binding event of peptides on PepTSt was first investigated using molecular dynamics (MD) simulations. The key residues involved in binding, as determined by the simulations, agreed well with mutagenesis experiments. Approximate binding energies of peptides into the binding cavity of PepTSt were calculated using fast scoring functions and end-point free energy methods i.e. Linear Interaction Energy (LIE) and Molecular Mechanics Poisson–Boltzmann/Generalized Born Surface Area (MM-PB/GBSA). Comparing the results with IC50 values from transport assays of PepTSt in liposome shows that the latter methods correlate well with experimental data, allowing them to be used to distinguish good substrates from poor substrates and non-transported peptides. To extend this work to the human peptide transporter, a full-length homology model of PepT1 was built using bacterial crystal structures as the templates. Although this model was stable when simulated and the key residues mapped well into the binding cavity, the ability to predict binding energy significantly decreased across all methods. Overall, our studies suggest that the approximate free energy methods like MM-PB/GBSA are capable of predicting substrates of the bacterial peptide transporters, but a high-resolution crystal structure is requisite to apply them to human PepT1 to predict drug transport.


Background: The serotonin transporter (SERT) on nerve cells mediates the reuptake of released serotonin from the synaptic cleft into the presynaptic compartment. In the adult brain, SERT is predominantly expressed in the serotoninergic neurons of the pontine raphe nuclei, which project to many brain areas including the cortex. Accordingly, during its synthesis, SERT is inserted into the endoplasmic reticulum (ER) of these neurons. To fulfill its namesake action, SERT must be exported from the ER and trafficked to the plasma membrane of the presynaptic specialization. The coatomer protein II (COPII) complex is responsible for recognition of cargo molecules like SERT at the ER membrane and generation of transport vesicles harboring this cargo. Previous work showed that the COPII component SEC24C directly interacts with the C-terminus of SERT by binding to an R607I608. In the absence of this interaction, SERT is slowly exported from the ER in a non-concentrative manner. In this work, we want to understand the mechanisms leading to ER Export of neurotransmitter transporters. Methods: Substrate uptake assays, plasmid- and siRNA-transfection, confocal microscopy and GST-pulldowns were performed as described in references 2 and 3. Results: In contrast to SERT which requires SEC24C, ER export of the dopamine transporter (DAT) and the norepinephrine transporter (NET) requires SEC24D. Our work shows that a single C-terminal amino acid accounts for this difference. Exchanging this appropriate residue between SERT and DAT/NET, alters SERT SEC24D-dependent and DAT/NET SEC24C-dependent GST-pulldown data suggest that SERT and other neurotransmitter transporters do not only interact with SEC24 but also with SAR1, the G-protein which initiates COPII assembly. Therefore the transporters might have a “landmark” function, showing SAR1 where to initiate COPII assembly. In neurons, YFP-SERT carrying a mutation in the RI-motif is excluded from the axonal compartment and redistributed to the MAP2-positive somato-dendritic compartments. The same phenotype was achieved by coexpression of wt YFP-SERT with a dominant negative version of SEC24C (CFP-SEC24C-VN), which blocked COPII mediated export of SERT. This shows that COPII-dependent concentrative export from the ER is a prerequisite for correct axonal targeting indicating that sorting decisions are already made on the level of the ER. Discussion: The present work shows that ER export of SERT is tightly regulated and controls the delivery of SERT to the plasma membrane. Surface levels of neurotransmitter transporters have been proposed to modify the susceptibility to mood disorders (e.g., depression). Understanding intracellular trafficking of these proteins can contribute to our understanding of how these disorders emerge. Acknowledgements: This work was supported by FWF, SFB35 and Cell communication in health and disease (CCHD)
unconjugated hyperbilirubinemia. If our hypothesis holds true, ABCB1 stimulation might become a feasible novel treatment strategy for patients with severe unconjugated hyperbilirubinemia.

PP-25 Neuronal Dopamine Transporter Trafficking Is Differentially-Dependent On Dynamin And The Actin Cytoskeleton

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The Na⁺/Cl⁻-dependent presynaptic dopamine (DA) transporter (DAT) limits synaptic DA availability and maintains dopaminergic tone by recapturing released DA. DAT is the primary target for therapeutic and addictive psychostimulants, such as methylphenidate (Ritalin), cocaine and amphetamine, and DAT plasma membrane availability directly impacts the efficacy of these psychoactive drugs. DAT dynamically shuttles between the plasma membrane and intracellular vesicles via the endocytic recycling pathway, and PKC activation acutely decreases DAT surface expression by rapidly accelerating DAT internalization and dampening DAT recycling. While considerable evidence supports regulated DAT trafficking in heterologous expression studies, PKC-stimulated DAT internalization is not readily observed in cultured dopaminergic neurons. In addition, a variety of endocytic mechanisms have been reported to govern DAT endocytosis, including classic clathrin/dynamin-dependent mechanisms, as well as membrane raft-dependent means. These studies primarily utilized either chronic gene disruption and/or dominant negative approaches, or were performed in cell lines and cultured neurons, yielding results difficult to translate to adult dopaminergic neurons. In the current study, we took advantage of several new pharmacological tools to acutely inhibit dynamin and test whether constitutive and PKC-stimulated DAT endocytosis are dynamin-dependent in adult dopaminergic terminals. Ex vivo biotinylation studies in mouse striatal slices revealed that PKC activation acutely depletes native DAT surface levels. Moreover, we observed that a limited DAT surface pool is capable of endocytosis, while the remaining surface population is resistant to both basal and PKC-stimulated internalization. Dynamin inhibitor studies revealed that dynamin activity is not required for constitutive DAT internalization, whereas PKC-stimulated DAT internalization is dynamin-dependent. Moreover, TIRF microscopy experiments demonstrated that constitutive DAT internalization occurs non-preferentially from both lipid raft and non-raft microdomains, whereas PKC-stimulated DAT internalization arises exclusively from lipid rafts. Finally, DAT endocytic recycling relies on a dynamin-dependent mechanism that acts in concert with the actin cytoskeleton. These studies are the first comprehensive investigation of native DAT trafficking, ex vivo in adult dopaminergic terminals, and reveal that complex multimodal mechanisms govern DAT surface dynamics.
PP-26  Probing the binding site of an antimicrobial ABC exporter

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Antimicrobial peptides are produced by Enterobacteriaceae under nutrient limited conditions for survival. Microcin J25 (MccJ25) is a modified antimicrobial peptide with a distinctive 3D lasso structure. MccJ25 inhibits bacterial growth by binding to RNA polymerase, stopping transcription. MccJ25 is secreted by McjD, an ATP binding cassette (ABC)-exporter, providing immunity. The crystal structure of McjD has been solved at 2.7 Å resolution, the first structure of a peptide ABC transporter. McjD is in a new ‘nucleotide-bound outward-occluded’ conformation, presenting the first insight into information of an ABC-exporter’s cavity when completely closed to both the periplasm and cytoplasm. MccJ25 was modelled into the cavity of McjD. The rigidity and shape of MccJ25’s lariat ring resulted in it only being able to be positioned in one orientation. To evaluate the model of the MccJ25-McjD complex cavity mutants were generated. All of the McjD mutants were shown to have no significant effect on the basal ATPase activity. However, the majority of the mutants showed significant effect on the ligand induced ATPase activity. The reduction in relative activity seen in these mutants in the presence of ligand provides evidence that there is a likelihood that these residues play a role in the binding of MccJ25. The mutants may cause MccJ25 to only loosely bind and weakly activate transport. McjD was also shown to be capable of distinguishing between different microcins and lasso peptides, shown from our ligand stimulated ATPase measurements. McjD showed preference to MccJ25 compared to other antibacterial peptides (MccB17 and capistruin). The MccB17 microcin is a long peptide containing thiazole and oxazole rings, which cannot fit inside the McjD cavity even if it adopted a more compact structure similar to that of MccJ25. Capistruin a lasso peptide possesses a longer C-terminal tail and a shorter loop than MccJ25, meaning that it may no longer fit as well within the cavity. Therefore, McjD exhibits evidence that it has high specificity to its native substrate with multiple residues involved in substrate recognition and/or transport.

PP-27  Ligand-induced conformation of the human Serotonin Transporter

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The human serotonin transporter (hSERT) regulates serotonergic signaling in the brain by actively regulating the concentration of serotonin in the synaptic cleft. hSERT mediates reuptake of serotonin into the pre-synaptic neurons. hSERT is a molecular target for antidepressant drugs and psychostimulants that act by altering the serotonin concentration in the synapses.

Conformational changes of hSERT occur during substrate and ligand binding and during translocation of serotonin. Overall, during the transport cycle, hSERT shifts from an extracellular-facing conformation through an occluded state ending in a cytoplasmic-facing conformation.

A bound ligand in a fixed concentration induces a given conformation of hSERT. It has previously been shown, that the substrate, serotonin, and the hallucinogenic alkaloid, ibogaine, both bind to hSERT and stabilize an inward-facing conformation of hSERT. However, the interplay between ligand binding, substrate transport, and hSERT conformation is not fully understood.

We have analyzed binding characteristics of serotonin and ibogaine and their effect on conformation of hSERT using the Substituted Cysteine Accessibility assay. Surprisingly, in a high-affinity state for serotonin and ibogaine binding these ligands induce an outward-facing conformation of hSERT, which is found to be ion-dependent. This novel dualistic nature of substrate and substrate analogs and the resulting conformational state might represent a hitherto undescribed step of the transport cycle.
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PP-28  The SLC35F1 transporter: expression and localization in brain
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Background: SLC35F1 belongs to the human SLC35 family of transporters some of that (subfamilies A-D) have been proposed to transport nucleotide-activated sugars from cytosol into the lumen of Golgi complex or endoplasmic reticulum and may thus be involved in the formation of proteoglycans and glycoproteins. So far no function has been identified for any of the five members of the SLC35F subfamily, however, high SLC35F1 mRNA levels have been reported especially in adult and fetal brain (Nishimura et al., Drug Metab. Pharmacokinet. 2009). To clarify the role of this transporter for physiological and pathological processes we have investigated the expression of SLC35F1 as well as its cellular localization in brain and have established cellular models to study its function.

Methods and results: The expression levels of SLC35F1 in human and murine brain were analyzed by means of real time RT-PCR and Western Blot. In accordance with the results of Nishimura et al. we detected high levels of SLC35F1 mRNA in brain of both species. Western Blot analysis of murine brain homogenates with a specific antibody indicated also high level of slc35f1 protein. Confocal fluorescence microscopy studies in murine brain sections revealed expression of slc35f1 in some neurons of cerebrum, in Purkinje cells of the cerebellum, as well as at the choroid plexus. In human brain sections SLC35F1 was also detected in some neurons of the cerebrum, whereas in human cerebellum staining was mainly observed in the granular layer. To investigate the function of this solute carrier we established and characterized MDCK cells stably transfected with the SLC35F1 cDNA. Confocal microscopy analysis of these cells indicated that the transporter is mainly localized at the plasma membrane and in some small intracellular vesicles. For transport studies in isolated membrane vesicles we expressed the human SLC35F1 protein in addition in Sf9 insect cells. Both systems were validated for screening of endogenous substrates of this transporter.

Conclusion: The localization of SLC35F1 in specific neurons in murine and human cerebrum and cerebellum indicate a role of this transporter in neuronal function or differentiation.

