SFB 35

# SFB-Symposium 10

## TRANSMEMBRANE TRANSPORTERS IN HEALTH AND DISEASE

SEPTEMBER 3. – 4. 2010

GREAT LECTURE HALL

INSTITUTE OF PHARMACOLOGY

## SCIENTIFIC SESSIONS AND POSTERSESSION

**Elizabeth DeLange,** University of Leiden,The Netherlands
**Gerhard Ecker,** University of Vienna, Austria
**Michael Freissmuth,** Medical University of Vienna, Austria
**Peter Hinterdorfer,** Johannes Kepler - University Linz, Austria
**Karam Caline S.,** Columbia University, New York, USA
**Hermann Koepsell,** University of Würzburg, Germany
**Adrian Lammertsma,** University Medical Centre, Amsterdam, The Netherlands
**Oliver Langer,** Medical University of Vienna, Austria
**Peter Larsson,** University of Miami, USA
**Maarten E. Reith,** University of New York, USA
**Gary Rudnick,** Yale University, USA
**Kazumitsu Ueda,** Kyoto University, Japan
**Harel Weinstein,** College of Cornell University, New York, USA

The number of participants is limited by 120.
We will award student travel grants!

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FWF Der Wissenschaftsfonds. | IMP Research Institute of Molecular Pathology | Vienna Kultur | universität wien | MFPL Max F. Perutz Laboratories | Stadt Wien | AstraZeneca | Alumni Club Medizinische Universität Wien | JKU Johannes Kepler University Linz | Medical University of Vienna

**The 3rd SFB35 Symposium 2010 gratefully acknowledges the support of the following sponsors:**





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# Programme - Overview

# *3rd SFB-Symposium '10, Vienna*

## Friday, 3rd of September

08:00 **Registration**

08:30 Welcome address

***Session 1:*** *(Chair: W. Donald Miller – University of Manitoba, Canada)*

08:45 Speaker 1: **Elizabeth DeLange** (University of Leiden, Netherlands)

*Impact of different functionalities of transporters at BBB and BSCFB on the predictive value of CSF for ECF pharmacokinetics: about P-glycoprotein*

09:25 Speaker 2: **Oliver Langer** (Medical University of Vienna, Austria)

*Imaging of ABC transporters with positron emission tomography*

10:05 Speaker 3: **Gerhard Ecker** (University of Vienna, Austria)

*Experimental data guided ligand docking – a versatile approach for elucidating the molecular basis of drug/transporter interactions*

10:45 Coffee/Tea break

11:15 ***Plenary lecture 1:*** (*Chair: Ulrik Gether – University of Copenhagen, Denmark)*

**Hermann Koepsell** (University of Würzburg, Germany)

*Attempts to elucidate the molecular mechanism of drug transport by polyspecific organic cation transporters*

12:15 **Reception at the Town hall of Vienna,**
**Welcome address from the Mayor of Vienna**

14:30 **Postersession** & Coffee/Tea

***Session 2:*** *(Chair: Arne Schousboe – University of Copenhagen, Denmark)*

16:00 Speaker 1: **Maarten E. Reith** (New York University School of Medicine, USA)

*Dopamine Transporter Structure and Function*

16:40 Speaker 2: **Gary Rudnick** (Yale University, USA)

*Mechanism and ion coupling in the neurotransmitter-sodium symporter family*

17:20 Speaker 3: **Michael Freissmuth** (Medical University of Vienna, Austria)

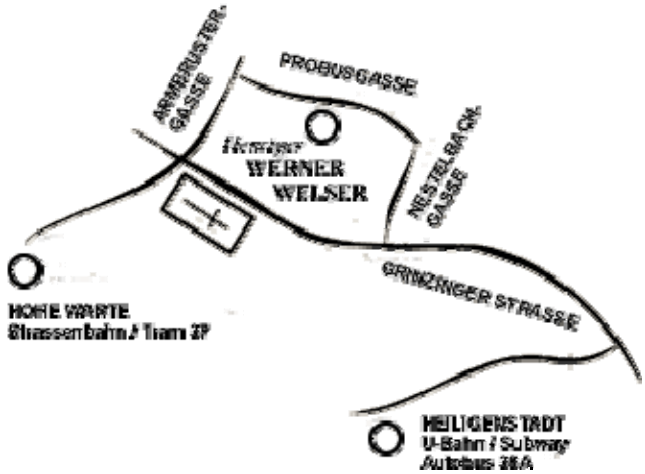
*More than loose ends: exploring the role of the N- and C-termini of the serotonin transporter in ER-export, folding and the action of amphetamines*

18:00 Speaker 4: **Caline S. Karam** (Columbia University, New York, USA)

*Delineating molecular mechanisms of amphetamine action in Drosophila melanogaster*

19:00 **Heurigen Restaurant "Ing. Werner Welser" - Probusgasse**

Probusgasse 12, A-1190 Wien,
Tel.: +43 (1) 318 97 97




***Public transport:***
*Take tram no. 37 in direction „Hohe Warte“ and get off at the last stop. From there walk down Hohe Warte (keeping the direction of the tram line), cross Grinzinger Straße, enter Armbrustergasse, first right is Probusgasse. Alternatively, you can take underground U4 (green line) until last Stop Heiligenstadt, then take bus no. 38A until Stop Armbrustergasse.*

## Saturday, 4th of September

08:30 **Registration**

09:00 ***Plenary lecture 2:*** (*Chair: Markus Müller – Medical University of Vienna, Austria)*

**Adrian Lammertsma** (University Medical Centre, Amsterdam, Netherlands)

*PET as an in vivo tool for measuring P-gp expression and function*

10:00 Coffee/Tea break

10:30 ***Plenary lecture 3:*** (*Chair: Aurelio Galli – Vanderbilt Kennedy Center, USA)*

**Harel Weinstein** (Cornell University, New York, USA)

*NSS transporter mechanisms in atomic detail: Allostery remodels the molecule and disturbs its environment*

11:30 **Josephinum: Guided Tour**

13:00 **Lunch** (*at the Institute of Pharmacology)*

14:00 **Postersession** & Coffee/Tea

***Session 3*** (*Chair: Karl Kuchler – Max F. Perutz Laboratory, Vienna, Austria* )

15:30 Speaker 1: **Peter Larsson** (University of Miami, USA)

*Mechanism of sodium-coupled amino acid uptake in glutamate transporters.*

16:10 Speaker 2: **Kazumitsu Ueda** (Kyoto University, Japan)

*Mechanism and regulation of HDL formation by ABCA1*

16:50 Speaker 3: **Peter Hinterdorfer** (Johannes Kepler University Linz, Austria)

*Single molecule force spectroscopy and recognition imaging*

17:30 **End of meeting**

# Posters (SHORT):

**P 1 ) Molecular determinants for selective recognition of antidepressants in the human serotonin and norepinephrine transporters**
*Jacob Andersen, Mette Thomsen, Lena Sørensen, Benny Bang-Andersen, Kristian Strømgaard, Anders S. Kristensen*

**P 2 ) Lithium modulates sodium-binding kinetics of phosphate cotransporters**
*Olga Andrini, Chiara Ghezzi, Anne-Kristine Meinild, Heini Murer and I. C. Forser*

**P 3 ) Transporter-mediated interaction of simvastatin and doxorubicin translates into topoisomerase II inhibition in human rhabdomyosarcoma cells.**
*Bihter Atil, Martin Werner, Evelyn Sieczkowski, Peter Chiba*, Martin Hohenegger*

**P 4 ) Characterization of FLIPW-CTYT FRET biosensor for measurement of real time tryptophan influx in live cells**
*Alok K. Behera, Monika Kretz, Benjamin Oberfeld, Simone M.R. Camargo, and François Verrey*

**P 5) LOCALIZATION OF THE ORPHAN CARRIER SLC10A4 IN THE PERIPHERAL NERVOUS SYSTEM AND ITS CO-EXPRESSION WITH VMAT-2 and VAChT**
*S. Burger, M. Moncada, S. Schmidt, R. Gerstberger, J. Geyer*

**P 6) Neurotransmitter Release through the Neurotransmitter Transporter GAT1**
*CHERUBINO F[1,2] , BERTRAM S[2] , BOSSI E[2] and PERES A[2]*

**P 7) ARG282 AND ASP341 ARE INVOLVED IN CHARGE MOVEMENT AND TRANSPORT FUNCTION IN THE OLIGOPEPTIDE TRANSPORTER PepT1**
*Francesca D'Antoni, Maria Daniela Renna, Rachele Sangaletti, Elena Bossi*

**P 8) Inverted re-entrant loops of the electrogenic $Na^+$-coupled inorganic phosphate cotransporter, NaPi-IIb interact during the transport cycle.**
*Chiara Ghezzi, Heini Murer and Ian C. Forster*

**P 9) Increased cortico-limbic coupling in remitted depressed patients**
*Hartinger B[1], Scharinger C[1], Diers, K[2], Kasess C[1,2], Huf W[1], Pail G[1], Brocke B[4], Kasper S[1], Moser E[2,3], Pezawas L[1].*

**P 10) Spatial Segregation of Emotion-Cognition-Interaction in Two Independent Samples Using Volume-Based fMRI Analysis**
*Huf W (a), Pail G (a), Chen G (d), Hartinger B (a), Kasess Ch (b), Kasper S (a), Mattay VS (e), Diers K (f), Brocke B (f), Meyer-Lindenberg A (ce), Moser E (b), Weinberger DR (c), Pezawas L (ac)*

**P 11) QSAR, Ligand efficiency (LE) and lipophilic efficiency (LipE) studies of a series of benzophenone-type inhibitors of the multidrug transporter P-glycoprotein**
*I. Jabeen[a], Z. Parveen[b], U. Rinner[c], P.Chiba[b], G. F. Ecker[a]*

**P 12) Serotonin Uptake in Blood Platelets is Related to Cingulate Cortex Activation**
*Kasess CH[1,2], Scharinger C[1], Rabl U[3], Hofmaier T[3], Hartinger B[1], Pail G[1], Huf W[1], Esterbauer H[4], Freissmuth M[4], Kasper S[1], Moser E[2,5], Sitte HH[4], Pezawas L[1]*

**P 13) Molecular structure-function analysis of Pdr12 and Snq2, two ABC transporters from *S. cerevisiae***
*Cornelia Klein, Martin Valachovic and Karl Kuchler*

**P 14) Substrate Interaction to SGLT1 on Living Cells probed at Different Temperatures – A Cooperative Radioactive Transport and Single Molecule Recognition Study**
*Isabel Neundlinger[1], Helmut Kipp[2], Linda Wildling[1], Wei Huang[3], Rolf K.H. Kinne[4], Hermann Gruber[1],Hermann Koepsell[2], Peter Hinterdorfer[1]*

**P 15) ELECTROPHYSIOLOGICAL CHARACTERISATION OF THE MAMMALIAN OLIGOPEPTIDE TRANSPORTER, rPEPT 1**
*Ayodele Oyadeyi, Maria Daniela Renna, Antonio Peres*

**P 16) Interaction of low molecular weight ligands with human P-glycoprotein**
*Zahida Parveen, Thomas Stockner, Edina Csaszar, Gerhard F. Ecker, Michael Freissmuth and Peter Chiba*

**P 17) Considerations Concerning Homology Modeling of ABC Transporters and Neurotransmitter transporters**
*Ravna AW, Gabrielsen M, Kristiansen K, Sylte I, Sager G, Dahl S*

**P 18) A UNIQUE KINETIC SCHEME CAN REPRODUCE FUNCTIONAL DIFFERENCES EXHIBITED BY THE OLIGOPEPTIDE TRANSPORTER PepT1 FROM DIFFERENT SPECIES**
*Maria Daniela Renna, Rachele Sangaletti, Antonio Peres*

**P 19) TRICYCLIC ANTIDEPRESSANTS COMPETITIVELY INHIBIT THE SEROTONIN TRANSPORTER VIA AN INTERACTION INVOLVING TWO DISCONTINUOUS BINDING SITES**
*Subhodeep Sarker[1], René Weissensteiner[2], Ilka Steiner[1], Harald H. Sitte[1], Gerhard F. Ecker[2], Michael Freissmuth[1]* and Sonja Sucic[1]*