PP-29  Variation of the detergent binding capacity and phospholipid content of membrane proteins when purified in different detergents
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Purified membrane proteins are ternary complexes consisting of protein, lipid and detergent. In the present study, three model membrane proteins of different oligomeric states were purified in nine different detergents and characterized biochemically and biophysically. Detergent binding capacities and phospholipid contents of certain model proteins were determined and compared. The insights on ternary complexes obtained from the experimental results, when put into a general context are summarized as follows: i.) The amount of detergent and endogenous phospholipid bound to membrane proteins is strongly dependent on the hydrophobic, lipid-accessible protein surfaces. ii.) The size of the detergent and lipid belt surrounding the hydrophobic, lipid-accessible surface of purified membrane proteins can be tuned by the appropriate choice of the detergent. iii.) The detergents n-nonyl-β-D-maltopyranoside and Cymal-5 have exceptional delipidating effects on ternary complexes. iv.) The types of endogenous phospholipids bound to membrane proteins can vary depending on the detergent used for solubilization. Furthermore, we demonstrate that size-exclusion chromatography is in general a suitable method to estimate the molecular mass of ternary complexes. Based on the presented experimental results, we propose a refined version of the prolate monolayer model that describes the protein, phospholipid and detergent interactions in the ternary complexes.
PP-30  The molecular basis for selective serotonin re-uptake inhibition by the antidepressant agent fluoxetine (Prozac)

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The human serotonin and norepinephrine transporters (hSERT and hNET, respectively) are important drug targets for medications used in the treatment of major depressive disorders, including tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs). In 1986, fluoxetine (Prozac) was one of the first SSRIs to be approved for the treatment of depression. Fluoxetine has been widely acknowledged as a breakthrough drug for depression, and has since its approval been one of the most widely prescribed antidepressant agents. X-ray structures of a bacterial transporter (LeuT) have suggested that SSRIs, including fluoxetine, bind to a site located in an extracellular facing vestibule (denoted the S2 site). In this study, the structural basis for high affinity binding in hSERT and selectivity over hNET for fluoxetine is delineated. A systematic structure-activity study allowed identification of the substituents that control activity and selectivity towards SERT over NET for fluoxetine, and we show that the affinity for hSERT and selectivity over hNET can be improved by subtle changes of the chemical scaffold. Binding of fluoxetine to models of hSERT was examined via induced fit dockings. In contrast to what was found in LeuT, fluoxetine was predicted to bind in the central substrate site (denoted the S1 site) of the human transporter. The predicted binding mode was verified by a comprehensive mutational analysis, and non-conserved residues within the central S1 site were identified as important determinants for the SERT/NET selectivity of fluoxetine. In contrast, the molecular determinants underlying the selectivity of nisoxetine, a structurally closely related analog of fluoxetine with selectivity for NET, was located in the S2 site of hNET, thus showing that the molecular determinants that define the selectivity of fluoxetine and nisoxetine are located in different regions of the transporters. This may indicate that the two inhibitors bind to distinct sites on SERT and NET, and emphasize the need for careful experimental verification when extrapolating findings from X-ray structures of a bacterial homolog to human transporters.

PP-31  Phosphorylation-mediated function of Serotonin transporter

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Phosphorylation regulates biological events of serotonin transporter (SERT) including surface trafficking, endocytosis and its catalytic activity. Although SERT has been proposed as a substrate of several kinases and phosphatases, such as protein kinase C (PKC), PKG, p38 MAPK and protein phosphatase (PP) 2A, with their implicated functions, the probability of orchestration of kinases and phosphatases in multiple signal cascades and lack of identified phosphorylation sites render understanding of many aspects of phosphorylation-mediated modulation of SERT difficult.

Therefore, we applied mass spectrometry (MS) to investigate kinase-specific phosphorylation sites in SERT functions. Immunoblot analysis revealed that the ER retention form of SERT was decreased by anisomycin, a p38MAPK activator, and increased upon PKC activation by β-PMA, respectively and further LC-MS/MS identified phosphorylation sites on SERT at serine (S) 52, S62 and threonine (T) 616 by anisomycin treatment and S52 by β-PMA in heterologous cells. In vitro phosphorylation assay with GST-fusion N- and C-termini of SERT and recombinant kinases showed T616 and T603 as a substrate for p38 and PKCα, respectively; however, no N-terminal phosphorylation site (i.e. S52 or S62) was detected. PP2A is known to be associated with SERT and thus phosphatases were inhibited by okadaic acid (OA) in cells expressing SERT. This induced phosphorylation at S52 and S62 on SERT. To address specific phosphorylation sites in the regulation of the ER retention of SERT upon anisomycin, the identified phosphorylation sites were replaced to alanine (A) and aspartic acid (D) and analyzed by immunoblotting. The dephosphomimetic T616A mutant reduced the effect of anisomycin on the ER retention compared to the SERT wild type and phosphomimetic T616D mutant.

We here identified kinase-specific phosphorylation sites in SERT and presented their implication in the ER retention of SERT, probably mirroring surface expression of SERT. Phosphorylation at the C-terminus by p38 MAPK, but not in the N-terminus, regulated ER retention of SERT showing a specific phosphorylation region for the control of SERT trafficking. Interestingly, in vitro phosphorylation with recombinant p38 and PKCα proteins did not induce the SERT N-terminal phosphorylation that was observed by in vivo phosphorylation upon activation of those kinases. However, the inhibition of phosphatases induced in vivo N-terminal phosphorylation of SERT. Therefore, it suggests that N-terminal SERT phosphorylation is modulated by concerted action of protein kinases and phosphatases PP1/PP2A.
PP-32  Conformational dynamics of the type I histidine ABC import system of *Salmonella enterica* serovar Typhimurium

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Recent findings with bacterial ABC import systems have stimulated the discussion about different transporter type-related mechanisms of signal/conformation coupling during the transport cycle. LAO/HisJ-QMP₂ is a relatively small type I ABC importer for the positively charged amino acids histidine, arginine, lysine and ornithine. Our interest is to unravel if its mechanism of transport is comparable to another type I transporter investigated in our laboratory, the maltose system, which possesses additional structural features that play a role in its proposed mechanism.

Different conformations were determined for the histidine transporter depending on the addition of ATP, Mg²⁺ and the substrate binding protein (SBP) LAO by limited proteolysis and fluorescence lifetime spectroscopy for the detergent stabilized and the reconstituted protein. Motional changes of specific sites, identified by mass spectrometry and mutational analysis, could be monitored and revealed signalling from the periplasmic to the cytoplasmic side of the transporter.

The data suggest that in general the histidine system resembles the maltose system in its mechanism despite their structural differences: (i) The SBP is in contact with the transmembrane domains (TMD) HisQM already in the apo state and (ii) closing of the nucleotide binding domains (NBD) HisP₂ is observed when both, SBP and nucleotide, bind. The protease cleavage site at the cytoplasmic end of the 5th helix of HisQ points to a localized rotational movement.

Moreover, limited proteolysis proved to be a useful tool for analysis of conformational defects of transporter variants.

PP-33  Investigating the role of EGFR in the regulation of glutamate mediated neocortical neurodegeneration

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The Epidermal growth factor receptor (EGFR) pathway is one of the critical genetic pathways that are required for the maintenance and proliferation of mammalian cells throughout development. While work is beginning to elucidate the role of EGFR in organs such as the skin, liver and the immune system much is still unknown about how EGFR regulates the development and regulation of the mammalian brain. Previously, Professor Maria Sibilia was successful in generating both an EGFR knock-out animal model as well as transgenic mice that have been useful in investigating the role of EGFR signalling in skin, liver and brain development. Early studies identified that loss of EGFR signalling in the developing brain led to a neurodegeneration of the frontal neocortex but not in the midbrain of developing mice. Utilising the EGFR knock out murine model we have shown that loss of EGFR in the cortical astrocytes, but not midbrain astrocytes, resulted in a loss in the number and function of glutamate transporters, the critical transport system that regulates the activity of the excitatory neurotransmitter glutamate. Furthermore, we identified that EGFR ablated cortical astrocytes, but not EGFR ablated midbrain astrocytes, were unable to keep cortical-derived neurons alive *in vitro*. Our continuing studies investigate the effects by which loss of EGFR in cortical astrocytes leads to neuron death both *in vitro* and *in vivo*. Here we show our preliminary findings that EGFR regulates the glutamate transporters in the developing neocortex and that deficient EGFR-induced uptake of glutamate in the synaptic cleft of cortical neurons leads to neurotoxic-induced neurodegeneration of the murine neocortex. Our research aims to provide considerable insight into the cellular and molecular mechanisms of neurodegeneration through defining the process by which EGFR regulates glutamate transport in cortical astrocytes.
PP-34 Probing the inward facing conformation of the leucine transporter
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The human monoamine transporters are involved in a variety of disorders and thus important medical targets. There are no available high-resolution structures of the transporters. The leucine transporter (LeuT) is a bacterial homologue of the human monoamine transporters with a sequence similarity of 20-24%. LeuT has been crystallized with a number of different ligands and in several different conformations. Most recently, an inward facing conformation was solved. The crystallization process required the use of four point mutations as well as the association of a FAB antibody fragment. We have applied computational methods to investigate whether the conformation of this manipulated protein is relevant for wild-type LeuT in a membrane environment. We have performed several 100 ns molecular dynamics simulations of both the mutated and wild-type transporter, and analyzed the stability through measurements of helix tilt angles. The simulations reveal that the mutant and wild-type systems types behave similarly and both maintain a relatively stable inward facing conformation. However, consistent local changes in the conformation with respect to the crystal structure are observed. To investigate the release of substrate and ions from the transporter, we have also performed simulations of wild-type LeuT in the inward facing conformation with sodium ions and either leucine or alanine bound. From this, we can conclude that one of the two sodium binding sites is fully disrupted in this conformation leading to the release of this ion in all of the simulations. Furthermore, in one of the simulations we observe release of alanine along with the second sodium ion, and are thus able to observe the final part of the translocation process in atomic detail.


PP-35 Regulation of the canalicular phosphatidylcholine transporter ABCB4/MDR3
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ABCB4 plays a key role in bile formation. While ABCB11 transport BS, ABCB4 secretes phosphatidylcholine (PC). PC and cholesterol form mixed stable micelles with BS which protects the biliary tree from their detergent effects.

To identify novel pharmacological strategies to regulate ABCB4 expression and activity, we screened a panel of nuclear receptor agonists and antagonists on human hepatocellular carcinoma-derived cells (HepG2, HuH-7 cells) and hepatocyte-derived cells (IH cells), which express ABCB4 endogenously. Among these, a range of farnesoid X receptor (FXR) and peroxisome proliferator-activated receptor (PPAR) agonists was identified to stimulate ABCB4 expression in line with published data. Notably, our screen uncovered thyroid hormone receptor (TR), which is activated by triiodothyronine (T3). T3 induced (almost 2.5 fold) ABCB4 expression after a 48 hours treatment at 20 µM in the three cell lines. Moreover, co-treatment with T3 and FXR agonists (CDCA or GW4064) resulted in an additive stimulation of ABCB4 expression in the above-mentioned cell lines. In order to explore potential future therapeutic applications, the undesirable effects of T3 on other systems (e.g. deleterious cardiac effects) have to be minimized. Since this can be achieved by cell/tissue specific TR stimulation, we next aimed to identify the hepatic TR isoform that increased ABCB4 expression in our experiments. RT-qPCR profiling showed that TRα1 and TRβ1 isoforms are expressed in our cell models, contrary to the TRα2 isoform. Furthermore, western blot analysis revealed that THβ1 protein is expressed while no TRα1 protein expression was detected. Moreover, we tested several specific agonists: sobetirome (GC-1), eprotirome (KB-2115), CGS23425 and KB-141 are T3 analogs mostly specifically targeting the TRβ1 isoform (the major isoform expressed in the liver). All these analogs resulted in an increase of ABCB4 expression, similarly to the native hormone and at a much lower concentration (1 to 2 µM). Other experiments, like TR overexpression (DNA transfection) or knockdown (RNA interference) are in progress. Further investigations are planned to precisely characterize the location of the TR response element in the ABCB4 promoter. Finally, an essential point is to determine the activity of ABCB4. To this end, in-vitro PC excretion will be measured to check the difference of activity after T3 and T3 analogs treatment. Moreover, proof of concept in vivo animal studies applying (cell) specific ligands in established models of cholestasis will be considered. Conversely, our findings may have important implication to understand thyroid hormone function (e.g. hypothyroidism frequently associated with PBC) as a potential determinant/modifier of bile duct injury in cholangiopathies.
PP-36  The Serotonin transporter and its dependence on different lipid raft components
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The importance of specific lipid-protein interactions of membrane-embedded proteins, such as the neurotransmitter transporters, is becoming increasingly clear. The serotonin transporter is distributed throughout the membrane but concentrated in membrane microdomains, lipid rafts. The distribution between raft associated and non-raft associated serotonin transporters are poorly understood, as is the specific consequences of the changes in the lipid composition around the transporters. Using the CHO-LY B cell line, we investigated how manipulation of the contents of the two primary raft components, cholesterol and sphingolipids, in the membrane affected basic properties of the serotonin transporter such as conformational equilibrium and binding of conformationally restrictive inhibitors.

PP-37  A link between the EGFR and ABC transporters?
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ABCB1 (Mdr1a/b), ABCC2 and/or ABCG2 (Bcrp) transporters play important roles in multi-drug resistance to chemotherapeutic agents, in the secretion of biliary components and clearance of carcinogens. It is known that most human HCCs are resistant to chemotherapy, likely because of a dysregulation in expression levels or function of ABC transporters in tumor cells. There is clinical evidence that there is a link between ABC transporter expression and/or function and EGFR expression. We would like to employ mice lacking the EGFR in the liver to investigate whether and how expression levels or function of ABCB1, ABCC2 and/or ABCG2 transporters are altered and how this possible alteration or functional dysregulation leads to a change in the susceptibility to anti-cancer treatment. We will investigate the functional consequence of a possible altered ABCB1, ABCC2 and/or ABCG2 transporter expression on the development of inflammatory liver diseases and HCC and investigate if and how EGFR signaling affects ABC transporter expression and/or function, which so far is poorly understood. The results of these experiments will not only provide important mechanistic insights into signaling pathways of EGFR and ABC transporter regulation, but may offer new therapeutic concepts for the treatment of liver diseases.
L-carnitine (3-hydroxy-4-N-(trimethylamino)-butyrate) is required for many physiological roles as participating β-oxidation. OCTN2 ("novel organic cation/carnitine transporter 2"; SLC22A5), and its rat ortholog Octn2 (rSlc22a5), are ubiquitously-expressed membrane proteins that are hypothesized to be sodium-dependent specific transporters of L-carnitine and related compounds. Although not generally regarded as a drug transporter, its role in drug pharmacokinetics has been clearly shown, particularly in relation to renal excretion.

Mildronate (3-(2,2,2-trimethylhydrazinium)propionate), is a carnitine congener which acts to inhibit fatty acid oxidation, and is thought to inhibit enzymes of the carnitine biosynthesis pathway. Mildronate has been shown to be transported by rat Octn2. However, characterization of kinetics of transport has not been described.

**Aims:** To correlate transport properties of rat Octn2 and human OCTN2 and investigate mildronate transport by Octn2/OCTN2 in particular. Identify and examine drugs inhibiting L-carnitine uptake, and to perform a species specificity analysis of the rat and human orthologs.

**Methods and results:** We utilized CHO-K1 cells overexpressing rat or human Octn2/OCTN2 to examine species differences between the two transporters. Cellular uptake assays were performed using CHO-K1 cell lines stably expressing human OCTN2 and rat Octn2. Uptake of L-carnitine was quantitated using radiolabelled compound, whereas the detection of mildronate was carried out using HPLC-MS. Among others amiloride, imatinib, mildronate, omeprazole, quinine, quinidine and vincristine were used to examine their influence on L-carnitine uptake.

**Conclusion:** Similarly to the human ortholog, rat Octn2 also transports mildronate high potency. However, L-carnitine was found to be a lower affinity substrate for rat Octn2 than for human OCTN2. Furthermore, many pharmacologically important drugs were shown to affect L-carnitine transport by Octn2/OCTN2, although several differences between rat and the human orthologs were also observed. In the case of rat Octn2 (compared to human OCTN2), significantly higher IC$_{50}$ values were observed for imatinib, mildronate, omeprazole, quinidine, and quinine, whereas significantly lower IC$_{50}$ values were observed for nelfinavir and vincristine.
PP-39  Conformational studies of the transmembrane helix 1A in the Leucine Transporter (LeuT)

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Neurotransmitter transporter are found on the presynaptic neurons and on glial cells. The function of the transporters is termination of neurotransmission by rapid removal of neurotransmitter molecules from the synaptic cleft. These transporters couple substrate transport to ion gradients of sodium and chloride. Structural studies often require the transporter to be removed from its physiological membrane, which can affect its structure or conformation. Crystal structures of the bacterial homolog LeuTαa (SLC6 family) were solved in the three states of the transport cycle: occluded, outward and inward [1, 2, 3]. The recent inward facing structure showed a conformation of the first transmembrane helix of LeuT (TM1A) that did not seem to be compatible with the membrane environment. We carried out molecular dynamics simulations using Gromacs [4], applying the OPLS force field on LeuT embedded in a membrane and a micelle environment to investigate the conformational behaviour of TM1A. We used POPC as membrane lipids, and build the micelle systems with three different protein-detergent ratios (1:120, 1:140, or 1:160) using the detergent n-Octyl-β-D-Glucopyranoside (BOG) molecules. We observed a rigid body motion of the TM1A helix in one of our three atomistic simulations, where the TM1A helix moves out of the hydrophobic part of the membrane. In contrary, TM1A was stable in its position in the three micelle simulations. This study suggests that the TM1A helix would not protrude into the membrane as we observe that the polar part of this helix moves out of the hydrophobic region of the membrane. As a consequence, there is no extensive opening of the inner vestibule as suggested by the crystal structure.

References:

Acknowledgements:
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PP-40  Converting the yeast arginine permease into a lysine permease

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In the yeast Saccharomyces cerevisiae, amino acid uptake is mediated by a family of about sixteen plasma membrane amino-acid permeases displaying various substrate specificity ranges. These transporters include a general amino-acid permease, Gap1, which mediates uptake of all amino acids (1), and several more specific permeases, eg. Can1 and Lyp1 transporting arginine and lysine, respectively (2,3). All these permeases are similar in sequence and belong to the APC superfamily (amino acid-polyamine-organocation) of transporters. To gain further insight into the structural basis of substrate recognition by yeast amino acid transporters, we have built a model of the Can1 tertiary structure using AdiC, a bacterial arginine-agmatine antiporter, as a template. The AdiC protein belongs to the APC superfamily and has recently been crystallized and its structure determined (4). We have used the model of the Can1 permease bound to arginine to define the residues of its substrate-binding pocket. All of these residues but two, a serine in TM3 and a threonine in TM10, turned out to be conserved between Can1 and Lyp1. We thus replaced one or both of these residues with those present in Lyp1 or with alanines. The subcellular location, transport activity and apparent Km of the mutant proteins were then compared to those of native Can1 and Lyp1.

One of the Can1 mutants displayed a gain of lysine transport, while another one showed a loss of arginine transport activity coupled to a gain of a specific lysine transport function. Our work demonstrates that differences in substrate selectivity between Can1 and Lyp1 can be attributed to two residues of the substrate-binding pocket of the permease. Docking of arginine or lysine in the native and the different mutant Can1 3D structures was also carried out to decipher the structural code of amino-acid recognition. In this combined computational and experimental work, we demonstrate that the AdiC structure represents a good working model of yeast amino acid transporters.

References:
6th SFB35 Symposium 2013, Vienna


PP-41 Lanthanide resonance energy transfer based distance measurements in the mammalian glutamate transporter excitatory amino acid transporter 3

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EAAT3 (Excitatory amino acid transporter 3) mediates the regulation of synaptic transmission by reuptake of glutamate in the synaptic cleft. EAAT3 belongs to the family of soluble carrier family 1 member 1 (SLC1A1) and is selectively enriched in the neurons of the hippocampus, cerebellum and the basal ganglia. The motivation of the study is to gain insight into the structure function relation of EAAT3. The project utilizes the high-resolution crystal structure of GltPh, the bacterial orthologue of Pyrococcus horikoshi to mammalian glutamate transporters. GltPh provides a structural framework for the determination of the helical movement in EAAT3. The structural rearrangement of the protein is caused by the helical movements which will be assessed by distance measurements using the technique of lanthanide resonance energy transfer (LRET).

LRET relies on Förster’s theory of resonance energy transfer where there is distance dependent transfer of energy between the donor fluorochrome to an acceptor fluorochrome. The protein is expressed in Xenopus laevis oocytes. Lanthanide binding tags (LBT) has been inserted into the protein to chelate the lanthanide terbium which serves as the donor element. Existing aminoacid residues in the protein are mutated to cysteine which then reacts to an acceptor dye (bodipy FL) and serves as acceptor. The energy transferred from the acceptor to the donor can be converted into a specific distance estimate.

The measured distances will allow us to obtain new insights into the structure function relationship of the glutamate transporters and can be further investigated using different substrates and inhibitors.

The results obtained in this project will allow us to better understand pathophysiological conditions associated with loss of function in EAAT3, for instance in ischemia or mutations associated in obsessive compulsive disorder.

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**PP-42**  Controlling synaptic function by glial GABA transporters

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Several studies demonstrated the ability of astrocytes to sense, respond to and regulate neuronal function. Importantly, astrocytes possess the complete set of membrane proteins to detect GABA, the major inhibitory neurotransmitter of the brain. In addition to ionotropic and metabotropic GABA receptors, astrocytes also express GABA transporters the role of which has long remained uncertain. In this lecture I will present evidence that activation of the glial glutamate transporters by their endogenous substrates triggers the reversal of the closely localized glial GABA transporter subtypes GAT-2 or GAT-3 and lead to an increase of the extracellular GABA level. GAT reversal is initiated by the elevation of the local intracellular Na⁺ concentration that subsequently turns back the driving force for the GABA transporters. In addition, we explored the potential physiological and pathophysiological role of the Glu/GABA exchange process in freshly isolated hippocampal slices and in the hippocampus in vivo. We demonstrated that the glutamate uptake-induced release of putrescine-derived GABA through astrocytic GATs has a direct impact on the excitability of pyramidal neurons in the hippocampus. The released GABA significantly contributes to the tonic inhibition of neurons in a network activity-dependent manner providing a tuneable, in situ negative feedback. We prove that the Glu/GABA exchange mechanism is functioning in the hippocampus under physiological conditions in vivo. Importantly, blockade of the mechanism increases the duration of seizure-like events and frequency of glial Ca²⁺ spikes in the low-[Mg²⁺] in vitro model of epilepsy, demonstrating that the negative feedback control of astrocytes on neuronal excitability offers significant neuro- and glioprotection in pathophysiologically overactivated states.

**PP-43**  Substrate specificity of lipid transporters, ABCA1 and ABCA7

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While cholesterol is crucial for proper cell function, perturbed cholesterol balance can be a cause of several diseases such as atherosclerosis and Alzheimer’s disease (AD). High-density lipoprotein (HDL) carries cholesterol back to liver from peripheral tissues to avoid abnormal accumulation of cholesterol. Therefore, the amount of HDL in blood inversely correlates with risk of atherosclerosis. ABCA1, a key molecule of HDL formation, transports cholesterol and phospholipid (PL) to lipid acceptor apoA-I to generate HDL. ABCA7, which shares 66.2% homology in amino acid sequence with ABCA1, has been reported to have a link with risk of AD. However, physiological function of ABCA7 is little revealed and the transport substrate of ABCA7 is also controversial; one group reported that ABCA7 transports cholesterol and PL, and another reported that ABCA7 transports only PL but not cholesterol.

In this research, we compare the cholesterol and PL efflux ability of humanABCA1 (hABCA1), humanABCA7 (hABCA7) and mouseABCA7 (mABCA7) under the same expression conditions. BHK clones were established for each ABC protein, in which the gene expression can be induced with the synthetic steroid (mifepristone). From BHK/ABCA1 clones, both cholesterol and PL were exported to the media in the presence of apoA-I after mifepristone treatment. On the other hand, significant amount of PL but only little amount of cholesterol was exported from BHK/ABCA7 clones. Now we are working on mABCA7 to clarify the species specificity. We expect our research will determine the transport substrate of ABCA7 and reveal the link between ABCA7 and Alzheimer’s disease.

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β-phenylethylamine (βPEA) is a trace amine present in the central nervous system of all animals tested to date. However, its function is still not fully understood. βPEA has been suggested to function as a neurotransmitter and/or to mimic the effect of amphetamine (Amph). In support of the latter is the observation that βPEA and Amph produce similar but not identical behaviors. Here we show that βPEA, like Amph, activates the dopamine transporter (DAT) and the amine-gated chloride channel LGC-55 to generate behaviors in C. elegans. However, while the Amph-induced behaviors occurred gradually during 10 minute treatment, βPEA induced maximal effects within 1 minute. Our data suggest that the activation of LGC-55 mostly accounts for the behavioral effects reached after 1 minute of treatment with βPEA. Moreover, both our in vitro and in vivo data demonstrate that Amph potentiated the effects of βPEA on the LGC-55. These data demonstrate important functional similarities and differences between Amph and βPEA, identify a new target for βPEA and suggest a novel mechanism of action of Amph. Furthermore, our results highlight C. elegans as a powerful genetic model for studying the effects of biogenic and synthetic amines both at molecular and behavioral level.

PP-45 Remitted Major Depressive Disorder with Adolescent Onset is associated with reduced Suppression of the Default-Mode Network during Working Memory Performance

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The proper performance of working memory (WM) tasks is dependent on neural activation of WM-related cortical brain areas as well as on suppression of the default mode network (DMN). The DMN has been related to self-referential processing and interacts with many brain areas including WM networks (WMN) during rest. Due to conflicting computations the DMN is inhibited during task performance. Its lacking suppression has been implicated in acute Major Depressive Disorder (MDD) and has been related to increased rumination. Moreover, acute MDD patients have shown to exhibit dysfunctional activation of the WMN, which goes along with cognitive symptoms occurring during depression. However, it is currently not clear, if all of these alterations are fully normalized in remitted MDD (rMDD).