**P 20) Occluded Leu transporter conformation-based homology modelling of GABA-Na+ symporter subtypes: druggability and function**
*Ágnes Simon[1], Ákos Bencsura[2], Anna Palló[1], Julianna Kardos[1]*

**P 21) Dopaminergic deficiencies and excesses in the neuronal Rictor deficient mouse: is Akt a negative regulator of dopamine signaling?**
*Michael Siuta, Adeola Davis, Roxanne Vaughn, Kevin Niswender, Aurelio Galli*

**P 22) Screening of intracellular regions in the human Serotonin Transporter for phosphorylation sites**
*Lena Sørensen, Troels R. Eskildsen, Kristian Strømgaard, Anders S. Kristensen*

**P 23) Calmodulin kinase II regulates amphetamine induced reverse transport in the dopamine transporter**
*Thomas Steinkellner, Ype Elgersma, Michael Freissmuth, Oliver Kudlacek, Harald Sitte*

**P 24) Conformation of the ATP-releasing state of membrane insert Pglycoprotein**
*Thomas Stockner[1] and Peter Chiba[2]*

**P 25) CONCENTRATIVE ER EXPORT OF THE SEROTONIN TRANSPORTER REQUIRES AN INTERACTION WITH SEC24C**
*Sucic S.[1], El-Kasaby A.[1], Marin P.[2], and Freissmuth M.[1]*

**P 26) Identification of quinidine as a probe substrate for P-glycoprotein at the blood-brain barrier.**
*Sziráki[1], I., F. Erdő[1], K. Herédi-Szabó[1], D. Méhn[1], M. Jani[1], E. Beéry[1], I. Wilhelm[2], I. Krizbai[2], T. Abonyi[1], P. Molnár[1], A. Szekeres[3] and P. Krajcsi[1],*

**P 27) Structure-function analysis of putative sterol importers in yeast.**
*Martin Valachovic, Cornelia Klein, and Karl Kuchler*

**P 28) INVESTIGATION OF DETERGENTS AT GABA TRANSPORTERS**
*Georg Höfner, Klaus T. Wanner*

**P 29) Single molecule recognition force spectroscopy (SMRFS) on SERT expressing living cells**
*Linda Wildling[1], Andreas Ebner[1], Christian Rankl[2], Thomas Haselgrübler[1], Hermann Gruber[1], Hans Oberleithner[3], Harald Sitte[4] and Peter Hinterdorfer[1]*

# Plenary lectures:

## PL 1) Hermann Koepsell

### Attempts to elucidate the molecular mechanism of drug transport by polyspecific organic cation transporters

*Hermann Koepsell, Christopher Volk and Thomas Müller, and Valentin Gorboulev*

Polyspecific organic cation transporters (OCTs) of the *SLC22* family play important roles in the elimination and tissue distribution of drugs. Functional studies on three main OCT subtypes OCT1-3 revealed polyspecific facilitated diffusion systems that translocate organic cations in both directions across the plasma membrane. Data were obtained suggesting a stoichiometry of 1 between translocated organic cations and translocated positive charges at -100 mV. Trying to understand how OCTs recognize substrates and inhibitors with different chemical structures and how substrate translocation is achieved without allowing extensive passive permeation of small ions, we modeled tertiary structures of OCTs and tried to verify the modeled substrate binding regions and presumed substrate permeation pathways by functional measurements on mutants. Modeling was performed using approved tertiary structures of lactose permease (LacY) and glycerol-3-phosphate transporter (GlpT) from *Escherichia coli* which belong to the same superfamily as OCTs. Functional measurements of OCT1 mutants provided validation of the modeled substrate binding regions within the outward-facing and inward-facing conformations of OCT1. Evidence was obtained that the outward-facing and inward-facing binding regions contain overlapping interaction domains for organic cations. This allows two or more organic cations to bind simultaneously and to exhibit allosteric interactions. Employing the nontransported inhibitor corticosterone which competes with transported substrates to measure inhibitor-substrate interaction from extracellular or intracellular, we observed that five amino acids may be exposed to the extracellular or intracellular aqueous phase. These five amino acids line the outward-open as well as inward-open binding clefts of the OCT1 models. Our data support the following transport mechanism: At negative membrane potential extracellular organic cations bind to the outward-open conformation of the unloaded transporter. Organic cation binding promotes a conformational change to the inward-open conformation. During this transition the translocation pathway remains closed suggesting an occluded intermediate state. The substrate binding region within the inward-facing conformation may have a lower affinity for organic cation substrates compared to the outward-facing binding region favouring intracellular substrate release. The unloaded or substrate loaded transporter may re-orient to the outward-facing conformation promoting electrogenic or electroneutral translocation, respectively.

# PL 2) Adriaan A. Lammertsma

## PET as an *in vivo* tool for measuring P-gp expression and function

*Adriaan A. Lammertsma*
*Department of Nuclear Medicine & PET Research*
*VU University Medical Centre, Amsterdam*

Positron emission tomography (PET) is a tomographic imaging technique, which allows for accurate non-invasive *in vivo* measurements of regional tissue function in man. By using different tracers, a multitude of physiological, biochemical and pharmacological parameters can be measured. These include blood flow (perfusion), blood volume (vascularity), oxygen utilisation, glucose metabolism, pre- and post-synaptic receptor density and affinity, neurotransmitter release, enzyme activity, drug delivery and uptake, gene expression, etc. In fact, PET represents the most selective and sensitive (pico- to nano-molar range) method for measuring molecular pathways and interactions *in vivo*. Apart from its capacity to provide new information on human disease, PET is also important for the objective assessment of therapeutic efficacy and could play an important role in the development of new drugs.

Based on the unrivalled sensitivity mentioned above, PET is also the method of choice for measuring P-gp activity in the brain. In theory, both expression and functionality of P-gp can be measured by selecting appropriate radiolabelled inhibitors and substrates of P-gp, respectively. Although several PET tracers (based on known P-gp substrates) have been developed and characterized in laboratory animals, the best validated PET tracer is [$^{11}$C]verapamil, which has already been used in several studies in humans. Initially, a racemic mixture was used, but later enantiomerically pure *(R)*-[$^{11}$C]verapamil was developed, enabling more accurate quantification. Using this tracer, subtle age effects on P-gp function have been demonstrated. In case of overexpression of P-gp, the already low *(R)*-[$^{11}$C]verapamil signal will be reduced even further, and a radiolabelled inhibitor would be a more suitable tracer. To this end, [$^{11}$C]laniquidar, a third generation P-gp inhibitor, was synthesised and its suitability as a tracer of P-gp expression investigated.

In this contribution, first the principles of PET will be discussed with emphasis on quantification. Next, an overview will be given of the current status of PET studies using *(R)*-[$^{11}$C]verapamil and [$^{11}$C]laniquidar.

# PL 3) Harel Weinstein

## NSS transporter mechanisms in atomic detail: Allostery remodels the molecule and disturbs its environment

*Harel Weinstein – Dept. Physiology and Biophysics,*
*and Institute for Computational Biomedicine, Weill Cornell Medical College,*
*Cornell University, New York, NY, 10065*

The protein family of neurotransmitter:Na+ symporters (NSS) includes the transporters for biogenic amines (dopamine, serotonin, norepinephrine), amino acids (GABA, glycine, proline, taurine) and osmolytes (betaine, creatine). The neurotransmitter transporters are of great interest because they terminate neurotransmission by reuptake of their substrates against the concentration gradient from the synaptic cleft into the pre-synaptic neuron, in a Na+ and Cl- dependent process. They are therefore primary targets for antidepressants (e.g., SSRIs) as well as psychostimulants such as cocaine and amphetamine, and have been implicated in a variety of disorders. Recent crystallographic studies revealed that five transporter families without much sequence similarities among them have similar structure folds to LeuT, a bacterial Neurotransmitter:Sodium Symporter (NSS) and enabled the detailed atomistic study of the transport cycle mechanism.

The presentation will focus on the sodium-coupled transport modulated by substrate in a process that involves its simultaneous binding in two sites with distinct properties. The mechanistic insights were obtained at the detailed molecular level from a combination of computational simulation studies analyzed and interpreted in the context of ongoing collaborative investigations of LeuT dynamics with single-molecule fluorescence (smFRET) and EPR. The component approaches have produced excellent agreement on the dynamic details that appear as well to be generalizable across the transporter families and to transcend sequence and motif similarities. Previously unidentified effects of the dynamic rearrangements required for the function of the transporters, on the organization and local structure of the surrounding lipid membrane were revealed from mesoscale simulation approaches. The results yield information about putative driving forces for intermolecular interactions (protein-membrane and protein-protein) in the cell membrane that are triggered by the structural rearrangements in the proteins and relate to cell signaling.

## Relevant Literature


Kniazeff J, Shi L, Loland CJ, Javitch JA, Weinstein H, Gether U. - An intracellular interaction network regulates conformational transitions in the dopamine transporter. **J Biol Chem, 2008** 283 (25):17691-701

Shi, L, Quick, M, Zhao, Y, Weinstein, H and Javitch, JA - The mechanism of sodium-coupled symport by a homolog of neurotransmitter transporters: Two substrates are required" **Molecular CELL, 2008** 30:667–677.

Beuming, T, Bergmann, ML, Shi, L, Gracia, L, Raniszewska K, Newman, AH, Javitch, JA, Weinstein, H, Gether U and Loland, CJ - The binding sites for cocaine and dopamine in the dopamine transporter are overlapping **Nature Neurosci, 2008** 11(7):780-9.

Quick M, Lund Winther A-M, Shi L, Nissen P, Weinstein H, Javitch JA - Binding of an octylgluco-side detergent molecule in the second substrate (S2) site of LeuT establishes an inhibitor-bound conformation. **Proc Natl Acad Sci 2009** - 106:5563–5568.

Khelashvili, G, Harried, D, Weinstein, H – Modeling membrane deformations and lipid demixing upon protein-membrane interaction: The BAR dimer adsorption. **Biophys J. 2009** Sep 16;97:1626-35.

Zhao Y, Quick M, Shi L, Mehler EL, Weinstein H, Javitch JA - Substrate-dependent proton antiport in neurotransmitter:sodium symporters. **Nature Chem Bio 2010** 6:109-116.

Zhao Y, Terry D, Shi L, Weinstein H, Blanchard, SC, Javitch JA – Single molecule studies of the allosteric modulation of intracellular gating in a neurotransmitter transporter homolog. **Nature 2010** May 13;465(7295):188-93.

Claxton DP, Matthias Quick M, Shi L, Delmondes de Carvalho F, Weinstein H, Javitch JA, Mchaourab HS – Ion/substrate-dependent conformational dynamics of a bacterial homolog of neurotransmitter:sodium symporters. **Nature Struct Mol Biol. 2010** Jul;17(7):822-9.

# Sessions:

## Session 1

### ***Speaker 1: Elizabeth DeLange***

### Impact of different functionalities of transporters at BBB and BSCFB on the predictive value of CSF for ECF pharmacokinetics: about P-glycoprotein

*Elizabeth CM de Lange*

*Head Target Site Equilibration Group, Department of Pharmacology, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands*
*Email: l.lange@lacdr.leidenuniv.nl*

In the clinical setting, the possibility of direct measurement of free drug concentrations in brain extracellular fluid (ECF), which is often closely linked to the brain target site, is highly restricted. As an alternative, free drug concentrations in lumbar or ventricular cerebrospinal fluid (CSF) are sometimes used. However, a generally applicable relationship between CSF and brain ECF does not exist.[1] The purpose of this study was to mechanistically investigate the passive and active transport processes that govern the relationship between brain ECF and CSF pharmacokinetics (PK)[2]. Especially the different functionalities of transporters at the blood-brain barriers: the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB) are of interest[3].

Using our recently developed technique of parallel implanted microdialysis probes in rats we investigated the blood to brain distribution in the lateral ventricle (LV), cistern magna (CM) and striatum (brain ECF) for two model compounds with different properties. Acetaminophen (i.v. infusion of 15 mg/kg in 10 min) was used as a model drug for passive blood-brain transport. Quinidine (i.v. infusion of 10 or 20 mg/kg in 10 min) was used as a model drug for active blood-brain transport by P-glycoprotein (P-gp). The specific impact of P-gp mediated transport was studied by pre-administration of the P-gp blocker tariquidar (i.v. infusion of 15 mg/kg in 10 min). Acetaminophen and quinidine concentrations in brain ECF, LV, CM and in plasma were determined by HPLC combined with electrochemical and fluorescence detection, respectively.

The acetaminophen concentration time profiles for the brain ECF, LV CSF, and CM CSF are similar. The corresponding brain to plasma ratios are 21.2%, 20.7%, and 21.1%, respectively. The quinidine concentration time profiles are different. The brain to plasma ratios for brain ECF, LV CSF and CM CSF without pre-administration of tariquidar are 3.8%, 7.2%, and 6.8%, respectively. With pre-administration of tariquidar, the ratios are 27.5%, 17.5%, and 17.4%, respectively.

This means that blocking active transport by P-gp results in a 7.3-fold increase in brain ECF exposure, whereas there is only a 2.4- to 2.6-fold increase in CSF exposure.

In the case of passive blood-brain transport, CSF PK is a good representative of brain ECF PK. However, in the case of active blood-brain transport, there is a difference between CSF PK and brain ECF PK. Therefore, the impact of active transport should not be underestimated.
Population-based PK analysis will be performed, using NONMEM®, to reveal the impact of active transport processes on the brain ECF – CSF relationships.

***References***


1) *De Lange, ECM, Danhof, M. Considerations in the use of cerebrospinal fluid pharmacokinetics to predict brain target concentrations in the clinical setting: implications of the barriers between blood and brain. Clin. Pharmacokin. 41, 691-703, 2002*

2) *De Lange ECM, Ravenstijn PGM, Groenendaal D, and van Steeg TS. "Toward the Prediction of CNS Drug Effect Profiles in Physiological and Pathological Conditions Using Microdialysis and Mechanism-Based Pharmacokinetic-Pharmacodynamic Modeling". AAPS Journal, 7(3) article 54, 2005*

3) *De Lange ECM. Potential Role of ABC transporters as a Detoxification System at the Blood-Cerebrospinal Fluid-Barrier. Adv Drug Del Rev 56(12); 1793-1809, 2004*

## ***Speaker 2: Oliver Langer***

### Imaging of ABC transporters with positron emission tomography

*Oliver Langer*

*Department of Clinical Pharmacology, Medical University of Vienna, Austria*

Adenosine binding cassette (ABC) transporters, such as P-glycoprotein (P-gp, ABCB1), breast cancer resistance protein (BCRP, ABCG2) and multidrug resistance proteins (MRPs) are expressed in high concentrations at various physiological barriers (blood-brain barrier, blood-testis barrier, blood-tumour barrier), where they impede the tissue accumulation of various drugs by active efflux transport. Changes in ABC transporter expression and function are thought to be implicated in various diseases, such as cancer, epilepsy, Alzheimer's and Parkinson's disease. The availability of a non-invasive imaging method which allows for measuring ABC transporter function and/or expression *in vivo* would be of great clinical use in that it could allow for indentifying those patients that would benefit from treatment with ABC-transporter modulating drugs. To date three different kinds of imaging probes have been described to measure ABC transporters *in vivo*: i) radiolabelled transporter substrates ii) radiolabelled transporter inhibitors and iii) radiolabelled prodrugs which are enzymatically converted into transporter substrates in the organ of interest (e.g. brain). The present talk will provide an overview of our efforts to visualize ABC transporters at the blood-brain barrier of animals and humans with positron emission tomography (PET).

## ***Speaker 3: Gerhard Ecker***

### Experimental data guided ligand docking – a versatile approach for elucidating the molecular basis of drug/transporter interactions

*Gerhard F. Ecker, Lars Richter, Freya Klepsch, Rene Weissensteiner, Zahida Parveen, Subhodeep Sarker, Sonja Sucic, Margot Ernst, Werner Sieghart, Peter Chiba, Harald Sitte, Michael Freissmuth*

With the almost exponential growth of X-ray structures deposited in the protein data bank, structure-based ligand design became a routine method in drug discovery programs. However, although most docking programs are able to retrieve the correct orientation of the ligand in the binding site, the correct pose is seldom ranked number one. This is mainly due to the challenging and unsolved problem of a correct prediction of binding free energy values. In case of transport proteins this is even more complicated, as they undergo major conformational changes during the transport cycle.

In our attempt to elucidate the molecular basis of drug/transporter interactions for ABCB1, SERT and $GABA_A$, we included experimental data derived from ligand-based drug design studies as well as from mutagenesis studies in order to guide the pose selection process. The main concept follows one of the basic paradigms in ligand-based drug design: structurally similar compounds show similar biological profiles and are supposed to exhibit similar binding modes. When docking individual compounds, hundreds of different potential orientations in the binding site are retrieved. However, when docking small compound databases with similar chemical scaffolds, clustering of all poses according to common scaffold RMSD values revealed only a few clusters which contain poses of all compounds docked. These candidate binding modes are further prioritized according to structure-activity data of the ligands (for ABCB1 inhibitors), site directed mutagenesis data (for SERT inhibitors) and performance in virtual screening runs (for $GABA_A$ ligands). In all three case studies the top ranked binding mode also conforms well to biochemical data.

Funded by the Austrian Science Fund (SFB35) and the Austrian Academy of Sciences (DOC stipendium to L.R.)

# Session 2

## ***Speaker 1: Maarten E. Reith***

### Dopamine Transporter Structure and Function

*Maarten E.A. Reith*
*Departments of Psychiatry and Pharmacology*
*New York University School of Medicine*
*New York, N.Y., USA*

The dopamine (DA) transporter (DAT), a target for psychostimulants such as amphetamine and cocaine, translocates DA along with $Na^+$ and $Cl^-$. Structural information relevant to DAT has come from the crystallization of the leucine transporter LeuT from Aquifex aeolicus. Molecular dynamics and computational modeling of LeuT revealed a permeation pathway for substrate with multiple interaction points. Two major interaction points stand out: the primary substrate site (S1) in the protein interior, and a more extracellularly located secondary site (S2) in the same vestibule where antidepressant drugs bind in LeuT. Although there is wide agreement that S1 of DAT formed by residues that correspond to those for S1 in LeuT, a role for S2 in DAT is a matter of speculation.

We developed bivalent ligands in which two DA-like "heads" (DA, phenethylamine, amphetamine) were linked by a methylene chain of varying length. Appreciable gains were noted in their binding affinity for DAT. Although substrate-derived, the compounds were bulky enough to be transport inhibitors rather than substrates. The spacers linking the active heads were not long enough to bridge sites in protomers within a DAT oligomer. One set of active compounds centered around an optimal distance between the heads of ~ 13 Å, consonant with the distance of 11-13 Å between S1 and S2 in LeuT (or in homology-modeled DAT). As substrate moves along its path from outside to inside, the transporter changes conformation. Crystal structures of LeuT so far have captured only outward ("open-to-out") and intermediate ("occluded"), but not inward ("open-to-in") states. Bivalent ligands appear to prefer inward-facing DAT.

Inward states can also be stabilized by non-cocaine-like compounds such as benztropine and analogs, bupropion, GBR 12909, and rimcazole analogs. This may be a factor in anti-cocaine activity observed for some of these compounds. Our recent results additionally indicate that modafinil, a promising treatment compound for cocaine addiction, stabilizes an inward form of DAT, as judged from experiments with outward-facing DAT constructs W84L and D313N, and from work with $Zn^{2+}$. Homology docking of modafinil also showed an un-cocaine-like effect on the distance between Asp79 and Tyr156, which is known to be conformationally sensitive.

Finally, the conformational state of DAT can be affected by its localization in the membrane. DAT in cholesterol-rich membrane domains displays a more rapid return step of empty carrier towards the outward state in the translocation cycle, consonant with a recent report by others on cholesterol promoting outward-facing DAT.

It is not known whether such effects are related to integrity of lipid rafts that contain DAT, or to direct effects of cholesterol on DAT (conformation, oligomers?). Taken together, recent structure-function studies on DAT show that conformational states are important not only during substrate translocation, but also in interaction with inhibitors. Binding of an inhibitor can influence the equilibrium between DAT conformations, which may affect its phenomenological effect.

Supported by NIH grants DA019676 and DA013261 (Reith), and MH083840 (Wang)

## ***Speaker 2: Gary Rudnick***

### Mechanism and ion coupling in the neurotransmitter-sodium symporter family

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*Yale University School of Medicine*

In ion-coupled transport proteins, occupation of selective ion binding sites is required to trigger conformational changes that lead to substrate translocation. These conformational changes probably involve rocking back and forth of a 4-helix bundle. Neurotransmitter transporters require $Na^+$ and $Cl^-$ for function. We recently proposed a chloride binding site in these proteins not present in $Cl^-$-independent prokaryotic homologues. I will describe conversion of the $Cl^-$-independent prokaryotic tryptophan transporter TnaT to a fully functional $Cl^-$-dependent form by a single point mutation, D286S. Mutations in TnaT-D268S, in wild type TnaT and in serotonin transporter provide direct evidence for the involvement of each of the proposed residues in $Cl^-$ coordination. In both SERT and TnaT-D268S, $Cl^-$ and $Na^+$ mutually increased each other's potency, consistent with electrostatic interaction through adjacent binding sites. These studies establish the site where $Cl^-$ binds to trigger conformational change during neurotransmitter transport.

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## ***Speaker 3: Michael Freissmuth***

### More than loose ends: exploring the role of the N- and C-termini of the serotonin transporter in ER-export, folding and the action of amphetamines

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Conceptually, the intracellular N- and C-termini of SLC6-family members are dispensable for the core function of the transporter, i.e. the translocation of substrate. In fact, the bacterial orthologue LeuT*Aa* has a very short N- and C-terminal extension. The N-terminus of the serotonin transporter SERT and of the dopamine transporter DAT is dispensable for substrate translocation: truncation by up to 64 residues has only modest effects on substrate uptake. However, the truncation abolishes transport reversal induced by amphetamine. In contrast, truncations at the C-terminus of SERT are much less tolerated: truncation of the last 17 amino acids results in a transporter that is not properly folded and trapped in the endoplasmic reticulum. Site-directed mutagenesis identified sequence spots ($PG^{601, 602}$, $RII^{607\text{-}609}$) within the C-terminus that were relevant for export of SERT from the endoplasmic reticulum (ER). $RI^{607,608}$ is homologous to the RL-motif that in other SLC6 family members provides a docking site for the COPII component SEC24D. The primary defect resulting from mutation at $PG^{601,602}$ and $RI^{607,608}$ was impaired folding, because mutated transporters failed to bind the inhibitor [$^3$H]imipramine. In contrast, when retained in the ER (e.g., by dominant negative Sar1) the wild type transporter bound [$^3$H]imipramine with an affinity comparable to that of the surface expressed transporter. SERT-$RI^{607,608}$AA and SERT-$RII^{607\text{-}609}$AAA were partially rescued by treatment of cells with the nonspecific chemical chaperone DMSO or the specific pharmacochaperone ibogaine (which binds to the inward facing conformation of SERT) but not by other classes of ligands (inhibitors, substrates, amphetamines). These observations (i) demonstrate an hitherto unappreciated role of the C-terminus in the folding of SERT, (ii) indicates that the folding trajectory proceeds via an inward facing intermediate and (iii) suggest a model where the RI-motif plays a crucial role in preventing premature Sec24-recruitment and export of incorrectly folded transporters.

While the RI-motif is conserved in all SLC6-family members, the flanking sequence is of SERT diverges from that of its close relatives (DAT, norepinephrine transporter NET, GABA-transporter-1 GAT1): a highly conserved D at the position +9 from the RI/RL-motif is replaced by a proline in SERT. This aspartate was proposed to be part of the SEC24-binding site. Accordingly, we examined which SEC24-isoform was required for ER-export of SERT. In contrast to NET, DAT, and GAT1, which required SEC24D for ER-export, ER export of SERT relied on SEC24C. This was confirmed by identifying SEC24C in pull-downs from brain lysates.