To address this question, we investigated fully remitted and drug-free MDD patients with adolescent- (n=42) and adult-onset (n=36), who were compared to healthy subjects lacking a life-time diagnosis of psychiatric illness (n=42). Subjects performed the classical digit variant of the n-back task during functional magnetic resonance imaging in order to assess neural activation of WM networks. At the behavioral level accuracy and reaction time have been recorded.

Behavioral analyses revealed a significant effect of WM load, while no significant group differences were found. At the brain systems level, all three groups showed a significant load-dependent activation of the WMN, which was most pronounced in the dorsolateral prefrontal cortex (DLPFC). Concurrently, a significant load-dependent deactivation was found in the DMN with punctum maximum in the anterior cingulate cortex (ACC). Importantly, in comparison to healthy subjects, rMDD patients experiencing adolescent-onset exhibited significantly reduced suppression of the DMN, which was most pronounced in the ACC. Interestingly, rMDD patients experiencing adult-onset demonstrated relatively decreased suppression of the DMN, which was non-significantly different from the remaining groups, but positioned in between adolescent-onset rMDD patients and healthy controls.

Our findings of deficient suppression of the DMN during WM performance in both rMDD subgroups may reflect insufficient inhibition of conflicting computations that occur physiologically during rest. This inability to down-regulate the DMN seems to persist in MDD patients even, when mood becomes euthymic. Observed pronounced effects in early-onset MDD patients hint at a neurodevelopmental origin of these findings.
PP-46  The SLC1A5 aminoacid transporter: functional properties of the human protein and perspectives of application in antitumor drug design.

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Amino acid transport systems play pivotal roles in mammalian cells. Some of these transporters share specificity for glutamine thus being involved in its homeostasis and trafficking in different tissues. Noteworthy, the expression of some glutamine transporters, SLC1A5 (ASCT2) and SLC7A5 (LAT1), is up-regulated in tumors. This phenomenon is linked to the altered metabolism of cancer cells which, differently from normal ones, rely mainly on glutamine supply for energy production. Therefore, the mentioned glutamine transporters are essential for cancer cell survival and, hence, represent potential targets for antitumor drugs. Large scale screening of potential inhibitors will have great importance for drug discovery and design. In vitro methodologies, using if possible human transporters, may help the prediction of efficacy and/or toxicity of hypothetical drugs before animal experimentation. The proteoliposome system constitutes a good alternative to cells, using purified transport proteins extracted from cells, tissue or produced by heterologous expression. In this work, the hASCT2 transporter has been heterologously over-expressed in \textit{P. pastoris} and purified by Ni\textsuperscript{2+}-chelating chromatography (yield of 10 mg/liter). Biochemical characterization has been performed by reconstituting purified hASCT2 in proteoliposomes with the same orientation it has in cell membrane. Na\textsuperscript{+}-dependent [\textsuperscript{3}H]glutamine/glutamine antiport has been measured at pH 7.0. The main novelty of this study is the functional asymmetry of hASCT2-mediated transport: Gln, Ser, Thr and Asn are bi-directionally transported, while Cys and Ala are only inwardly transported. Moreover, the transport mechanism, still controversial from studies conducted in cell system, has been investigated by pseudo-bi-reactant analysis. A random simultaneous mechanism has been demonstrated. The reconstituted protein has been further tested to identify amino acid residues important for its function. These inhibition studies showed that some SH-reagents strongly impaired glutamine uptake. From dose-response analysis, IC50 values below 10 \textmu M were calculated for mersaryl and methanesulphonates. The data suggested Cys residues as target of specific inhibitors. The homology model of the hASCT2 protein, built using as template the GltPh of \textit{P. horikoshii}, showed that some Cys residues can become externally exposed, explaining the reactivity towards SH reagents. A pilot study on the rat isoform of ASCT2, extracted in native form from kidney, has been conducted to screen a large number of SH reagents (dithiazoles) with antitumor activity identifying some specific and potent inhibitors of rASCT2 (IC50 values below 20 \textmu M). These molecules were not able to inhibit, at the same extent, the human isoform of ASCT2. Interaction studies with the human protein are in the course for designing potent inhibitors of the hASCT2 with potential antitumor activity.

PP-47  SERT/NET selectivity of tropane compounds

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The main target for modern antidepressant is the serotonin transporter, SERT, either selectively (SSRIs) or in combination with the norepinephrine transporter, NET. Interestingly, the selectivity towards SERT can be altered towards NET if only minor structural modifications are introduced in the ligand.

To help the development of more specific antidepressants and to be able to tune the selectivity the interaction points with the transporter needs to be investigated. In this project a structural group of tropane compounds with a range of selectivity profiles have been studied. The molecular determinants for selectivity were studied by introducing reverse single mutations at non-conserved residues between SERT and NET, mainly within the S1 site. We hypothesize that the NET-like binding pocket in SERT will result in decreased affinities of the tropane compounds while SERT residues in the NET binding pocket will produce higher affinities compared to NET.
PP-48  Distribution of glutamate transporters – an overview
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Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and is inactivated by cellular uptake catalyzed by glutamate transporter proteins. The mammalian genome contains five genes encoding transporters that are referred to as GLAST (EAAT1; slc1a3), GLT1 (EAAT2; slc1a2), EAAC1 (EAAT3; slc1a1), EAAT4 (slc1a6) and EAAT5 (slc1a7). GLT1 is the most important subtype in the mature brain. It is strongly expressed representing about 1% of total adult brain protein (1) and accounts for around 95% of the total glutamate uptake activity in the forebrain (2). GLT1 is predominantly expressed in astrocytes (3), but about 10% of GLT1 is expressed in axon terminals, at least in the hippocampus, where it accounts for the glutamate uptake by glutamatergic nerve terminals (4). In contrast, GLAST is selectively expressed in astrocytes in the brain (3). The highest levels are found in the cerebellum, but forebrain levels are also substantial (1). In agreement with the importance of GLT1, GLT1 deficient mice become epileptic at around P21 and half of them die before P30 (2). EAAC1 appears to be selective for neurons and is only found in the somato-dendritic compartments and thereby in the parts that do not express GLT1. However, the total levels of EAAC1 in hippocampal tissue are about 100 times lower than those of GLT1 (5). EAAC1 is neither present in terminals nor in glia. EAAT4 is mostly in cerebellar Purkinje cells, but there is also some in the forebrain (6).

References

PP-49  Rescue of ABCB1 domain interface mutants by folding correctors
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Folding defects of proteins caused by mutation and subsequent impaired trafficking is the pathogenic principle for a number of monogenic diseases, such as cystic fibrosis and nephrogenic diabetes insipidus. Aberrant folding leads to retrotranslocation to the cytosol 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. A first pharmacochaperone for the treatment of phenylketonuria has been introduced to the market. PCs bind to the respective client proteins and correct mutation-induced aberrant folding. Non-synonymous mutations at the TMD/NBD interface lead to misfolding of at least three ABC-transporters and human disease. Mutations in ABCG2 lead to cystic fibrosis, those in ABCG2 to gout and that in ABCB1 to intrahepatic cholestasis. The analogous trafficking deficient ABCB1 mutant ΔY490 was used, because of the ability of this transporter to interact with a large complement of small solute-like molecules. A number of solute analogues related to the lead compound propafenone have been synthesized and evaluated for their ability to elicit a trafficking rescue. Several compounds are able to restore surface expression to wild type levels. The potency to recover surface expression correlates with the IC50 values for daunomycin efflux inhibition. Therefore IC50 values predict the ability of a compound to rescue trafficking. This allowed their use as a surrogate parameter in database screening.

A previous study in our group demonstrated the ability of substrates to interact with ABCB1 in two rotationally symmetric positions, which involve pore exposed tyrosine residues (Parveen et al, Mol Pharmacol 2011). Among those Y953 located in transmembrane helix 11 in the preferred propafenone site (site 2) has been shown to form hydrogen-bonds with propafenone analogues in the intact protein (see poster Dönmez et al).
ABC5B1 mutant ΔY490-Y953F was used to examine if propafenones use the same site in the protein to act as pharmacocchaperones than they use for the binding to the mature protein. The answer of this question will help us to improve our understanding how PCs enable to rescue trafficking.

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PP-50  Binding interactions between the dopamine transporter and the cocaine-like photo affinity ligand RTI 82

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Cocaine elicits its primary psychotropic and addictive effects by targeting the dopamine neurotransmitter transporter (DAT) thereby altering dopaminergic neurotransmission. However, the molecular determinants important for cocaine binding to DAT remain poorly understood. Availability of crystal structures from a bacterial Leucine transporter (LeuT) homologous to DAT provides a scaffold for unraveling the structural and molecular details of drug-DAT interactions. In this study, comparative models of DAT were constructed based on the LeuT ‘occluded’ and ‘open-to-out’ structures (PDBID: 2A65 and 3F3A, respectively) followed by iterative model refinement and energy minimization in the molecular modeling package Rosetta 3.1. The twenty best scoring (lowest energy) DAT structures (10 from both 2A65 and 3F3A) were carried forward for flexible ensemble docking of RTI 82 using RosettaLigand. In addition, Na+ and Cl– were placed in their reported binding sites in the 3F3A and 2A65-based Rosetta 3.1 models and analyzed by induced fit docking (IFD) (Schrödinger Molecular Modeling Suite). The top-scoring models from both RosettaLigand and IFD docking supported crosslinking between the azido group of RTI 82 with residue F319. This prediction was verified through site-directed mutagenesis (SDM) and biochemical crosslinking studies. Our DAT/RTI 82 docked complexes place the ligand in the S1 binding pocket is a pose similar to that reported for CFT by Beuming et al. 2008. In addition to the docking analysis, the best scoring poses from each docking method were refined by molecular dynamic simulations. Interestingly, refinement of IFD (but not Rosetta)-docked poses resulted in a small shift of RTI 82 downward in the binding pocket that did not affect the pi-pi interactions between F319 (TM6) and the aryl azide on RTI 82. To investigate this difference in refined poses, we generated mutations at F319 which eliminate the aromatic side chain while maintaining hydrophobicity and found no significant changes in the potency of RTI 82. These results suggest that the best pose generated by RosettaLigand, which does not show aromatic ring stacking between RTI 82 and F319, may better represent cross-linking and site-directed mutagenesis studies. Overall, these findings provide insight into two distinct small molecule docking methodologies as well as convincing evidence that our comparative models and docking methodologies have predictive value for analyzing other small molecules that interact with DAT including typical and atypical antagonists.
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PP-51 Probing serotonin transporters on cells using AFM cantilevers with bi-valent branched crosslinkers with MFZ2-12 or citalopram

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Atomic force microscopy (AFM) can not only get sub-nanometer resolution topography of biological samples in physiological liquid, but can measure the molecular interaction force with pico-Newton sensitivity. The recently developed simultaneous topography and recognition (TREC) imaging can use a force sensor (i.e. cantilever) tethered with a ligand to map the distribution of the ligand-binding molecules on the sample surface. Here, we used AFM to probe the serotonin transporters (SERT) on live CHOk1-YFP-hSERT cells using the cantilever tip conjugated with bi-valent branched crosslinker and MFZ2-12 (a potent cocaine analog) or citalopram (an anti-depressant drug). From the force-distance curve measurements, we observed both single unbinding events and sequential multiple unbinding events. Increasing the contact time of the tip on cellular surface increased the probability of multiple unbinding events. The tips with bivalent citalopram showed higher relative probability of multiple sequential binding events than the tips with bivalent MFZ, possibly due to the vestibule binding site in the SERT specific for citalopram but not for MFZ. The distance between the two sequential unbinding events had a broad distribution with a peak around 100nm. The first unbinding event tended to have higher unbinding force than the second one when the distance between them was short. The existence of the sequential two unbinding events indicated that the pulling force was not equally shared by the two pairs of SERT-ligand. We used cantilever tips functionalized with the monovalent racemic citalopram to measure the TREC images on fixed CHO k1-YFP-hSERT cells, which revealed widely distributed recognition spots (binding sites). Injection of 90 µM racemic citalopram resulted in partial block of the recognition, which supported the specificity of the recognition spots.

PP-52 Role of low molecular weight compounds (pharmacological chaperones) in the correction of mutation-induced misfolding of the bile salt export pump.