## ***Speaker 4: Caline S. Karam***

**Delineating molecular mechanisms of amphetamine action in Drosophila melanogaster**

*Presenter: Caline S. Karam, PhD*

*Lab: Jonathan Javitch*

Abstract:

The dopamine transporter (DAT) plays a critical role in dopaminergic signaling by mediating the inactivation of released dopamine (DA) through reuptake. DAT is a primary molecular target of psychostimulants, such as amphetamine (AMPH) and methylphenidate (MPH). While both drugs elevate extracellular DA levels by inhibiting its uptake, AMPH also acts as a substrate of DAT, depletes DA from intravesicular stores, and elicits reverse transport (efflux) of cytoplasmic DA through DAT. Recent studies have shown that phosphorylation of the DAT N-terminus is required for AMPH-stimulated DA efflux, but many of the interacting proteins and molecular mechanisms that coordinate efflux and the ensuing behavioral effects remain elusive. The goal of this study is to develop a genetic model, using Drosophila larvae, to investigate the molecular determinants of DAT-mediated, psychostimulant-induced behavior. Our data show that larvae display acute hyperlocomotion in response to AMPH and MPH. These responses are absent in dDAT null mutants, and in larvae expressing dDAT RNAi in dopaminergic neurons, suggesting that dDAT is the primary target for AMPH and MPH in Drosophila. Using this assay we identify, for the first time, a role for the membrane raft protein Flotillin1 (Flot1) in AMPH-induced efflux and behavior. In contrast, Flot1 is not required for MPH-stimulated, efflux-independent behavior. Taken together, our results establish Drosophila as a powerful experimental system to investigate the mechanisms by which DAT regulates dopaminergic neurotransmission and to identify specific signaling pathways that contribute to the distinct molecular actions of these two classes of psychostimulants.

# Session 3

## ***Speaker 1: Peter Larsson***

### Mechanism of sodium-coupled amino acid uptake in glutamate transporters.

*H. Peter Larsson, Associate Professor*
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*University of Miami, Miller School of Medicine*

Excitatory Amino Acid Transporters (EAATs) remove glutamate from synapses thereby maintaining an efficient synaptic transmission and prevent glutamate from reaching neurotoxic levels. Glutamate transporters couple the uptake of one glutamate to the co-transport of three sodium ions and one proton and the counter-transport of one potassium ion. The molecular mechanism for this coupled uptake of glutamate and its co- and counter-transported ions is not known. In a crystal structure of the bacterial glutamate transporter homologue, GltPH, only two cations are bound to the transporter and there was no indication of the location of the third sodium site. In experiments using Voltage Clamp Fluorometry and simulations, we have located the third sodium binding site in EAAT3. Interestingly, the sodium bound at this third binding site forms part of the binding site for the amino acid substrate, which suggests explanations for both the strict coupling of sodium transport to uptake of glutamate and the ion selectivity of the affinity for the transported amino acid in EAATs. Using Electron Paramagnetic Resonance, we also show that the external gate opens upon binding of sodium, thereby allowing glutamate access to its binding site. Using our data, in combination with the recent crystal structure of the inward state of GltPh, we propose a model for the whole transport cycle in glutamate transporters.

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## ***Speaker 2: Kazumitsu Ueda***

### Mechanism and regulation of HDL formation by ABCA1

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ATP-binding cassette protein A1 (ABCA1) transfers cellular free cholesterol and phospholipids to an extracellular acceptor, apolipoprotein A-I (apoA-I), to generate high-density lipoprotein (HDL) and maintain cholesterol homeostasis. In addition, ABCA1 has been also proposed to be involved in reorganizing lipid microdomains in the plasma membrane. However, the mechanism by which ABCA1 moves lipids and mediates HDL formation remains unclear. To reveal the mechanism, the dynamics of ABCA1 was examined at the level of single molecules by using total internal reflection fluorescence (TIRF) microscopy. ABCA1 showed the unique dynamics on the plasma membrane. Mechanism of HDL formation will be discussed.

Cholesterol is essential for cell survival and proliferation, but at high levels it can cause cellular toxicity; therefore, the ABCA1-mediated secretion of cholesterol is highly regulated at the transcriptional level with a nuclear receptor, LXR. Here, we show that in addition to its well-defined role in transcription, LXRbeta directly binds the C-terminal region of ABCA1 to mediate its post-translational regulation. In the absence of cholesterol accumulation, the ABCA1-LXRbeta complex stably localizes to the plasma membrane, but is repressed in proper ATP binding, and thus inert in apoA-I binding and cholesterol secretion. When cholesterol accumulates, oxysterols bind to LXR□ and LXR□ dissociates from ABCA1 protein. Then, ABCA1 immediately restores the activity and cholesterol is secreted. This is the first study to show that a protein-protein interaction suppresses the function of ABCA1 by inhibiting ATP binding and, through its interaction with ABCA1, LXRbeta has at least two distinct roles in controlling cholesterol homeostasis.

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## ***Speaker 3: Peter Hinterdorfer***

### Single molecule force spectroscopy and recognition imaging

*Peter Hinterdorfer*

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In single molecule force spectroscopy, a ligand-containing tip is approached towards the receptors on the probe surface, which possibly leads to formation of a receptor-ligand bond. The tip is subsequently retracted until the bond breaks at a certain measurable force (unbinding force). The attachment of human rhino virus 2 (HRV2) to the cell surface, the first step in infection, was characterized at the single molecule level. Sequential binding of multiple receptors was evident from recordings of characteristic quantized force spectra. This suggests that multiple receptors bound to the virus in a timely manner. Unbinding forces required to detach the virus from the cell membrane increased within a time frame of several 100 ms. The number of receptors involved in virus binding was determined and estimates for on-rate, off-rate, and equilibrium binding constant of the interaction between HRV2 and plasma membrane anchored receptors were obtained. We then present a strategy to measure distances between the unique 5-methylcytidine bases in individual DNA strands with a resolution of 4 Å, thereby yielding the DNA methylation pattern, which is important for the epigenetic control of gene expression.

The new detection system is operated by an atomic force microscopy cantilever that carries a tethered antibody. The two Fab arms of the antibody are able to bind two 5-methylcytidine bases of a surface-immobilized DNA strand. Retracting the cantilever sequentially dissociates the biomolecular interactions, resulting in a unique rupture signature that reveals the spacing between the two tagged bases. Furthermore, we show a method for the localization of specific binding sites and epitopes with nm positional accuracy. A magnetically driven AFM tip containing a ligand covalently bound via a tether molecule is oscillated at a few nm amplitude, during scanning along the surface. In this way, topography and recognition images on membranes and cell surfaces were obtained simultaneously with nm resolution. Finally, we introduce a new high-speed Bio-AFM with which we are able to resolve that antibodies exhibit a bipedal walking on bacterial surfaces. The walking speed depends on the lateral spacing of antigens on a 2D-crystalline bacterial surface; thus steric strain appears to be the main reason for this short-lived bivalent binding.

# posters:

## P 1) Molecular determinants for selective recognition of antidepressants in the human serotonin and norepinephrine transporters

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The monoamine neurotransmitters serotonin and norepinephrine are involved in control of human mood and behaviour. The closely related serotonin transporter (SERT) and norepinephrine transporter (NET) belong to the family of Na+/Cl- coupled neurotransmitter transporters and mediate the re-uptake of synaptically released serotonin and norepinephrine in the brain. Competitive inhibitors of SERT and NET regulate the extracellular levels of serotonin and norepinephrine and are used as therapeutics for a wide range of affective disorders, including major depressive disorder (MDD) and attention-deficit hyperactivity disorder (ADHD). The majority of currently approved antidepressant drugs are SERT and/or NET inhibitors and are categorized as selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors (NRIs), or dual-acting serotonin-norepinephrine reuptake inhibitors (SNRIs); depending on their selectivity profile at SERT and NET.

Difference in SERT/NET activity among antidepressants is a potential contributing factor for differences in therapeutic efficacy in various disease types. Fine-tuning of the SERT/NET selectivity profile is thus an important factor to consider for development of novel antidepressant drugs. At present, the understanding of the molecular determinants in SERT and NET for the selectivity of most antidepressants is limited; including the structure of the SERT and NET drug binding pockets, potential drug orientations in these pockets and the specific residues that determine drug selectivity. The present study examines the role of a subset of amino acid residues within the antidepressant binding pocket in human SERT and NET for potency and selectivity of a range of clinically relevant antidepressant drugs. Previous mutational analyses of residues within the SERT substrate-binding pocket have identified several specific residues that are critical for antidepressant potency (i.e. mutation of these residues promote more than 10-fold loss of inhibitory potency)[1-3]. Here, we have focused on a subset of these mutations in SERT and introduced the equivalent mutations in NET, and determined the effect of these mutations on the inhibitory potency of 15 clinically used antidepressants; including prototypical SSRIs, NRIs, and SNRIs. The resulting mutational dataset provide a basis for construction of models for the structural basis for antidepressant selectivity at monoamine transporters, which potentially is useful in rational approaches for the development of future SERT and NET ligands with tailor-made selectivity profile.

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## P 2) Lithium modulates sodium-binding kinetics of phosphate cotransporters

*Olga Andrini, Chiara Ghezzi, Anne-Kristine Meinild, Heini Murer and I. C. Forser Institute of Physiology, Zürich Center for Integrative Human Physiology (ZIHP), University of Zürich.*

### Introduction:

Sodium-phosphate coupled cotransporters belong to the SLC34 gene family and are essential for phosphate (Pi) homeostasis in the body. They are responsible for Pi absorption in the gut and Pi reabsorption in the nephron at the proximal tubule. Both the electrogenic (NaPi-IIa,b) and electroneutral (NaPi-IIc) members of SLC34 family preferentially cotransport divalent Pi (H2PO4 2-) and Na+ with strict stoichiometries of 1:3 and 1:2, respectively. Transport of Pi appears to be solely dependent on Na+ ions, yet biophysical studies suggest that also Li+ ions can interact with the electrogenic and electoneutral tranporters in absence of Pi. The interaction has not been studied in detail therefore some questions are still open: what are the characteristics of the Li+ interaction; what do they tell us about the how cations drive the transport?

### Methods:

NaPi-IIb and NaPi-IIa, cloned from flounder and human respectively, were expressed in *X. laevis* oocytes and studied using radioactive tracer and electrophysiology under different superfusion conditions.

### Results:

Double uptake experiments performed using 32P and 22Na suggest that the stoichiometry of NaPi-IIb in presence of Li+ does not change from the established value of 3:1 (Na+:Pi). This demonstrates that Li+ is most likely not cotransported by NaPi-IIb. However, steady-state kinetics of voltage-clamped oocytes revealed that a 50% replacement of Na+ with Li+ (50 mM final concentration) reduced the maximum transport rate by _ 50% and surprisingly, increased the apparent affinity for Pi. To investigate further how the external cations could alter the kinetics of NaPi-IIb, we applied rapid changes in membrane voltage to induce presteady-state charge relaxations. These reflect the movements of cations to and from their binding sites within the protein and the movement of the empty transporter in the plasma membrane. In the absence of Pi, the release of Na+ was slowed significantly by Li+: this suggests that Li+ altered the equilibrium between conformation states associated with Na+ binding our experimental findings could be verified by simulations using a 10 state model of the electrogenic transport cycle.

### Conclusion:

Ion substitution experiments and detailed presteady-state analysis support the notion that Li+ modulates the 1st Na+ ion binding kinetics, but does not replace Na+ as a cotransported substrate.

## P 3) Transporter-mediated interaction of simvastatin and doxorubicin translates into topoisomerase II inhibition in human rhabdomyosarcoma cells.

*Bihter Atil, Martin Werner, Evelyn Sieczkowski, Peter Chiba*, Martin Hohenegger*
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Cancer treatment is often impaired by lack of responsiveness of the tumor. The development of chemoresistance involves a broad spectrum of molecular mechanisms. In particular, multidrug resistance due to up-regulation of ATP binding Cassette (ABC)-transporters is a common problem seen in repetitive applications of many chemotherapeutics. We could demonstrate in human rhabdomyosarcoma (RD) cells that the anti-tumor activity of HMG-CoA reductase inhibitors, such as simvastatin, enhanced the caspase 3- and 9-mediated intrinsic pathway of apoptosis when co-administrated with the anthracycline doxorubicin [Werner et al., 2004].

The aim of the here presented study was to identify the mechanisms behind this drug interaction. Doxorubicin is a fluorescent drug which is therefore easily traced within a cell. The simultaneous application of simvastatin led to an enhanced accumulation of doxorubicin in the nucleus. This effect was not HMG-CoA reductase dependent and is most like explained by a direct inhibition of ABCB1 (P-glycoprotein). Consequently, nuclear extracts from simvastatin and doxorubicin treated RD cells showed a significant inhibition of topoisomerase II activity compared the compounds alone. These data for the first time show that the co-administration of a statin with an anthracycline translates into a significant inhibition of topoisomerase II-mediated catenation activity. In the line of these findings, colony formation was synergistically inhibited by the co-application of simvastatin with doxorubicin.

Doxorubicin may also accumulate in the acidic compartment of lysosomes which may crucially be linked to cellular clearance from the anthracycline. However, size and intensity of doxorubicin containing lysosomes did not change upon co-administration with simvastatin. Finally, the direct inhibition of the ABCB1 transporter by simvastatin was seen at equimolar concentrations as with the first generation inhibitor verapamil, measuring daunorubicin and rhodamine 123 efflux. Beside this immediate effect, simvastatin leads also to a down-regulation of the ABCB1 transporter after exposure times longer than 24 hours.

Based on these findings, HMG-CoA reductase inhibitor simvastatin seems to be a promising candidate as an adjuvant chemotherapeutic drug to reduce transporter mediated multidrug resistance.

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## P 4) Characterization of FLIPW-CTYT FRET biosensor for measurement of real time tryptophan influx in live cells

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**Background and Aim:**

The functional cooperation between amino acid transporters plays an important role for cell homeostasis by controlling the efflux and exchange of amino acids. To characterize these functional interactions, a suitable method is required that allows the continuous measurement of local amino acid concentrations in live cells. The use of FRET amino acid biosensors could be an attractive solution. A tryptophan biosensor (FLIPW-CTYT) has been shown by others to allow the measurement of tryptophan steady-state levels and flux in real time in cultured mammalian cells.

**Results:**

We tested the FLIPW-CTYT biosensor *in vitro* and in live cells to establish techniques for reliable real time measurements. The signal to background ratio of this biosensor turned out to be not sufficient. Specifically, its signal is very sensitive to other parameters than tryptophan concentration such as for instance pH. For *in vitro* FRET measurements, MOPS buffer pH 7.0 in the absence of protease inhibitors was for instance shown to be the best condition. The FLIPW-CTYT biosensor efficiency and specificity was not influenced by the presence of a mixture of other amino acids. Interestingly, the presence of Na+-, K+- ions and the nature of their counter-ions influenced the fluorescence intensity of the acceptor of FLIPW-CTYT biosensor *in vitro*, an effect that was not observed *in vivo*. We further investigated the real time change in tryptophan uptake in the cos-7 cells expressing the facilitated diffusion transporter TAT1 (Slc16a10) by using the FLIPW-CTYT biosensor. We observed that the influx of tryptophan was much faster and increased when TAT1 was expressed. Aiming at having a biosensor with higher FRET efficiency and presumably higher FRET signal to background ratio, we constructed a series of FLIPW-CTY mutants and tested in COS-7 cells. Two of the new FLIPW-CTY variants showed in a first round of tests a much higher normalized FRET ratio in the presence of tryptophan in cos-7 cells (19 and 26%) when compared with the previously used FLIPW-CTYT biosensor (8%). Further characterization experiments and the construction of additional variant are ongoing.

**Conclusion:**

The FLIPW-CTYT biosensor is being ameliorated to become a tool that will allow us to follow the local concentration of tryptophan in living cells and thus to understand better the cooperation of amino acid transporters.

## P 5) LOCALIZATION OF THE ORPHAN CARRIER SLC10A4 IN THE PERIPHERAL NERVOUS SYSTEM AND ITS CO-EXPRESSION WITH VMAT-2 and VAChT

*S. Burger, M. Moncada, S. Schmidt, R. Gerstberger, J. Geyer*

The solute carrier family SLC10 comprises two well established sodium-dependent bile acid transporters, the $Na^+$/taurocholate cotransporting polypeptide (NTCP; SLC10A1) and the apical sodium-dependent bile acid transporter (ASBT; SLC10A2). These carriers maintain the enterohepatic circulation of bile acids in the liver (NTCP) and the intestine (ASBT) [1].

A third member of the SLC10 family was recently identified and was termed sodium-dependent organic anion transporter (SOAT; SLC10A6). SOAT does not transport bile acids but sulfoconjugated steroid hormones in a sodium-dependent manner [2].

In 2004, a further new member of this carrier family was cloned from rat adrenal gland and is referred to as SLC10A4. Although SLC10A4 shows the highest phylogenetic relationship to NTCP, it showed no transport activity for bile acids and sulfoconjugated steroid hormones when expressed in *Xenopus laevis* oocytes or HEK293 cells [3].

Gene expression analysis by real-time quantitative PCR revealed that SLC10A4 expression is highest in the brain. Immunofluorescence co-localization studies with antibodies directed against a C-terminal epitope of the rat SLC10A4 protein, against the cholinergic marker protein VAChT, and against the monoaminergic marker protein VMAT-2, revealed, that SLC10A4 is expressed in cholinergic and monoaminergic neurons and innervations of the rat central and peripheral nervous system as well as in the epithelium of the urinary bladder. Additionally, SLC10A4 is expressed in granules of rat peritoneal and tissue associated mast cells, what has been verified by immunofluorescence and electron microscopic examinations. Western blot and immunoprecipitation experiments revealed that SLC10A4 is expressed in synaptic vesicles of the rat brain. This vesicular expression pattern can also be seen in rat PC12 cells, human SH-SY5Y cells and in stably transfected SLC10A4-HEK293 cells. Transport studies were performed with [$^3$H]choline and [$^3$H]dopamine, but in contrast to the *high affinity choline transporter* CHT1 or the *dopamine transporter* DAT, respectively, SLC10A4 showed no transport activity when expressed in HEK293 cells. Due to its expression pattern in the neuronal and non-neuronal cholinergic system, in monoaminergic neurons and granules of mast cells as well as in neuronal cell cultures, we assume a specialized function of SLC10A4 in the regulation, release or storage of neuronal components like neurotransmitters or neuropeptides or in the exocytosis and retrieval of vesicles and granules.

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## P 6) Neurotransmitter Release through the Neurotransmitter Transporter GAT1

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The reuptake of Neurotransmitters (NTs) by specific transporters in the CNS insures termination of synaptic signalling, the maintenance of low basal extracellular levels of NTs and their recycling. There are evidences that some NT transporters can also release the NT from the presynaptic membrane, but results are still controversial.

The reverse mode of operation of the GABA transporter GAT1 was investigated in *Xenopus laevis* oocytes transfected with the transporter's cRNA .

In oocytes injected with GABA dissolved in NaCl solution (final GABA concentration about 10 mM), the transport current elicited by 300 µM external GABA showed an apparent inward behavior not present in non-injected oocytes.

In the same injected oocytes and in absence of external GABA, an outward current was visible that was blocked by the addition of the GAT1 inhibitor SKF89976-A .

Subtracting the I/V curves in presence of blocker from those in its absence gives the outward current due to the reverse transport of GAT1.

The outward current obtained in this way was not observed in oocytes injected only with the NaCl solution without GABA.

The intracellular injection of GABA together with its inhibitor SKF89976-A seems to show that this molecule is not able to block GAT1 from the inner side of membrane.

These initial results, indicate that GAT1 is able to operate the reverse transport and enhance the understanding of the conditions for its establishment.

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## P 7) ARG282 AND ASP341 ARE INVOLVED IN CHARGE MOVEMENT AND TRANSPORT FUNCTION IN THE OLIGOPEPTIDE TRANSPORTER PepT1

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The proton-dependent di- and tripeptide transporter PepT1 represents the major route of dietary amino acid intake in the intestine of many species. This transporter belongs to the solute carrier family SLC15 and, because of its electrogenic properties, it may be studied both through electrophysiological and radiotracer uptake experiments. In addition to the physiological relevance, understanding of the details of its mechanisms of operation is important since it appears to be involved in the absorption of many important, orally administered, drugs.

PepT1 has been cloned from various mammalian and non mammalian species showing high degree of amino acid similarity among the different isoforms. From the functional point of view, all the studied PepT1s are able to transport di- and tripeptides with varying degrees of efficiency, and they share the substantial inability to transport tetra- (or larger) peptides, as well as single amino acids

Significant evidences of the interaction of protons with the transporter protein arise from mutational studies: histidine 57 in the second transmembrane domain of PepT1 is required in order for the transporter to be functional, and this observation suggests that protonation of this residue is a necessary step in the transport cycle. Tyrosine residues around His57 have also been shown to stabilize proton binding in rabbit PepT1. Other residues have been found to affect PepT1 activity in interesting ways; in particular Arg282 and Asp341 have been reported to form a charge pair that may break and reform during the transport cycle. Interestingly, mutation of Arg282Glu has been reported to convert the cotransporter to a substrate-gated, rather unspecific cation channel. In addition, this mutation appears to cause loss of sensitivity to pH.

We have studied the effects of mutations in the charge pair residues R282 and D341 of the rabbit oligopeptide transporter PepT1 using the two-electrode voltage-clamp technique in transfected *Xenopus laevis* oocytes.

Mutations R282D and D341R, in which the sign of the electrical charge is reversed, produced opposite changes in the characteristics of charge movement with respect to the wild-type form. R282D increased the inward unidirectional rate of charge movement, and decreased the outward rate. Conversely, D341R increased the outward and decreased the inward rate. Analogous effects were seen in the corresponding mutants in the seabass PepT1 (K285D and D344R). These alterations suggest that these residues take part in the complex of intrinsic charges moving in the membrane electrical field during the transport cycle. The charge movement characteristics of the mutants remained pH-sensitive, as well as the transport-related currents. In the presence of saturating substrate, strong outward currents were generated during short voltage-pulses in the R282D form. The dependence on substrates concentrations of the reversal potential and slope conductance indicate that this mutant translocates protons and peptides as a complex, and confirms the two-fold role of protons in PepT1 function: on one hand they provide the neutralizing charge during the inward translocation of the substrates, while on the other they cause a slowing down of the overall cycling rate.

## P 8) Inverted re-entrant loops of the electrogenic Na+-coupled inorganic phosphate cotransporter, NaPi-IIb interact during the transport cycle.

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Sodium-coupled inorganic phosphate cotransporters of the SLC34 family (NaPi-IIa/b/c) are characterized by a common structural feature: a pair of inverted regions in the N- and C-terminal halves of the protein. These regions may form re-entrant loops that allow access of the substrate from either side of the membrane. Inverted repeats are not exclusive to SLC34 proteins: the crystallization of genetically unrelated transporters (e.g. LeuT, $Glt_{Ph}$, vSGLT) has shown that an inverted topology is a common motif involved in substrate translocation. We investigated the role of inverted repeats in SLC34 proteins to determine if these regions interact during the transport cycle. With this aim we introduced novel cysteines for the purpose of fluorescent labelling and cross-linking at selected sites associated with the predicted re-entrant regions of the flounder NaPi-IIb and analysed the behavior of single and double mutants using electrophysiology and voltage clamp fluorometry. Voltage clamp fluorometry (VCF) combines electrophysiology with simultaneous fluorescence measurements from fluorophore-labelled amino acids. The fluorescence emitted by the fluorophore depends on its micro-environment so that a change in the fluorescence, induced by voltage or different substrate concentration, reflects local conformational changes.

Analysis of the fluorescence data indicated that the two loops interact during transport cycle and the voltage dependence of fluorescence changes reported by the fluorophore label in the C-terminal half was influenced by specific residues in the N-terminal re-entrant segment.

To confirm our fluorescence data, we applied an indirect method: cross-linking of cysteines. If two cysteine are close enough, formation of a disulphide bridge can be promoted by applying an oxidating agent (e.g. Copper (II)(1,10-phenanthroline). Experiments indicate that this interaction is dependent on the conformational state of the protein. If sodium was not present in the extracellular medium, cross-linking was undetectable, but was observed in presence of external sodium. This suggested that external sodium increased the probability that the protein occupied a conformation favouring interaction between the two re-entrant regions.