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The bile salt export pump (BSEP/ABCB11) is located at the canalicular membrane of hepatocytes and is the major transport system of bile salts into bile. A number of disease causing mutations have been documented, which result in the misfolding of ABCB11 and cause progressive familial intrahepatic cholestasis type II (PFIC II). E297G and D482G contribute to approximately 60% of PFIC II cases in the European population. Other mutations, which are interesting because of their position in the protein are R517H (equivalent to position 507 in CFTR, a known disease causing mutation in cystic fibrosis) and R1153C (a residue that in wild type protein is likely to form a charge interaction with residue E297 of intracellular loop 2 in TMD1, thereby possibly contributing to the correct formation of the transmission interface). This research project focuses on the elucidation of some small molecular weight compounds (pharmacological chaperones) that can rescue the mutation-induced misfolded ABCB11 from endoplasmic reticulum (ER) to their final destination (Plasma membrane) and eradicate or curtail the phenotype of disease. We generated these mutations in pENTR4 plasmid containing the WT-BSEP insert using primer extension PCR and mutated inserts were transfected to the gateway compatible proprietary pCEP4d destination vector by exploiting the unique recombination properties of bacteriophage lambda integrase in the commercially available LR-reaction kit (Invitrogen). HEK293 cells were transfected with these destination plasmids and stable cell lines expressing BSEP mutants are presently generated under hygromycin B selection conditions. Incubation of these cell lines at low temperature (28°C), which favors protein folding, showed increased expression of these BSEP mutants at the plasma membrane. These cell lines are being used for identification of pharmacological chaperones that can correct trafficking of mutants. Drugs that bind to ABCB11 and cause drug induced intrahepatic cholestasis are used as starting points in our search for correctors of mutation-induced misfolding variants of ABCB11.

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PP-53  Relaxing a spring-loaded transporter: Increasing the length of intracellular loop 5 domain impacts the overall function of the human serotonin transporter

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The serotonin transporter (SERT) is a monoamine transporter and belongs to a large gene family of neurotransmitter sodium symporters (NSS). The main function of the SERT is to reuptake the released serotonin from the synaptic cleft between the neurons in the central nervous system, thus regulating serotonergic neurotransmission.

Dysfunction of the serotonergic system has been associated to various mood disorders; therefore SERT has become a major target for drugs chiefly in the treatment of depression. Exploring the many features of SERT and understanding of its functions may provide clues for designing novel drugs.

We have inserted a tetracysteine (CCPGCC) and a tetraalanine (AAPGAA) motif into the intracellular loop 5 (IL5) domain of the human serotonin transporter (hSERT). Both insertions into the IL5 have large impacts on many functional aspects of SERT in terms of substrate functional affinity, turnover rate, inhibitor binding and conformational equilibrium. These functional consequences highlight that the IL5 plays an important role in the overall function of SERT.

PP-54  The Aspartate transporter in motion – combining steered molecular dynamics with Lanthanide Resonance energy transfer based distance measurements

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Excitatory amino acid transporters (EAAT’s) or glutamate transporters belong to the SLC1 family of the neurotransmitter transporters and mediate the re-uptake of glutamate from the synaptic cleft. Transporter dysfunction results in pathological conditions such as stroke, epilepsy and other neurodegenerative diseases. Crystal structures of GltPh, an archael homologue of the mammalian glutamate transporter, have been solved in several states, providing a starting point for understanding the conformational changes that accompany substrate transport. In this study we aim to integrate molecular dynamics simulations and Lanthanide Resonance Energy Transfer (LRET) based distance measurements to study the molecular motions that accompany substrate transport. Steered Molecular Dynamics (SMD) simulations were used to obtain insights into the transition path that lead to internalization of substrate from the extracellular milieu. Our simulations revealed the existence of an intermediate state along the transition path from the outward-occluded to the inward-occluded conformation. Local structural rearrangements at the Na2 binding site, as well as the large scale rearrangements at the transport-domain and the trimerization-domain interface accompany the transition into the inward-occluded conformation. Our simulations highlighted the existence of gatekeeper interactions at the transition from the intermediate state to the inward-facing state. Based on the dynamics observed from our simulations, cysteine mutants were designed to observe the conformational changes in vitro. Site directed mutagenesis was used to insert genetically encoded lanthanide binding tags (LBT) and also cysteines which act as fluorophore docking sites to perform LRET based distance measurements, thus generated LBT mutants were expressed and purified. The wild type and mutant proteins were expressed and purified using affinity column chromatography, donor decay signals were recorded for LBT insertion mutants to confirm the insertion of tags. Furthermore radioligand binding assays were performed with the mutants and they were found to be functional. The distance measurements made with LRET were compatible with the distances observed in the crystal structure. Efforts are being made to perform the distance measurements in reconstituted proteins in proteoliposomes.
PP-55 Molecular modeling of mammalian ABCG2 - suitable for a better understanding of structure-function relationships?

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ATP-Binding Cassette transporters (ABC-transporters) constitute a large superfamily of integral membrane proteins that are found in all living organisms from microbes to humans. These transporters mediate the translocation of diverse substrates across cellular membranes by utilizing the energy derived from ATP hydrolysis at nucleotide binding domains (NBDs) to drive conformational changes at the transmembrane domains (TMDs). In mammalian cells, the basic structures of functionally active ABC-transporters consist minimally of four domains, two NBDs and two TMDs. Mutations in these proteins have been causally implicated in the etiology of severe diseases. Importantly, members of three mammalian subfamilies, ABCB, ABCC and ABCG, are considered relevant contributors to clinical multidrug resistance (MDR) in human cancer.

We focus on the molecular structure-function analysis of ABCG2 (also known as Breast Cancer Resistance Protein, BCRP) which is originally identified from drug cancer resistance cell line. ABCG2 is a half transporter consisting of a large cytoplasmic N-terminal region containing NBD, followed by 6 transmembrane segments at C-terminus. ABCG2 has to form a homodimer to yield a functional transporter. However, the understanding of dimerization and subsequent cellular trafficking is still incomplete.

Here, we combine a theoretical and experimental approach to study the function of ABCG2. Computational methods provide important strategies to predict protein structures and their functions. For example, homology modeling is a valuable tool for prediction and understanding the structure of proteins. We have created a low resolution model of ABCG2.

This model will be used to generate hypothesis, which will lead to experimental testing and validation of a predicted structure and may be valuable for the development of pharmacological approaches to identify ABCG2-specific substrates as well as inhibitors.

PP-56 LC-MS/MS uptake assay for the evaluation of hGAT1 inhibitors

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γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in CNS. It is mainly released into the synaptic cleft from vesicles upon a depolarization of the presynaptic neuron and can then bind to specific receptors on the postsynaptic neuron to transduce the signal. Termination of signal transduction is performed by reuptake into the presynaptic neuron or into glia cells via specific GABA transporters. GABA transporters are potential drug targets for diseases like Morbus Parkinson, schizophrenia or neuropathic pain, which are associated with a dysbalance of neurotransmitter levels in the synaptic cleft. Four subtypes of these transporters are known which are located in different tissue. Tiagabine, an approved antiepileptic that inhibits GAT1, is the only example of a drug on the market. There clearly is the need for new GAT inhibitors.

To identify potential GAT inhibitors, different screening methodologies can be used. The transporter affinity of an inhibitor can be determined by ligand binding assays, employing a known marker substance which is displaced by the potential inhibitor. Although MS-binding assays are a powerful approach, they do not provide any information on biological activity. Furthermore, a selective marker substance with distinct affinity has to be provided for screening applications. Therefore, the use of uptake assays for the characterization and identification of potential GAT inhibitors are elemental. To identify new GAT inhibitors, radiolabel uptake experiments are widely used which are sensitive and robust, but have all the disadvantages of using radioactivity, like high cost, strict regulations and health and safety issues. For GABA uptake experiments, the substrate has to be quantified with high selectivity and sufficient sensitivity.

A non-radioactive GABA uptake assay is presented for the screening of hGAT1 inhibitors. The assay uses COS-7 cells stably expressing hGAT1 in combination with (1H)3GABA as substrate to differentiate between transported and endogenous GABA, which is present in the cell culture in significant amount. Substrate quantification is performed using (1H)3GABA as internal standard on a YMC-PVA-Sil column with isocratic elution coupled to an API5000 mass spectrometer without any sample preparation. The assay is capable of measuring 96 samples in less than 6 h and is well suited for screening applications. Results from these experiments were reproducible and are in good agreement with results obtained by [3H]GABA uptake assays in HEK cells. Moreover, competition experiments with known GAT inhibitors led to IC50 values that are in good accordance with IC50 values derived by radioactive uptake assays. The established procedure is very versatile and is also appropriate to study (1H)3GABA transport via GAT2, GAT3 and BGT1, as exemplified with hGAT3.
PP-57 Combining in vitro with in silico studies to obtain insights into substrate transport by the multidrug resistance protein P-glycoprotein

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The human genome contains 48 ATP-Binding Cassette (ABC) proteins, 44 of which are membrane transporter proteins. Several of the ABC transporter are causally related to disease. We focus on the multidrug resistance transporter P-glycoprotein, which is particularly important in the protection of barrier tissues. P-glycoprotein is expressed at the blood-brain-barrier, in the intestine, kidney, liver and in macrophages and transports an extraordinarily diverse range of structurally unrelated drugs, xenobiotics and endogenous substrates. Cancer cells acquire resistance to chemotherapy when expressing P-glycoprotein or the ABC transporter breast cancer resistance protein (ABCG2). It is well established that binding of ATP and its subsequent hydrolysis drives the transport process in all ABC transporters, including P-glycoprotein. In contrast, the details of how substrates are translocated by ABC exporters remain largely unknown. In fact, most details regarding the mechanism of substrate recognition, uptake and binding to P-glycoprotein, and the mechanism by which substrate binding in the transmembrane domains is coupled to ATP hydrolysis in the nucleotide binding domains remain unknown.

The bacterial homologue of P-gp, Sav1866 (Staphylococcus aureus), was the first ABC exporter crystalized and showed an unexpected domain interlinking architecture. The same fold was later observed in other transporter of the ABCB family, suggesting a conserved architecture across the ABCB exporter family. Although ABC exporters have now been crystalized in several conformations, uncertainty remains regarding the physiological conformation of these structures. None of the crystal structures are fully compatible with all biochemical evidence. The observed conformation of the ATP-bound state might be artificial and a consequence of the crystallization procedure or conditions. We combined modeling with experiments to address these issues. Homology modeling and MD simulations were used to determine the equilibrium conformation of ATP-bound P-glycoprotein in a membrane environment. In contrast to the conformations observed in crystal structures, the wing shape structure is unstable in the membrane environment. The conformation observed by MD simulations is devoid of the wing-shape, but in agreement with the bulk of the biochemical data. Acknowledgements: The research was funded by the Austrian Science Fund (FWF): P23319-B11

PP-58 The puzzling C-tail and the part it plays in the folding and ER export trajectory of the serotonin transporter

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The serotonin transporter (SERT) mediates the reuptake of its eponymous substrate serotonin from the synaptic cleft into presynaptic specialisations. Like other membrane proteins, SERT is co-translationally inserted into the ER membrane. After adopting its folded state, it must reach the ER exit sites to recruit components of the COPII coat, i.e., the SEC23/SEC24-dimer to allow for their export from the ER. Interestingly, SERT recruits SEC24C, although its close relatives (DAT, NET, GAT1) all require SEC24D (1). Amino acid residues flanking the ER export motif on SERT (R607I608) must specify/direct SERT exit from the ER compartments. Mutations of interest were generated by site-directed mutagenesis using the QuikChange™ kit (Stratagene) and wild type human SERT as template. HEK293 cells were transfected with plasmids encoding the wild type and mutant SERTs by lipofection (Invitrogen). All constructs had yellow fluorescent protein tags on their N-termini to allow visualization by confocal laser scanning microscopy. Functional activity was assessed by radiolabeled serotonin uptake assays. Radiolabeled imipramine binding was used to examine protein folding. To address SEC24-dependence, we used siRNAs to knock-down SEC44 isoforms A-D, as described previously (1, 2). The replacement of an aromatic Phe 604, and of nonpolar aliphatic Ile residues 608 and 612, by polar uncharged Gln residues (i.e. mutants F604Q, I608Q and I612Q, respectively) lead to marked reductions in [3H]-5-HT uptake levels, compared to wild type SERT. Confocal microscopy experiments revealed that the impaired functional activity resulted from intracellular retention of the SERT protein. The mutants could become targeted to the plasma membrane upon an introduction of a second site suppressor E136A mutation. E136A locks the transporter in an inward-facing conformational state; albeit perfectly expressed at the cell surface, it lacks any appreciable uptake due to the defective transport cycle. Non-conservative mutations of F604, I608 and I612 cause ER retention of SERT by inducing folding defects, but not due to the loss of interaction with the COPII component SEC24C. Residue 604 must be hydrophobic for SERT to fold correctly, and although the F604Q mutant is retained in the ER, it can be rescued by various chemical and pharmacological chaperones. Current experiments begin to shed new light onto the folding trajectory and ER export of monoamine transporters. This work was supported by SFB35.