## P 9) Increased cortico-limbic coupling in remitted depressed patients

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Introduction. Over the last decade a tremendous amount of data has been collected in studies of acute Major Depressive Disorder (MDD), repeatedly implicating the amygdala and the anterior cingulate cortex (ACC) in the neurobiological underpinnings. Both, the amygdala and ACC are key players in the processing of negative emotions and further comprise a well studied neural circuitry, which has been related to anxious temperament and increased risk of MDD. While there are sufficient data available for acute MDD, knowledge regarding remitted MDD (rMDD) is sparse. While acute MDD has been associated with increased amygdala activation and diminished coupling between amygdala and ACC, findings in rMDD are limited and inconclusive. Hence, we have initiated a study with the goal to investigate the specific role of this neural circuitry in rMDD in order to elucidate its underlying neurobiology in a remitted state.

Methods. Structural and functional magnetic resonance images (MRI) have been obtained from 38 healthy subjects without any psychiatric life-time diagnosis except nicotine dependence and 38 gender-matched drug-free remitted MDD patients at two study sites (Vienna, Dresden) sharing the same type of scanner and study protocol. During functional MRI (fMRI) scanning subjects underwent a neuropsychological paradigm comprising two emotional tasks (angry/fearful faces and fearful/threatening scenes matching) and a control task (shape matching). Additionally, functional and structural connectivity has been calculated by using extracted activation or volume data of both amygdalae as seed. FDR correction has been applied, if necessary. Furthermore, correlation analyses have been performed with clinical variables such as accumulated depressive days adjusted for illness duration.

Results. Local fMRI results indicate a significant decrease of bilateral amygdala reactivity in rMDD patients as compared to controls. Functional connectivity results demonstrate significantly increased coupling between amygdala and sACC in rMDD patients compared to controls. Similarly, structural connectivity results show increased association between amygdala and sACC volume in rMDD patients compared to controls. On a clinical level we have found a significant positive correlation between functional connectivity of this neural circuitry and accumulated depressive days adjusted for illness duration.

Discussion. Our findings support the notion that remission of MDD has to be maintained by an active counter-regulation of a dysfunctional amygdala-ACC circuitry as frequently being reported in acute MDD. Hence, we propose a mechanistic model of depression, which suggests that the absence of symptoms in rMDD is achieved by active and adaptive neurobiological processes moving this neural system to a labile equilibrium. Consecutively, it also explains, why rMDD patients exhibit a significant higher recurrence rate of acute MDD than normal controls, which share the same absence of depressive psychopathology.

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2 *Pezawas, L. et al. 5-HTTLPR polymorphism impacts human cingulate-amygdala interactions: a genetic susceptibility mechanism for depression. Nat Neurosci **8**, 828-834 (2005).*
3 *Cremers, H. R. et al. Neuroticism modulates amygdala-prefrontal connectivity in response to negative emotional facial expressions. Neuroimage **49**, 963-970 (2010).*

## P 10) Spatial Segregation of Emotion-Cognition-Interaction in Two Independent Samples Using Volume-Based fMRI Analysis

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### Introduction

Emotion and cognition domains are intimately linked on a brain systems level [1], which has been observed in lesion experiments as well as in neuroimaging studies. Underlying brain mechanisms, however, are still under debate. To study the impact of emotion-laden stimuli on cognitive domains, we developed a working memory paradigm utilizing stimuli of varying emotional valence. The goal of this study was to identify spatial properties of medial frontal regions exhibiting interaction between emotional and cognitive networks using standard 3D volume-based analysis (VBA) of functional magnetic resonance imaging (fMRI) data.

### Methods

Eighty healthy volunteers (US sample) recruited at NIMH, NIH, Bethesda, MD, USA, and eighty-eight healthy volunteers (EU sample) recruited at Medical University of Vienna (MUV), Austria, and at Dresden University of Technology (TUD), Germany, performed two cognitive tasks, comprising a 2-back working memory task and a low-level control task within a mixed event-related fMRI design. Positively, neutrally, and negatively valenced and equally balanced stimuli taken from the International Affective Picture System (IAPS) were presented during the working memory and control task. Valences were presented in a pseudo-random fashion within each block.
Brain activation, as measured at 3 T, was first analyzed on single subject level using the standard GLM approach in AFNI [2] and the SPMG3 hemodynamic response model. Regression coefficients were then analyzed on the group level using a linear mixed effects model with group, task and valence as fixed factors, and subject as random factor. Separate models were applied for pleasant and unpleasant stimuli reflecting different neural systems being involved. Calculations were performed using the free sofware R [3]; coordinates are given in Talairach-Tournoux space and RAI order.

## Results

Dissociated interaction effects between cognitive load and emotional valence were found in the medial prefrontal cortex for the model comprising unpleasant (US sample: 1, -65, 5; EU sample: 1, -50, -1) and for the model comprising pleasant (US sample: -14, -32, 11; EU sample: -10, -35, 23) stimuli. Thus, Euclidean distances between cluster peaks were 37 mm for the US sample and 30 mm for the EU sample.

## Conclusions

Euclidean distances between corresponding cluster centers found were way outside the width of the applied Gaussian smoothing kernel (10 mm FWHM). Hence, our preliminary data support the idea of valence-specific regional segregation of interaction between emotion and cognition in the medial prefrontal and anterior cingulate cortex.

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## P 11) QSAR, Ligand efficiency (LE) and lipophilic efficiency (LipE) studies of a series of benzophenone-type inhibitors of the multidrug transporter P-glycoprotein

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P-glycoprotein (P-gp) is an ATP dependent efflux transporter often linked to multidrug resistance. A better understanding of the structural requirements and lipophilic behavior of P-gp inhibitors will aid in the understanding of the molecular basis of ligand recognition. QSAR studies using a data set of benzophenones highlight the importance of partial charge and hydrophobic polar surface area descriptors for high activity. In addition, binding energy of the ligand per atom (ligand efficiency), and lipophilic efficiency were compared with those of P-gp inhibitors which entered clinical studies. For benzophenones, smaller compounds generally exhibited higher LE values. Interestingly, although P-gp inhibitors are highly lipophilic, they showed LipE values below the threshold considered to be necessary for promising drug candidates. This might be due to the fact that the ligand-protein interaction takes place directly in the membrane bilayer.

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## P 12) Serotonin Uptake in Blood Platelets is Related to Cingulate Cortex Activation

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**Objective:** The cingulate cortex (CC) plays a crucial role in emotion processing as well as in depression and is divided into four pharmacologically distinct regions. Each region consists of two subregions comprising several cytoarchitectonically different areas. All sub-regions have been related to specific aspects of emotional behavior in animal models or human studies. Interestingly, it has been shown that the four region model of the CC is closely related to findings in neuroimaging studies. The anterior CC (ACC) is of major importance for depression research since it has been related to feelings of sadness as well depressive mood and antidepressive treatment. It is noteworthy that the ACC exhibits the highest 5-HTT density in the human cortex in post mortem and *in vivo* human PET/SPECT studies. Recent clinical and preclinical research indicates that increases of neural activation of the ACC are caused to a major extent by reduced serotonin (5-HT) turn over rates. Such assumptions are based on studies showing an increase of blood oxygen-level dependent (BOLD) signal after fenfluramine release as well as after transporter inhibition by selective serotonin reuptake inhibitors (SSRIs) in medial prefrontal cortical areas in human and animal studies. Further evidence stems from neuroimaging studies of 5-HTTLPR, which subserves as model of variable 5-HT availability. Except from brain tissue, 5-HTT is also present in blood platelets and sufficient evidence exists that peripheral 5-HT uptake is closely linked to synaptosomal 5-HT uptake in neurons. Hence, we have initiated a pioneer study aiming to explore the possibility to utilize peripheral 5-HT uptake in blood platelets as predictor of ACC activation in healthy controls.

**Methods:** Fourty-eight healthy volunteers underwent a classical functional magnetic resonance imaging (fMRI) paradigm presented within a block design and involving fearful and unpleasant stimuli. Only medically healthy subjects without any life-time psychiatric diagnosis except nicotine dependence have been included in this study. Maximal 5-HT uptake velocity (Vmax) was determined in blood platelets. A correlation analysis between platelet Vmax and contrast estimates of the activated network restricted to the CC was performed. False discovery rate (FDR) correction was applied.

**Results:** Correlation analysis revealed significant clusters of negative correlation ($r \leq -0.45$) within the subgenual and pregenual portion of the ACC (both $p=0.031$) and posterior CC (PCC) ($p=0.031$). No significant correlations could be found for the MCC.

**Conclusions:** Our preliminary results are in accordance with pharmacological treatment and animal studies indicating a tight relationship between 5-HT turn over rates and ACC activation. Furthermore, our data demonstrate the feasibility to utilize peripheral platelet measures as potential predictors of ACC activation in healthy subjects.

## P 13) Molecular structure-function analysis of Pdr12 and Snq2, two ABC transporters from *S. cerevisiae*

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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 *S. cerevisiae* Pdr5 confer resistance to a vast variety of drugs. This phenomenon resembles drug resistance in cancer cells conferred by proteins such as P-glycoprotein.

However, little is known about physiological substrates for ABC transporters or the molecular mechanism of function, mainly due to a lack of structural information. To gain insight into the principles underlying transport through ABC proteins we have applied two strategies. First, as used successfully for Pdr5 (Ernst R. *et al*, 2008), we have overexpressed the yeast ABC transporters Pdr12 and Snq2 under the control of the *PDR5* promoter, which is driving high-level protein expression in the presence of the gain-of -function form of its dedicated transcription factor Pdr1. Furthermore, a 14-Histidine tag has been fused to the N-terminus of the proteins to allow for their rapid purification by affinity chromatography. Purified ABC transporters will be subjected to reconstitution experiments, as well as structural studies, including cryo-electron microscopy. A mutational analysis of the transporters will link their primary structure to function and drug transport, and define the molecular basis of substrate specificity in the context with ATP consumption.

*Ernst, R. et al., (2008) Proc. Natl. Acad. Sci. U.S.A.* ***105****, 5069*

## P 14) Substrate Interaction to SGLT1 on Living Cells probed at Different Temperatures – A Cooperative Radioactive Transport and Single Molecule Recognition Study

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The sodium glucose co-transporter SGLT1 plays a crucial role in serious diseases such as congenital glucose-galactose-malabsorbtion. Furthermore, members of the SGLT transporter family have recently become a therapeutic target for the treatment of hyperglycemia in type 2 diabetes. Thus, it is of particular importance to study substrate/SGLT1 unbinding forces and to gain kinetic and structural parameters of the binding pocket in order to achieve insights into the dynamical behaviour of SGLT1 during glucose and phlorizin binding, Single molecule force spectroscopy (SMFS) measurements in which the tip–retraction velocity was varied were performed. Additionally, ancillary transport studies using isotope labelled glucose derivatives were carried out at distinct temperatures in order to discriminate between the binding sites addressable by SMFS.

For both methods living CHO cells stably expressing SGLT1 were used. As a result, transport of $C^{14}$-AMG was nearly abolished below 10 °C, but the isotope labelled inhibitor phlorizin still bound to SGLT1. Accordingly, we assumed that only extracellular binding sites of SGLT1 are addressable at 10 °C as substrate translocation was impeded. For SMFS three glucose derivatives (1-thio-ß-D-glucose, amino-glucose and phlorizin) were coupled to the AFM tip through several PEG [poly ethylene (glycol)] - crosslinkers varying in length to probe substrate-carrier interaction. Binding probabilities, interaction forces as well as kinetic off rates and structural parameters of the binding pocket were gained. The results might indicate that different binding sites of SGLT1 for both glucoses as well as for phlorizin were recognized when the transport protein was probed at 10 °C and 37 °C.

## P 15) ELECTROPHYSIOLOGICAL CHARACTERISATION OF THE MAMMALIAN OLIGOPEPTIDE TRANSPORTER, rPEPT 1

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### ABSTRACT

Pept 1 is an electrogenic, proton-coupled, plasma membrane transporter which absorbs di- and tri-peptides derived from protein digestion. It is also of clinical importance because it transports beta lactam antibiotics, anti-cancer drugs, renin inhibitors, and other therapeutic drugs. It consists of 707 amino acids arranged in 12 transmembrane domains (TMDs) and a large extracellular loop between the TMDs 9 and 10.

We studied the electrophysiological characteristics of the transporter using the two-electrode voltage clamp and current clamp techniques; with a glycine-glutamine (G-Q) substrate. The results showed that, in addition to the transport-associated current, the transporter also had capacitive-like pre-steady state currents which represent the first steps of the transport cycle before substrate binding.

To characterize the transient currents, we analysed the time constant (τ) and charge movement (Q). The τ / V curve, at pH 6.5, had a bell shape with a maximum at about -100mV, while at higher pH values (7, 7.5, 8) it was flatter and leaned towards more negative potential values. At the pH values tested, the Q / V curve had a sigmoidal pattern with saturation at positive voltages.

The transport-associated current showed a slight reverse current, more evident at higher pH values. However, voltage- and current-clamp experiments at positive potentials suggest that this effect may not have functional significance in physiological conditions.

## P 16) Interaction of low molecular weight ligands with human P-glycoprotein

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**Background:**
P-glycoprotein (P-gp,ABCB1) is a multispecific drug efflux transporter that causes drug resistance and modulates the pharmacological profile of systemically administered medicines (Giacomini et al., 2010). It is a member of the ATP binding cassette (ABC) superfamily of membrane transporter. It has arisen from a homodimeric ancestor by gene duplication. Crystal-structures of mouse MDR1A (Aller et al., 2009) indicate that P-gp shares the overall architecture with two homodimeric bacterial exporters, Sav1866 and MsbA, (Dawson and Locher, 2006; Dawson and Locher, 2007; Ward et al., 2007), which have complete rotational symmetry. For ABC-transporters nucleotide binding occurs in two symmetric positions in the motor domains (Higgins and Linton, 2004; Hvorup et al., 2007; Karpowich et al., 2001; Pinkett et al., 2007; Schmitt et al., 2003). Based on the homology with entirely symmetric half-transporters, the present study was designed to understand the interaction between P-gp and ligands with candidate pharmacochaperone properties.

**Methodology and principal findings**:
P-gp was photolabelled with propafenone analogs, purified, digested proteolytically and peptide fragments were identified by high-resolution mass-spectrometry. Labeling was assigned to regions in the protein by projecting data into homology models. Subsequently, symmetric residue pairs in the putative translocation pathways were identified and replaced by site directed mutagenesis. Transport assays demonstrate the existence of two pseudosymmetric interaction regions. While rhodamine 123 preferentially interacts with one, verapamil and propafenones are privileged to interact with the other.

**Conclusions/significance:**
Two major findings ensued from this study: the existence of two interaction regions as a reflection of evolutionary origin from a homodimeric ancestor and selective but not exclusive interaction with one of them. The pseudosymmetric behaviour reconciles kinetic and thermodynamic data and reflects the transporter's homodimeric origin. It represents a starting point for the understanding of solute/ inhibitor like pharmacochaperones with P-glycoprotein.

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## P 17) Considerations Concerning Homology Modeling of ABC Transporters and Neurotransmitter transporters

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Molecular models of transporters with unknown structure may be constructed by homology modeling using a transporter with a known 3D crystal structure with a sequence similarity, so called homology, to the drug target as a template. When constructing homology models of transporters, there are several pitfalls in the homology modeling procedure. Special challenges are that there are few templates available, if any, and that the resolution of these templates is generally low.
Furthermore, the homology between the target transporter and the template may also be low. An identity between the target protein and the template below 30% may be considered "borderline" of what can be considered as a basis for constructing realistic models, and structure-based drug design based on low homology models may not be as applicable as for models with identities above ~50%. But even though homology may be low, the 3D structure of homologous proteins is more conserved than sequence, so low homology models may be useful for assignment of protein fold and function.
Such models also provide tools for suggesting candidate residues for mutagenesis experiments, and active sites can be identified when combining molecular modeling and site directed mutagenesis studies. Here we present molecular models of ABC transporters and neurotransmitter transporters based on various templates. We have constructed outward-facing molecular models of ABCB1 (P-glycoprotein), ABCC4 and ABCC5 based on the *Staphylococcus aureus* ABC transporter Sav1866, which has been crystallized in an outward-facing ATP-bound state, and inward facing models of ABCB1, ABCC4 and ABCC5 based on a wide open inward-facing conformation of *Escherichia coli* MsbA. After the models were constructed, we got a unique opportunity to test our methodology when the X-ray crystal structure of the *Mus musculus* ABCB1 in a drug-bound conformation was published. Amino acids that formed a putative substrate recognition site in the ABCB1 models were confirmed by the ABCB1 X-ray crystal structure.
We have also constructed models of the dopamine transporter (DAT), the serotonin transporter (SERT), and the noradrenalin transporter (NET) based on *Aquifex aeolicus* LeuTAa crystal structures in substrate-bound and inhibitor-bound conformations. The models were also compared with site directed mutagenesis data. The transporter models are examples of how structural information and insights can be obtained even for transporter models which are based on low homology and low resolution templates. Transporters may undergo substantial conformational changes during the transport cycle, and when interpreting homology models of transporters and performing docking studies on such models, the structural flexibility of transporters should be considered. These models should be considered as working tools for generating hypotheses and designing further experimental studies related to ABC transporter and neurotransmitter structure and function, and their limitations due to uncertainties should be kept in mind.

## P 18) A UNIQUE KINETIC SCHEME CAN REPRODUCE FUNCTIONAL DIFFERENCES EXHIBITED BY THE OLIGOPEPTIDE TRANSPORTER PepT1 FROM DIFFERENT SPECIES

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Amino acid absorption in the intestine occurs mostly through the operation of PepT1, a transporter belonging to the SLC15 family. In contrast to other cotransporter families that rely for their functioning on the $Na^+$ electrochemical gradient, the members of the SLC15 family are proton-dependent (and Na+ independent). Low pH appears to enhance the uptake of substrates due to the presence of an acidic microenvironment in the vicinity of the absorptive enterocytes, generated by the activity of the $Na^+/H^+$ exchanger in the apical membrane.

The enhancing effect of acidic pH on substrate uptake however shows some contradictory aspects in fish species and in rabbit, as electrophysiological determinations reported a substantially pH-independent maximal rate of transport, or even a potentiating effect of alkaline pH. These differences contrast with the high homology among the various transporter isoforms that show amino acid identities around 56 – 58% and similarities around 72 – 74% between mammalian and fish species.

Although kinetic models have been proposed for hPepT1, none has been tested thoroughly on rbPepT1 to verify the ability to simulate all main aspects of the transporter functioning.

The partial and complete cycle of the intestinal pH-dependent oligopeptide transporter PepT1 from three species (seabass, zebrafish and rabbit) were studied using an electrophysiological approach and a biophysical analysis, in order to identify similarities and differences. On the whole, the presteady state currents of the fish transporters were similar to each other, while presenting some quantitative differences with respect to rabbit PepT1. This last form showed slower decaying currents and the charge vs potential (Q/V) and time constant vs potential (t/V) curves shifted to more positive potentials All isoforms were similarly affected by external pH; showing acidity-induced slowing of the transients and positive shifts Q/V and t/V curves. Analysis of the pH dependence of the unidirectional rates of the intramembrane charge movement suggested that it is generated by the displacement of intrinsic charges of the proteins, and that external protonation of the protein limits the speed of this process. The complete cycle of the transporter was studied using the neutral dipeptide Gly-Gln. Michaelis-Menten analysis confirmed that in all species the apparent affinity for the substrate is significantly increased by acidity, while the maximal transport current is not strongly affected.

A kinetic model incorporating the slowing effects of external protonation, and in which the intramembrane charge movement is due to intrinsic protein charges, was constructed. Numerical simulations using this model were able to reproduce the experimental data for all three species both with respect to the presteady-state and transport currents. This result indicates that the reported functional differences among the various PepT1 isoforms do not require substantial changes in the kinetic scheme, but can be accounted for by relatively modest changes in the values of few parameters.

## P 19) TRICYCLIC ANTIDEPRESSANTS COMPETITIVELY INHIBIT THE SEROTONIN TRANSPORTER VIA AN INTERACTION INVOLVING TWO DISCONTINUOUS BINDING SITES

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### ABSTRACT

The structure of the bacterial leucine transporter $LeuT_{Aa}$ has been used as a model for mammalian $Na^+/Cl^-$-dependent transporters, in particular the serotonin transporter (SERT). The crystal structure of $LeuT_{Aa}$ liganded to tricyclic antidepressants predicts simultaneous binding of inhibitor and substrate. This is incompatible with the mutually competitive inhibition of substrates and inhibitors of SERT. We explored the binding modes of the tricyclic ring by rigid docking. Two approaches were used subsequently to differentiate between four groups of high-ranked docking poses: (i) a diagnostic $SERT^{Y95F}$ mutation, which greatly reduced the affinity for [$^3$H]imipramine but did not affect substrate binding. (ii) Competition binding experiments in the presence and absence of carbamazepine (i.e, a tricyclic imipramine analog with a short side chain that competes with [$^3$H]imipramine binding to SERT).

Binding of releasers (*para*-chloroamphetamine, methylene-dioxy-methamphetamine/ecstasy) and of carbamazepine were mutually exclusive, but Dixon plots generated in the presence of carbamazepine yielded intersecting lines for serotonin, $MPP^+$, paroxetine and ibogaine. These observations are consistent with a model, where (i) the tricyclic ring is docked into the outer vestibule and the dimethyl-aminopropyl side chain points to the substrate binding site; (ii) binding of amphetamines creates a structural change in the inner and outer vestibule that precludes docking of the tricyclic ring. (iii) Simultaneous binding of ibogaine (which binds to the inward-facing conformation) and of carbamazepine is indicative of a second binding site in the inner vestibule, consistent with the pseudo-symmetric fold of monoamine transporters. This may be the second low-affinity binding site for antidepressants.

## P 20) Occluded Leu transporter conformation-based homology modelling of GABA-Na+ symporter subtypes: druggability and function

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Ambient level of γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter of the brain is mediated by GABA transporters (GATs), members of the neurotransmitter-sodium symporter (NSS) family. The neuronal GABA transporter subtype (GAT-1) has already been proven to be a target for the antiepileptic drug Tiagabine, whereas druggability of glial GATs (GAT-2/3) is yet to be understood. In order to explore structure-function relationships for the orthosteric ligand recognition, we built up homology models of human GATs (hGATs) based on the structure of the bacterial orthologue Leu transporter in the occlude-state. A characteristic structural feature of the occluded conformation of hGATs is that similar extra- and intracellular gates are formed by middle-broken TM1 and TM6 helices, whose residues orient GABA to fit in a TM1-bound way. Similarly, a favored accommodation of substrate inhibitors with high docking score predicts efficient inhibition of transport through hGAT-1 if the TM1 binding prerequisite is added. Docking, molecular dynamics (MD) and functional data indicate, that amino acids participating in substrate binding of hGAT-1 and glial (hGAT-2, hGAT-/3) subtypes are different.

By contrast, substrate binding crevices of hGAT-2/3 cannot be distinguished, avoiding sensible prediction of efficient substrate inhibitors. MD calculations disclose the formation of major half-extended and minor ring-like conformations of GABA in complex with $Na^+(1)$ in all hGAT subtypes. The unique ring-like conformation of $Na^+(1)$-GABA complex in the occluded binding crevices anticipates NSS family member symporters exploring chemiosmotic energy *via* reversible chemical coupling of $Na^+$ ion. Emerging crystallographic data disclosing the open-to-out and open-to-in conformations of bacterial transporter homologues together with MD simulation of human transporters may lead to a detailed understanding of transport mechanisms at the molecular level, allowing the design of subtype specific inhibitors in the future.

## P 21) Dopaminergic deficiencies and excesses in the neuronal Rictor deficient mouse: is Akt a negative regulator of dopamine signaling?

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Synaptic dopamine (DA) is an established negative regulator of protein kinase B (Akt) signaling. Akt is a protein kinase that is fully activated through phosphorylation at two key residues: Threonine-308 by the enzyme phosphointositide-dependent kinase 1 (PDK1) and Serine-473 by the mammalian target of rapamycin complex 2 (mTORC2), a multi-protein structure requiring the protein Rictor to perform its kinase function. In animal models, inhibition of Akt phosphorylation is seen in "hyperdopaminergic" states, here defined as conditions of excess extracellular dopamine. Either pharmacological inhibition of the DA transporter (DAT), or genetic deletion of this protein, results in increases in extracellular DA and decreases in Akt phosphorylation. In a parallel fashion, genetic or pharmacological inhibition of DA signaling, particularly by targeting D2 and D3 receptors, leads to increases in Akt phoshorylation.