**PP-59  Structure of a nucleotide-bound vitamin B12 transporter BtuCD in an outward facing conformation**

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Uptake of vitamin B12 in *Escherichia coli* and other bacteria is mediated by BtuCD-F, a complex formed by a type II ABC importer, BtuCD, and its cognate periplasmic binding protein, BtuF. We have recently solved a structure of BtuCD-F bound to a non-hydrolysable ATP analogue, AMPPNP. This structure revealed a large intramembrane cavity formed by the dimer of BtuC subunits. This lead us to propose a peristalsis-like mechanism of vitamin B12 transport via BtuCD-F. Here, we report the X-ray structure of disulphide-stabilized BtuCD in the absence of BtuF, with two AMPPNP molecules bound in the nucleotide binding sites, at 2.8-3.7Å resolution. The structure shows closure of the cytoplasmic gate II (residues N83-L85 of BtuC) concomitant with formation of a nucleotide-bound closed sandwich dimer of BtuD subunits. Contrary to the BtuCD-F/AMPPNP structure, in the absence of BtuF the periplasmic gate region of BtuCD creates an opening towards the periplasmic space, consistent with the available biophysical data. Thus, the structure of BtuCD bound to AMPPNP provides new insights into the coupling between the nucleotide binding and transmembrane domain rearrangements in type II ABC importers.

**PP-60  Direct inhibition of the norepinephrine transporter by the cocaine adulterant levamisole**

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**Methods: Uptake and Release Assays** HEK293 cell lines stably expressing the human isoforms of SERT, NET, or DAT were used for both assays. For inhibition experiments, specific activity of the tritiated substrate was kept constant: [3H]DA, 0.1 µM; [3H]MPP+, 0.015 µM; [3H]5-HT, 0.1 µM. For release studies, cells were preloaded with 0.4 µM [3H]DA, 0.1 µM [3H] MPP+, or 0.4 µM [3H]5-HT for 20 min at 37 °C. IC₅₀ values were calculated using non-linear regression fits performed with Prism (GraphPad 5.0).

**Homology modelling and docking** Both the neutral and protonated levamisole structures were built and minimized with QSite (vers. 5.8, Schrödinger) applying the 6-31G* basis set. **Results:** The observed IC₅₀ values for cocaine were 1.8± 1.12 µM (SERT), 1.0 ± 1.07 µM (NET), and 0.56 ± 1.12 µM (DAT) respectively. 1512± 1.09 µM (SERT), 74.5 ± 1.12 (NET), 209.9 ± 1.31 µM (DAT) for levamisole. Uptake-inhibition experiments indicated no allosteric effect. Furthermore, releasing properties of levamisole showed a slightly increased efflux in NET but not in SERT or DAT. The levamisole metabolite aminorex preferentially blocked substrate uptake by NET (IC₅₀: 0.33 ± 1.07 µM) and DAT (IC₅₀: 0.85 ± 1.20 µM), while SERT was inhibited only at 20-fold higher concentrations (IC₅₀: 18.39 ± 1.12 µM). The pattern of inhibition (NET>DAT>>SERT) was similar to levamisole, but the inhibitory potency of aminorex was comparable to that of cocaine. We observed significant efflux in SERT, slight efflux in NET but no efflux in DAT for aminorex. Using a ligand –docking approach levamisole was predicted to has the highest affinity for NET (charged: -830 kcal/mol; neutral: -820 kcal/mol), followed by DAT (charged: -792 kcal/mol neutral: -792 kcal/mol) and SERT (charged: -697 kcal/mol neutral: -683 kcal/mol).

**Discussion:** Testing the effects of levamisole on the serotonin transporter (SERT), norepinephrine transporter (NET) and the dopamine transporter (DAT), levamisole was about 100-fold less potent than cocaine in blocking substrate uptake in all three transporters. Moreover, levamisole did not trigger substrate efflux and did not enhance the inhibitory action of cocaine. Since levamisole is metabolized to aminorex, classified as amphetamine-like substance, we examined the uptake-inhibitory and efflux-eliciting properties of aminorex and found it to exert strong effects on all three neurotransmitter transporters in a manner similar to amphetamine. We therefore conclude that while the adulterant levamisole itself has only moderate effects on neurotransmitter transporters, its metabolite aminorex may exert distinct psychostimulant effects by itself. **Acknowledgements:** The work of was financially supported by the Austrian Science Foundation/FWF (grant F35).
Development of a LC-ESI-MS/MS method for quantification of Indatraline and its application to MS binding assays addressing the human norepinephrine transporter

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The neuronal systems of monoamines [i.e. dopamine (DA), norepinephrine (NE) and serotonin (5 HT)] are involved in different mental disorders such as depression, Parkinson’s disease, and schizophrenia. They are also associated with drug abuse. Since DA, NE, and 5-HT are removed from the synaptic cleft by the corresponding transporter proteins (DAT, NET, and SERT), inhibitors that prevent the reuptake of those monoamines represent relevant drugs for these targets.

Therefore, our aim was to develop a screening technique to identify potential reuptake inhibitors of the human monoamine transporters using the established approach of mass spectrometry based binding assays (MS binding assays). This concept of MS binding assays employs a native marker (i.e. a non-labeled ligand) which acts as competitor for the tested substances. We decided to use Indatraline [trans-3-(3,4-dichlorophenyl)-N-methyl-1-indanamine] (figure 1), a non-selective inhibitor of the monoamine transporters, as native marker. Firstly, we developed a sensitive LC-ESI-MS/MS method for quantification of Indatraline with a poly-deuterated internal standard (d7-Indatraline), followed by a validation according to the FDA guidance.

Preliminary MS binding assays performed with membrane preparations stably expressing hNET of HEK293 cells and the (1R,3S)-enantiomer of Indatraline, the assumed eutomer, confirmed the feasibility of this approach.

Literature:

Role of pore exposed tyrosine residues in drug interactions

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The multispecific drug efflux transporter P-glycoprotein (P-gp, ABCB1) is a member of the ABC transporter family. Its two transmembrane domains (TMDs) form composite solute translocation paths at their interface, which allow for movement of a wide variety of substrates across biological membranes. Availability of several ABC efflux transporter crystal structures and a large body of biochemical data improved our understanding of the overall architecture and structure of the TMDs. In the development of drug candidates an improved understanding of P-gp drug interactions transport process would be beneficial. By using a combination of photolabeling and mass spectrometry our group recently demonstrated the ability of substrates to interact with P-gp in two rotationally symmetric modes, which are related to each other by pseudosymmetry and involve pore exposed tyrosine residues in a conserved YxSYA motif in helices 5 and 11 (Parveen et al, Mol Pharmacol 2011).

Y953 was identified as being photolabeled by analogs of propafenone (a class 1c antiarrhythmic drugs) in previous experiments and together with residue Y950 is located in transmembrane helix 11, which contributes to binding of propafenones in mode 2. These two tyrosines are considered as potential interaction partners with propafenones and were mutated in this study to phenyalanine to assess the contribution of tyrosyl hydroxyl groups to hydrogen bond formation with these ligands. Y953 is shown to form hydrogen-bonds with propafenone analogs, but also with the preferred site 1 substrate rhodamine. Moreover, an accessory role of residue Y950 for binding of selected propafenone analogs is demonstrated. In addition, arginines were introduced below and outside the binding sites in order to direct charged compounds towards one or the other binding mode by charge repulsion. The present study presents evidence for a direct involvement of pore exposed tyrosines in drug binding.

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Vesicular glutamate transporters (VGLUTs) are known to be important in the uptake of glutamate into vesicles in the presynaptic terminal, and thereby play a role in maintaining normal synaptic function. VGLUT dysfunction has also been suggested in the pathophysiology of neurological and psychiatric disorders such as epilepsy and schizophrenia. A number of compounds have been identified as VGLUT inhibitors; however, little is known as to how these compounds affect synaptic transmission. We therefore investigated the effects of structurally unrelated VGLUT inhibitors on synaptic transmission in the mouse prefrontal cortex (PFC) and hippocampus. In the in vitro hippocampal slice preparation, field excitatory postsynaptic potentials (fEPSPs) were evoked in response to Schaffer collateral/commisural (Scc) pathway stimulation whilst recording from the CA1 region. These fEPSPs were mediated predominately by AMPA receptors. Application of the VGLUT inhibitors Rose Bengal (RB), Congo Red (CR) or Chicago Blue 6B (CB) to the bathing medium resulted in a concentration-related reduction of fEPSP amplitudes (up to 100% reduction). In Mg2+-free bathing solution containing NBOX (20 µM), Scc pathway stimulation evoked NMDA-receptor-mediated responses. RB (30 µM) or CR (300 µM) depressed the NMDA receptor-mediated response by an amount not dissimilar to that observed on the fEPSP under control conditions (up to 90% reduction). The kynurenine Xanthurenic Acid (XA), which occurs naturally in the CNS, is reported to be a VGLUT inhibitor. Consistent with the observed effects of the other VGLUT inhibitors, we found XA attenuated both AMPA and NMDA receptor-mediated synaptic transmission. The potency order of the VGLUT inhibitors was consistent with literature K_i values for VGLUT inhibition. Impaired glutamatergic neurotransmission is believed to contribute to schizophrenia, and VGLUTs have also been implicated in this disease. We therefore investigated the effect of VGLUT inhibition in the PFC. fEPSPs were evoked in the in vitro slice by stimulation in the forceps minor and recorded with an electrode positioned in layer V/VI. These fEPSPs were mediated mainly by AMPA receptors with a small NMDA receptor-mediated component. Application of RB or CB resulted in a concentration-dependent depression in fEPSP amplitude (up to 90% reduction). We conclude that VGLUT inhibitors can modulate glutamatergic synaptic transmission in the PFC and hippocampus. This could be important in the pathophysiology of nervous system disorders and might represent a target for developing novel pharmacological therapies.

PP-63 Effect of Vesicular Glutamate Transporter (VGLUT) inhibitors on glutamatergic synaptic transmission in the prefrontal cortex and hippocampus

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Genetic variants as well as stress exposure have been highlighted as interacting factors underlying hippocampal volume loss, which is associated with major psychiatric disorders. Here, we investigated a gene-environment interaction between COMT Val158Met, a functional polymorphism of the dopamine-degrading enzyme COMT, and chronic stress assessed by the life event questionnaire (LEQ) in a large sample (n=153) of healthy volunteers. Genotyping for COMT Val158Met resulted in 30 Val/Val, 85 Val/Met and 38 Met/Met carriers. The distribution of 5-HTTLPR, a polymorphism in SLC6A4, which has also been reported to impact on stress responsivity and hippocampal volume, did not differ significantly between Val/Val, Val/Met and Met/Met carriers (χ^2 = 1.79, p = 0.41). Bilateral hippocampal volumes were larger in the COMT Val158Met Met/Met group (4164.41 ± 332.4 mm^3) than in the Val/Val genotype, but did not reach significance (4056.89 ± 223.36 mm^3, t(64.51) = 1.59, p = 0.12). However, we found a significant gene x environment interaction between COMT Val158Met genotype and LE (B = 1597.81, SE = 502.25, t = 3.18, p = 0.002). Specifically, we found a negative correlation (r = -0.36, p = 0.03) between hippocampal volume and stress level in humans homozygous for the low expressing Met allele, whereas Val homozygotes exhibited opposing effects (r = 0.44, p = 0.02). A detailed analysis of hippocampal subfields showed that these effects have been driven by CA2/3 (explained variance s^2 = 0.18, p = 0.004), CA1 (s^2 = 0.16, p = 0.009), subiculum (s^2 = 0.14, p = 0.017) and CA4/dentate gyrus (s^2 = 0.14, p = 0.027) on the right hemisphere. Analogous results were found regarding cortical thickness of the medial prefrontal cortex, which is known to be equally responsive to cortisol and has been implicated in hypothalamus-pituitary axis regulation similar to the hippocampus. Our results underline the importance of gene-environment interaction in the pathogenesis of major psychiatric disorders.
interactions even on an intermediate phenotype level and suggest strong interactions between the stress system and the dopaminergic system.

PP-65  The role of OAT1, OAT3 and BCRP transporters in the transport of chlorothiazide, a potential BCRP probe across intestinal and kidney barriers investigated in vitro and in vivo

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Background:
Chlorothiazide (CHT) is a diuretic and antihypertensive agent, with known adverse effects such as renal failure, renal dysfunction and interstitial nephritis. CHT exhibits low passive permeability and solubility, is poorly metabolized, and eliminated rapidly by the kidney, which implicates the role of transporters in the process. We previously showed that CHT is a substrate of organic anion transporter 1 (OAT1/SLC22A6), organic anion transporter 3 (OAT3/SLC22A8) and breast resistance cancer protein (BCRP/ABCG2). We hypothesized that BCRP works in concert with OAT1 and/or OAT3 to transport CHT across the proximal tubule cells thus contributing to its rapid renal clearance.

Aim:
The present study focused on the further investigation of CHT transport across biological barriers, and on the role and the hypothesized interplay of OAT1, OAT3 and BCRP transporters in the CHT absorption and renal clearance, using in vitro and in vivo models.

Being a clinically relevant and commercially available BCRP substrate, CHT seems to be a potential BCRP probe. Therefore we also wanted to evaluate how CHT meets the other two criteria of an ideal probe substrate: specificity, and applicability both in vitro and in vivo.

Methods and results:
In vesicular transport assays, the inhibition of ATP specific transport of radiolabeled probe substrates by CHT was studied using membrane vesicles from transfected cells overexpressing BCRP (ABCG2), MDR1 (ABCB1), MRP2 (ABCC2) or MRP4 (ABCC4) transporters. CHT showed a high specificity to BCRP.

CHT vectorial transport was well detectable on Caco-2, as well as on MDCKII-OAT1/BCRP and MDCKII-OAT3/BCRP double-transfected monolayers from the basolateral (B) to apical (A) direction. Efflux ratios were superior to 80, 10 and 100 fold, respectively. Both Ko143, a BCRP specific inhibitor, and probenecid, an OATs specific inhibitor, were able to fully inhibit chlorothiazide vectorial transport.

In vivo absorption and renal excretion of CHT were measured on rats using metabolic cages. The preliminary results show a 61% decrease of CHT concentration in the urine two hours after CHT i.v. injection when rats were pretreated with specific BCRP inhibitor Ko134.

Conclusions: The results of our in vitro and in vivo experiments are well correlated, and suggest that BCRP plays an important role both in the intestinal absorption and - through the concerted action of basolateral OAT1/3 and apical BCRP - the renal clearance of CHT. These results also demonstrate that CHT is an ideal BCRP probe candidate.
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PP-66 Development of a zinc-dependent serotonin transporter: modelling, simulations and experimental scrutiny

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Neurotransmitter:sodium symporter (NSS) are localized on pre-synaptic neurons. These transporters are responsible for fast termination of neurotransmission by removing the neurotransmitter dopamine (DAT), serotonin (SERT) and noradrenaline (NET) from the synaptic cleft, thereby terminating neurotransmission. Abnormal transporter function is associated with different disease states such as depression, attention deficit hyperactivity disorder, autism and bipolar disorder. Several drugs for the treatment of these diseases that target NSS have been developed. Illicit life style drugs such as cocaine, amphetamine and ecstasy interfere with normal transport function. The structural determination of the bacterial homolog LeuT has provided considerable new insight into both structure and function, allowing to investigate the molecular details of NSS function at atomic resolution.

The high-resolution crystal structure of LeuT has been used as a template for development of a refined homology model of DAT. The much larger extracellular loop 2 of the NSS has been modelled and the endogenous zinc-binding site of DAT structurally characterized (Stockner et al., PLoS Comput. Biol., 2013). Development of a zinc-dependent SERT has been unsuccessful, in part because of limited structural knowledge. We have made use of our DAT transporter model to infer the corresponding site in SERT. Sequence alignments, homology modelling and simulations identified residues F213 and R390 as candidate residues. Position R390 in the extracellular loop 4of SERT corresponds to H375 in DAT. The R390H mutation is predicted to contribute a zinc coordinating histidine. Simulations identified residues F213 as having the highest probability of occupying the position, which is reserved to H193 in DAT. Experimental scrutiny of the double SERT mutant F213H and R390H by uptake inhibition assays confirmed the zinc dependency of this construct, validating the predictions.

PP-67 Differential rates of glutamate transporter proteolysis may lead to erroneous conclusions about transporter distributions

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The glutamate transporters (GLT-1, GLAST, EAAC1, EAAT4 and EAAT5) are highly interesting from a neurophysiological point of view because they keep excitatory amino acids under control (1). Therefore it is of interest to determine if their expression levels or distributions are changed in human diseases. Human brain tissue, however, is rarely obtained fresh. Further, it is currently discussed whether the transporter distributions in rodents differ from those in man as some researchers, have found a patchy labeling in human tissue and astrocytes expressing only GLAST. Here we have used mice brains to study how fast glutamate transporters are degraded after death (2). Immunoblots showed that epitopes on the termini of GLT-1 (EAAT2), the C-terminus in astrocytes expressing only GLAST. Here we have used mice brains to study how fast glutamate transporters are degraded after death (2). Immunoblots showed that epitopes on the termini of GLAST (EAAT1) degraded, but at a slightly slower rate. In contrast, epitopes within the central parts of GLT-1 and the C-terminus of EAAC1 (which is expressed in neurons) were readily detectable after 72 hours. The relatively fast decline of immunoreactivity to the termini of GLT-1 and GLAST in gial cells was also seen in tissue sections, but the proteolysis did not happen synchronously in all cells. In neocortical tissue fixed immediately after death GLAST and GLT-1 were found to colocalize. In contrast, cells only positive for GLAST were abundant in tissue stored for 12 hours prior to fixation. This uneven disappearance of labeling was not observed with the antibodies to GLT-1 residues 493-508, which correlated better with the glutamate uptake activity (3). Conclusions: Transporters present in vivo may evade detection in autopsy material when antibodies to the termini are used. Disease related changes in transporter immunoreactivity may need to be considered before concluding about in vivo transporter levels. In agreement with others (4) we find that differences in glutamate transporter distributions in man and in rodents are small and suggest that the large differences suggested in other studies may represent post mortem artifacts.

References
PP-68  A quantitative model of amphetamine action in SERT

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BACKGROUND AND PURPOSE  Amphetamines bind to the plasmalemmal transporters for the monoamines dopamine (DAT), norepinephrine (NET) and serotonin (SERT); influx of amphetamine leads to efflux of substrates. Various models have been put forth to account for this amphetamine-induced reverse transport in mechanistic terms. A most notable example is the molecular stent hypothesis, which posits a special amphetamine-induced conformation that is not foreseen in alternate access models of transport. The current study was designed to evaluate the explanatory power of these models and the molecular stent hypothesis.

EXPERIMENTAL APPROACH  Xenopus laevis oocytes and HEK293 cells expressing human (h)SERT were voltage clamped and exposed to serotonin (5-HT), p-chloroamphetamine (pCA) or methylenedioxyamphetamine (MDMA).

KEY RESULTS  In contrast to currents induced by 5-HT, pCA-triggered currents through SERT decayed slowly (i.e., with a half-life of » 20 s at 3 micromolar) in Xenopus laevis oocytes once the agonist was removed (consistent with the molecular stent hypothesis). However, when SERT was expressed in HEK293 cells, currents induced by 3 micromolar or 100 micromolar pCA decayed 10 or 100 times faster, respectively, after pCA removal.

CONCLUSIONS AND IMPLICATIONS  This discrepancy in decay rates is inconsistent with the molecular stent hypothesis. In contrast, a multi-state version of the alternate access model accounts for all the observations and reproduces the kinetic parameters extracted from the electrophysiological recordings. A crucial feature that explains the action of amphetamines is their lipophilic nature, which allows for rapid diffusion through the membrane.

PP-69  Exploring candidate proteins involved in methylmercury (MeHg) transfer at the human materno-fetal barrier: expression of the organic anion transporter (OAT4/SLC22A11)

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Background: The organic mercury compound MeHg is a prevalent environmental toxicant, which is formed in aquatic systems and biomagnified along aquatic food chains. Consequently, humans are exposed to MeHg by consumption of contaminated fish and seafood. Ingested MeHg actively traverses the placenta, accumulates in fetal tissues and behaves as a neurodevelopmental toxicant. As deduced from other organs and species, multiple proteins may be involved in the complex MeHg toxicokinetics at the human placental barrier, which is composed of two cellular layers, the epithelial syncytiotrophoblast (STB) and fetal endothelial cells (FEC). However, still very little is known on the human placental mechanisms involved in mercury toxicokinetics. Aim: Among the candidate proteins for cellular uptake of MeHg are members of the organic anion transporter (OAT) family. OAT1 has been demonstrated to be involved in renal uptake of mercury species. In the human placenta, only OAT4 is expressed exhibiting basolateral localization in STB. To clarify the role of OAT4 in placental MeHg mercury toxicokinetics, we established conditions to detect OAT4 in human placenta in situ and analyzed various cell lines for their endogenous expression of OAT4. Methods: Tissue collected from term healthy human placentas was either immediately frozen or HOPE-fixed and paraffin-embedded. The expression of OAT4 protein was analyzed using a polyclonal rabbit-anti-human OAT4 antibody (HPA026076) from Sigma. For western blotting experiments, total protein lysates as well as crude membrane fractions were prepared. Localization of protein was studied by immunofluorescence microscopy (IFM) on 2-4 µm tissue sections. Cell lines investigated were CaCo2, Q1, n-tera and HeLa cells. Results: Among all tested cell lines, only Caco2 cells exhibited significant levels of OAT4 protein expression in western blotting experiments. In accordace with literature, OAT4 expression was demonstrated in enriched membrane fractions of human placenta and was localized to the basal membrane of placental STB. Conclusion: We demonstrated that OAT4, which is a candidate protein of placental MeHg toxicokinetics, is expressed in CaCo2 cells as well as at the basal membrane of human placental STB in situ. CaCo2 cells as well as isolated placental trophoblasts will serve as in vitro models to study the role of OAT4 in MeHg uptake following siRNA transfection. Supported by LifeScience2010, NFB
PP-70  Calmodulin kinase II regulates amphetamine-induced reverse transport in the serotonin transporter

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The transporters for dopamine (DAT), serotonin (SERT) and norepinephrine (NET) mediate the reuptake of the respective monoamines previously released into the synaptic cleft. They hence regulate the neurotransmitter content available for synaptic transmission. Certain stimuli, such as changes in ionic composition of the extracellular fluid or psychostimulants (e.g. amphetamines) are able to induce outward transport of substrates and thus increase extracellular monoamine concentrations. Influx and efflux of substrate via DAT, SERT and NET are thought to be asymmetrical processes regulated by intracellular kinases. It has been demonstrated that removal of N-terminal serines ablates amphetamine-induced reverse transport in the DAT. Similarly, truncation of the SERT-N-terminus ablated amphetamine-triggered efflux. Besides, the Ca2+/Calmodulin dependent protein kinase II (aCaMKII) has been demonstrated to bind to the DAT C-terminus and to phosphorylate N-terminal serines. Pharmacological inhibition or genetic deletion of aCaMKII dramatically reduces amphetamine-induced efflux in cells and ex vivo brain preparations. Herein, we wanted to test whether the closely related serotonin transporter (SERT) is also regulated by aCaMKII. Co-immunoprecipitation experiments show that SERT and aCaMKII are found within the same protein complex in mouse brain homogenates. Additionally, in vitro binding of GST-purified SERT N- and C-termini demonstrates that aCaMKII can bind the SERT-C but not the SERT-N terminus, and that the interaction of aCaMKII is weaker for SERT compared to DAT. Mass spectrometry revealed phosphorylation of C-terminal residues of SERT by aCaMKII in vitro. Using superfusion studies, we show that aCaMKII regulates amphetamine-induced SERT-mediated efflux in cells transfected with SERT and aCaMKII. Additionally, mice lacking aCaMKII display a significantly reduced amphetamine-induced substrate efflux, but have normal serotonin uptake and SERT protein levels. Interestingly, in vivo administration of the SERT-preferring amphetamine-derivative 3,4-methylenedioxymethamphetamine (MDMA, ‘ecstasy’) induces less locomotor-activation in mice lacking aCaMKII suggesting that CaMKII-regulation of amphetamine-triggered SERT-mediated reverse transport is contingent on aCaMKII also in vivo.

PP-71  Determination of the oligomeric state of the human serotonin transporter using single molecule imaging and brightness analysis

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Using state of the art single molecule fluorescence microscopy, we have evaluated the oligomerization state of the human serotonin transporter (hSERT) in this study. The formation of dimers of hSERT in the plasma membrane has already been indicated by various approaches, including optical methods such as Förster resonance energy transfer (FRET) and classical biochemical ensemble analysis. However, neither application is suitable to yield quantitative interpretation and decipher the exact configuration of the oligomerization states; additionally, biochemical methods do not take the influence of the membrane environment into account. In contrast, the techniques applied in this study allow for identification and quantitative evaluation of subpopulations of hSERT complexes exhibiting different degrees of oligomerization in a living cell. A human embryonic kidney (HEK) cell line was transfected with an inducible expression vector carrying hSERT. The cocaine derivate JHC 1-64, conjugated to the fluorescent dye rhodamine red, was used as a high affinity probe to specifically label single hSERT molecules. The use of an inducible expression vector and short expression time ensured low density of the transporter on the membrane, thereby enabling imaging of single diffraction limited fluorescent spots. The statistical distribution of fluorescently labeled hSERT per diffraction limited spot was determined by single molecule brightness analysis. We found a variety of oligomerization states of membrane associated transporters, revealing molecular associations larger than dimers and demonstrating the coexistence of different degrees of oligomerization in a single cell. To determine a possible effect of the labeling using JHC 1-64 the results were confirmed using a fusion construct of mGFP and hSERT. Both labeling methods showed the same distribution of oligomeric states in the cell. Further on, we developed a special bleaching protocol to decipher the interaction kinetics of hSERT oligomers. The complexes were found to be stable over at least 10 minutes in the live cell plasma membrane.
PP-72  The role of heat shock proteins in ER export mechanisms of the human serotonin transporter
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ER export of solute carrier-6 (SLC6) family members relies on the recruitment of the COPII (coatamer protein II) coat to the transporter. The COPII component SEC24 binds to an ER export motif (RL/RI/KL) located on the C-terminus of the cargo proteins. The serotonin transporter (SERT) is exported from the ER compartments by recruiting the SEC24 isoform C to its C-terminus [1]. The same region also provides a docking site for various proteinaceous chaperones (heat shock protein (HSP)-isoforms), that assist in protein folding [3]. Based on these observations, we postulate sequential exchange of the chaperone(s) for the COPII coat, as a mechanism to prevent premature ER export of partially folded SERTs. In order to examine the exact functional role/s of HSPs in regulating the folding of SERT proteins, and the formation of COPII vesicles, we treated HEK293 cells expressing wild type SERT or several C-terminal mutants (including the RI-607,608-AA mutant, which is located at the putative ER export motif site of SERT) with different HSP inhibitors (e.g. 17-DMGA and geldanamycin), as well as with an ERAD inhibitor (kifunensine) or chemical - (such as 4-PBA and DMSO) and pharmaco-chaperones (e.g. noribogaine). We determined the effects of these compounds on transporter surface expression levels by: (i) measuring specific [3H]5-HT uptake, (ii) immunoblotting and (iii) confocal laser scanning microscopy. We subsequently replicated the same series of experiments in JAR cells, a human choriocarcinoma cell line which endogenously expresses the SERT. Further experiments are underway, optimising the conditions for mass spectrometry studies. These data will collectively help us elucidate the specific HSP isoforms associated with and regulating the ER export of SERT, as well as other members of the SLC6 transporter family.

This work was supported by SFB35.

References:

PP-73  New scaffolds for an old target – pharmacophore screening for novel GAT-1 inhibitors
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Designing inhibitors for the gamma-aminobutyric acid transporter (GAT) system is limited by the comparably small chemical space tolerated by the target proteins.

In order to searching for new chemical scaffolds of GAT-1 inhibitors, crucial binding features of known inhibitors were abstracted to pharmacophore models. Feature selection and deduction of the most probable steric arrangement was derived from molecular docking results. Subsequent virtual screening of a large open-source database yielded a small, chemically diverse set of substances as first guesses in the search for novel bioactive compounds.

Although no highly active molecule could be identified in pharmacological tests, new insights could be gained about allowed modifications of the known inhibitor scaffold and thus also regarding the micro-topology of the binding event. In addition, further investigation of dismissed screening hits pinpointed necessary improvements in the workflow, like the enhanced assignment of protonation states in the screening databases, alternative approaches for the consideration of steric restrictions and exhaustive sampling of highly flexible compounds. This resulted in a new generation of promising screening hits, for which pharmacological testing now is in progress.

We acknowledge financial support provided by the Austrian Science Fund, grants F03502 and W1232.
PP-74 Computational studies of ion and substrate recognition in alternate conformations of BetP

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The osmotic-stress regulated BetP is a betaine and sodium symporter from the (BCCT) family of betaine/choline/carnitine transporters that shares the same fold as LeuT. Recently, atomic structures of BetP in different conformational states have been resolved, providing important mechanistic insights into the alternating access cycle of this transporter. However, a key state, namely substrate-bound outward-open state of BetP was lacking; we will show this unpublished structure obtained from a choline transporting BetP mutant (G153D). We present the result from molecular dynamics simulations of this structure, as well as of previously-reported structure, outward-open apo state, to explore the formation of the central betaine-binding site and of the two sodium-binding sites. These results provide insight into the key events required for substrate and co-substrate binding to BetP, and into the sequential order of these binding events, enhancing our understanding of the mechanism of the transition from the outward-facing state to the closed state during the alternating-access cycle of transport.

PP-75 Activity of brain regions related to working memory are modulated by a SNP in the serotonin 1A receptor gene

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Background: The serotonin (5-HT) 1A receptor acts as a presynaptic somatodendritic autoreceptor in raphe cells and as a postsynaptic heteroreceptor on interneurons and pyramidal cells in cortico-limbic areas. The autoreceptor regulates serotoninergic projections from the dorsal raphé nuclei (DRN) towards cortico-limbic areas via negative feedback inhibition. The (-1019)G allele of the functional single nucleotide polymorphism rs6295 (-C[1090]G) in the promoter region of the 5-HT1A receptor gene HTR1A derepresses autoreceptor expression in the DRN. This leads to an increased negative feedback inhibition and decreased serotoninergic transmission to limbic and cortical regions. Previous studies associated the G allele with major depressive disorder (MDD) and completed suicide. Furthermore, functional magnetic resonance imaging (fMRI) studies showed that HTR1A modulates cortico-limbic areas in panic disorder, MDD patients and healthy subjects. But so far, no study to our knowledge examined the impact of rs6295 on working memory-related brain activity during performance of the working memory paradigm n-back. Thus, we initiated a cross-sectional fMRI study with the goal to investigate the influence of rs6295 on activation of working memory networks in healthy subjects.

Materials and Methods: 118 healthy subjects aged between 18 and 45 years without previous or current psychiatric or somatic illnesses were recruited and underwent fMRI scanning while performing the classical digit variant of the n-back task with two complexity conditions (0-back, 2-back). After genotyping, G allele carriers were compared to C/C genotype subjects.

Results: G allele carriers showed decreased activation of working memory-related brain regions with peaks in the DLPFC and posterior parietal regions compared to C/C genotype subjects. Results were independent of gender, age and intelligence.

Discussion: A decreased serotoninergic innervation of working memory-related brain regions as a consequence of increased negative feedback via 5-HT1A autoreceptors probably leads to the observed decreased working memory-related activation in G allele carriers. Therefore, rs6295 might impact serotoninergic tone in cortical areas. Investigation of this polymorphism could elucidate mechanisms of increased MDD risk in G allele carriers.
PP-76  A 2D-QSAR model for prediction of high-affinity SERT substrates

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Selective tool compounds are valuable for understanding the molecular mechanisms of transmembrane transporters. In this study, we employed a Hansch analysis for predicting such compounds by focusing on two different classes of SERT substrates: the cathinones and the phenylpiperazines (PPs). Hammett, lipophilicity, molar refractivity and Taft size descriptors were defined for substitutions on the aromatic ring and on the cationic nitrogen. Via a backward selection method, descriptors contributing most to affinity were defined, while inhibitory values obtained from in-house pIC50 data, as well as pK data from ref. [1] were used as dependent variables in a PLS regression.

The obtained QSAR models were of very acceptable quality (cathinones: R² = 0.78, Q² = 0.62; PPs: R² = 0.72, Q² = 0.63) and led to the observation that e.g. a lipophilic para-substitution had a beneficial effect on cathinone affinity, whereas an electronegative group on the same position would increase PP affinity.

Novel combinations of compounds with the same scaffold were generated, whereby 954 cathinones and 37 PPs were predicted as being more active than the most active compound of the training set. Binding modes were generated by a virtual screening docking into a validated hSERT homology model [2] while employing flexible side chains. Predicted IC50 values of down to 0.5 nM were found for the cathinones, while the cathinone with the lowest RMSD (1.99 Å) to a validated mephedrone binding mode had a predicted IC50 of 4.8 nM.

Unfortunately none of the compounds predicted as high-affinity substrate could be purchased, so further steps in this study would be the synthesis of selected hits.

We acknowledge financial support provided by the Austrian Science Fund, grants F03502 and W1232.

[1] Severinsen et al., 2012

PP-77  Influence of the lipid environment on the activity of the human ABCG2 multidrug transporter purified and reconstituted in liposomes

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Multidrug transporters of ABC membrane transporter family are involved in the chemical defense mechanism of cells by pumping out several different, mostly hydrophobic, but otherwise chemically unrelated toxic compounds. These transporters have important roles in developing multidrug resistance, which is a major problem in the therapy of several diseases, including tumors or chronic infections. ABCG2 is one of the three important multidrug transporters localized in several therapeutically important tissues, e.g. the intestine, the blood-brain barrier, tumor cells, stem cells, hepatocytes and kidney cells. We have studied the substrate interactions, transport cycle and structure-activity relationships of the ABCG2 protein. Earlier we established in vitro experimental systems by producing the human ABCG2 protein in insect cell cultures. Isolated membrane fractions of these cells were suitable for measuring ABCG2 ATPase and drug transport activity. Recently we established a new in vitro system by using a purified, reconstituted ABCG2 protein. Polyhistidine tagged ABCG2 was solubilized from insect cell membranes and isolated by affinity purification. We found that the use of mild detergents and excess lipids was the key factor in retaining the activity and the homodimer structure of ABCG2 throughout the process. The transporter had no measurable activity in a detergent, but regained activity after reconstitution in a proper lipid environment. We found that optimum transporter activity was obtained in natural lipid extracts, obtained from either E. coli or mammalian brain. Moreover cholesterol was an essential component for obtaining maximum ABCG2 activity. In insect cell membranes ABCG2 shows a significant drug-independent, so called “basal” ATPase activity that can be increased by substrate drugs. Experiments by using the reconstituted ABCG2 clarified that this basal activity is not caused by an unknown drug-substrate present in the insect membrane, but it is an intrinsic feature of the ABCG2 protein. We found that this basal ATPase activity was reduced by different sterol-derivatives, e.g. bile acids, while substrate-stimulated ATPase activity was unaltered. All these findings show that the ABCG2 multidrug transporter activity is fine-tuned by the lipid environment. Our new in vitro system establishes new functional assays for assessing the lipid effects. The ABCG2 protein reconstituted in liposomes can be directly used in selective vesicular transport assays. The generation of the functional, isolated and purified ABCG2 protein is an important step for allowing structural studies.
Electronic notebooks - A call for action

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It is becoming more challenging for leaders of biomedical research laboratories to stay in control of the research activity. This is due to increased complexity and increased speed. For instance, a mouse is no longer just a mouse. To handle genetically modified animals, it is not sufficient to record species, strain, gender, age and treatment. A number of other parameters must also be recorded such as genotype, genetic background and generation number. Similarly, most laboratories use a large number of different reagents and often receive or export samples to or from other laboratories. Computerized equipment makes it easy to generate large amounts of data. These data have to be stored somewhere. That is usually easy. The challenge is to keep track of what belongs to what. Immunocytochemistry can be used as an illustration: Tissue samples come from somewhere and have to be properly documented (species, strain, age, gender, organ, genotype, treatment/disease etc), mode of collection and preparation (surgery, fixation etc). Then the tissue may be prepared for cutting and labeled with antibodies and other reagents. These also have to be documented. But to do this, the researcher has to (a) find the correct material and (b) retrieve the documentation. This is not always easy. Freezers tend to fill up. Then images are collected (microscope settings, magnification, motive, etc) and analyzed. Who did what when with which material using which procedure? One thing is if one PhD student do all required work alone and manages to complete the work before the employment contract expires. A very different situation occurs when several people are involved and those who started cannot finish the job. On top of it all, the group leader must ensure that the various people in the lab use appropriate procedures that permit high quality data to be obtained without posing risks to humans or the environment. And, the group leader often has to teach, give seminars and also write grant applications as well as to keep track of funding. No wonder why we are busy. A good database system can make life easier. But databases like predefined categories and constant data relationships. And this is the clue: both the categories and the data relationships are evolving. Here we show how to get around the problem on a relatively low budget. A key point is that we, the scientists, take the initiative and do not wait for others to define the way they think we should work.
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Veranstaltung SFB35 Symposium 2013

TOTAL: 0,00 EUR

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