Importantly, pharmacologic and genetic models are beginning to suggest that Akt may also be a negative regulator of DA signaling. Pharmacologic studies reveal that Akt activity promotes DAT surface expression, suggesting Akt acts to limit the presence of DA at the synapse. In a genetic model, animals lacking the isoform Akt1 are more sensitive to behavioral disruptions following challenge with dopamine agonists like amphetamine (AMPH), although the exact mechanisms contributing to this phenotype are unknown. Therefore, we have generated another animal model with prominent neuronal defects in Akt signaling through a conditional model that deletes the mTORC2 protein Rictor in Nestin-expressing cells. The Rictor-deficient mouse has profound defects in Akt phosphorylation at the Serine-473 residue, and importantly an increase in striatal DA D2 receptors as well as a decrease in total DA and its metabolites DOPAC and HVA. At a behavioral level, the Rictor mouse shows hyperactivity in the open field. These phenotypes together (downregulation of DA synthesis, Akt phosphorylation deficits, and open field hyperlocomotion) parallel those observed in the DAT knockout mouse, suggesting that the Rictor mouse is a model of striatal hyperdopaminergia. Unlike the DAT knockout mouse, however, the Rictor neuronal knockout is hypersensitive to the locomotor stimulating effects of AMPH, suggesting that dysfunction in other physiological and biochemical mechanisms that regulate synaptic dopamine (firing rate, transmitter clearance, and receptor function) contribute to the observed phenotype. Together with previously published reports, this evidence suggests that impairment in networks that regulate Akt leads to hyperactivity of striatal dopamine networks.

## P 22) Screening of intracellular regions in the human Serotonin Transporter for phosphorylation sites

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The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) is involved in neuronal signalling in brain pathways that control behavioural functions such as mood, appetite, and reward. The serotonin transporter (SERT) is responsible for re-uptake of released 5-HT into nerve terminals. SERT is the therapeutic target for drugs used in the treatment of numerous neurological diseases such as major depressive disorder, anxiety and obsessive-compulsive disorder and for drugs of abuse, most notably cocaine and 3,4-methylenedioxy-*N*methylamphetamine (MDMA or "ecstasy").

Spatiotemporal modulation of SERT function and surface localization appear involved in modulation of 5-HT signalling. Several protein kinases, phosphatases and interacting proteins have been identified to participate to regulation of SERT activity by multiple pathways. In particular, activation and/or inhibition of kinases including PKC, PKG, p38MAPK and CamKII have been found to modulate SERT activity and trafficking (*1-7*). However, the cellular or molecular mechanisms by which kinase activity control these processes is poorly understood, including whether these effects involves direct phosphorylation of SERT and the identity of the specific amino acid that are phosphorylated.

As one approach to identify potential phosphoryalation sites in human SERT (hSERT), we have used solid-phase peptide synthesis to generate peptide fragments corresponding to the predicted intracellular regions of hSERT (N- and C-termini and intracellular loops). These peptides have been used as substrates in *in vitro* phosphorylation assays with a panel of kinases, which are either known to be involved in SERT regulation or for which canonical phosphorylation sites are predicted to exist. Using HPLC and tandem mass spectrometry to identify and quantify site–specific phosphorylation within peptides, we have identified 5 residues located in the N- and C-termini and in intracellular loop 1 and 2 of hSERT as potential sites for phosphorylation by the serine/threonine kinases p38MAPK, PKC and CamKII. We are currently investigating possible regulatory roles of the identified phosphorylation sites using phospho mimicking mutations and co-transfections of hSERT and constitutively active kinase mutants in a heterologous expression system.

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## P 23) Calmodulin kinase II regulates amphetamine induced reverse transport in the dopamine transporter

***Authors**:*
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The dopamine transporter (DAT) mediates dopamine (DA) reuptake once DA gets released into the synaptic cleft; thereby, the DAT regulates the DA content available for synaptic transmission. Upon certain stimuli, e.g. a change in the ionic composition of the extracellular fluid or certain psychostimulants like amphetamine can induce the reverse operation and induce outward transport, thereby increasing extracellular dopamine concentrations. Increases of DA in the synaptic cleft are associated with symptoms of psychosis and believed to underlie the initiation of drug addiction. Influx and efflux of substrate via the DAT are thought to be asymmetrical and were shown to possess consensus sites for the regulation by intracellular kinases.

It was demonstrated that the loss of N-terminal serines ablates amphetamine-induced reverse transport in the DAT and that Ca2+/Calmodulin dependent protein kinase II a (CaMKIIa) can physically bind the DAT C-terminus and phosphorylate N-terminal serines (Fog et al.2006). Pharmacological inhibition of CaMKIIa with KN93 dramatically reduces amphetamine-induced efflux in both cells stably transfected with the human DAT and in rat striatal slices.

Here, I show for the first time, that CaMKIIa is regulating amphetamine induced DAT mediated efflux in mice with different mutations in the gene of CaMKIIa and in a mouse model of Angelman Syndrome. Either pharmacological inhibition or genetic ablation of CaMKIIa function reduces reverse transport. As CaMKIIa is one of the brain's most abundant proteins involved in a plethora of regulatory processes it is not possible to pharmacologically target it in human AS patients. However, the DAT would be a possible target also for these patients and it might be promising to further investigate potential DAT influencing medications to treat Angelman Syndrome.

## P 24) Conformation of the ATP-releasing state of membrane insert Pglycoprotein

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ABC proteins form a large protein family. The human genome contains 48 ABC proteins, most of them coding for membrane transporter. First structures of ABC exporters have been solved in recent years showing 12 TM helices arranged in two bundles. The TMDs reaching over to the second NBD at the TMD-NBD interface. Some uncertainty remains with respect to conformation of the transporter, which seems not to be fully compatible with all biochemical evidence. Crystallization might have an influence on the transporters' conformation. We focus on the multidrug resistance transporter P-glycoprotein (P-gp, ABCB1), which is expressed at the blood-brain-barrier, in the intestine, kidney, liver and macrophages. P-gp inhibits xenotoxic compounds from entering the body or penetrating into the brain. In cancer cells, P-gp is often over-expressed and thereby confers resistance to chemotherapy.

We added experimental data from cysteine cross-linking at the step of homology modelling[1] and used MD simulations to identify the equilibrium conformation of the membrane inserted transporter. MD simulations are used to characterize the equilibrium structure of P-gp in the membrane environment. The results indicate that the membrane environment does have and influence on the transporter's most stable conformations, which shows a structure without wings. This conformations is in good accordance with experimental findings.

### *References*

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## P 25) CONCENTRATIVE ER EXPORT OF THE SEROTONIN TRANSPORTER REQUIRES AN INTERACTION WITH SEC24C

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The transporters for serotonin (SERT), dopamine (DAT) and noradrenaline (NET) belong to the monoamine neurotransmitter transporter family. They are primarily responsible for the rapid termination of neurotransmission *via* reuptake of neurotransmitters from the synaptic cleft. We have previously shown that the C-terminus of SERT plays a crucial role in trafficking and folding of the transporter. Mutations in this region of the protein (namely amino acid residues $PG^{601-602}$ and $RI^{607-608}$) cause intracellular retention of the SERT, and hence abolish substrate uptake and reduce inhibitor binding. In the present study, however, we were interested in isoform-specific interaction of Sec24 proteins with SERT, to study the nature of ER export of the transporter. Our initial studies involving mass spectrometry revealed that SERT directly interacts with Sec24 isoform C. To confirm these results, we subsequently used the siRNA approach to individually knock down the four mammalian Sec24 isoforms A, B, C or D in an immortalised cervical cancer cell line HeLa. The cells were transfected with YFP-tagged transporter plasmids (48h following siRNA transfections), and substrate uptake assays were performed after an additional 24h. Interestingly, knocking down Sec24A, B or D led to no changes in SERT function, while the knock-down of Sec24C isoform alone dramatically reduced serotonin uptake. Evidently, SERT specifically requires Sec24C in order to be exported from the ER and reach the cell surface. Our further data suggest that residues $RI^{607-608}$ may be the ER export motif on SERT C-terminus that mediates the interaction with Sec24C and in turn formation of the COPII coat. Moreover, the related transporters, DAT and NET, require Sec24D for ER export, consistent with reports available in the literature for other NSS transporters (*e.g.* GAT-1 and GLYT). In conclusion, ER export and trafficking of the SERT appears to occur in a unique manner judged by its exclusive interaction with Sec24C, and not Sec24D, different to many other NSS transporters that do require Sec24D.

## P 26) Identification of quinidine as a probe substrate for P-glycoprotein at the blood-brain barrier.

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Several studies suggest that the entry of quinidine (QND) to the brain is controlled by P-glycoprotein (P-gp), a major efflux pump at BBB. Yet, QND is classified as P-gp inhibitor even in the most recent reviews on drug-drug interactions at the BBB and on membrane transporters in drug development. The aim of this study was to provide evidence that QND can be used as probe substrate for P-gp by a complex test system including *in vitro* assays and dual/triple probe microdialysis (MD) assays in rats. Clinically relevant inhibitors, PSC-833 and LY-335979 were used to study substrate/modifier interactions.

Effects of QND and subtrate – inhibitor interactions on P-gp were tested in a membrane assay, in monolayer assays (MDCK-II, and rat brain capillary endothelial cell (RBEC)). QND increases the ATPase activity in membranes from insect cells expressing human MDR1 suggesting that the drug is a substrate for P-gp. In MDR1-MDCK-II monolayer a ~8-fold, in RBEC monolayer a ~2.0-fold efflux ratio ($P_{app}$ basal-to-apical/$P_{app}$ apical-to-basal) was observed indicating the role of P-gp in the transcellular transport of QND. LY-335979 and PSC-833 antagonized the effect of OND on ATPase activity. The vectorial transport of QND also was inhibited in the presence of LY-335979 and PSC-833 in a concentration-dependent manner. LY-335979 was more active than PSC-833 in both *in vitro* assays utilizing human P-gp. In the RBEC monolayer assay the two P-gp inhibitors were equiactive in inhibiting the transcellular transport of QND.

Brain penetration of QND was tested in MD experiments in rats by simultaneous assessing of unbound levels of QND in blood and brain. MD samples were collected at every 30 min from -120 min till +210 min in anesthetized rats dosed with QND (5 mg/kg; iv). In the control group the rats received saline-injection, in the PAG-vehicle group they received saline with a mixture of polyethyleneglycol-alcohol-glucose 20 min prior to QND treatment. The results are summarized in the table below.

**Pharmacokinetic parameters of unbound QND in brain and blood obtained by analysis of dialysate samples.**

| Groups | Brain | | Blood | | Brain/Blood | |
|---|---|---|---|---|---|---|
| | AUC (pmol/ml*h) | $C_{max}$ (pmol/ml) | AUC (pmol/ml*h) | $C_{max}$ (pmol/ml) | AUC | $C_{max}$ |
| Control | 103±15 | 56±9 | 328±35 | 173±11 | 0.32±0.04 | 0.34±0.06 |
| PAG-vehicle | 117±11 | 55±6 | 280±39 | 130±18 | 0.38±0.09 | 0.44±0.04 |
| PSC-833 (2x2 mg/kg) | 228±42**,+ | 107±13**,++ | 208±38 | 100±17**,+ | 1.24±0.31**,++ | 1.25±0.34*,+ |

Values are means ± SEM, n=5. *,** Significant differences between control vs. PSC-treated groups at a level of $p < 0.05$ and $p < 0.01$, respectively. $^{+,++}$ Significant differences between PAG-vehicle treated vs. PSC-treated groups at a level of $p < 0.05$ and $p < 0.01$, respectively.

To further investigate the inhibitor-substrate interactions on P-gp at the BBB, PSC-833 was locally applied via retrodialysis in anesthetized and in freely-moving rats. PSC-833 (10 mM) was perfused via the MD probe in the left frontal cortex (FC), starting 60 min before systemic administration of QND (5 mg/kg; iv). The ECF levels of QND in the left FC were much higher than in the right FC perfused with vehicle, indicating that P-gp was inhibited at BBB of the PSC-833 perfused side.

## P 27) Structure-function analysis of putative sterol importers in yeast.

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The utilization of external sterols in yeast involves passage of sterol molecule through the cell wall, entrance into the plasma membrane and actual internalization (integration into lipid turnover). Two plasma membrane proteins, Aus1 and Pdr11, members of the ATP-binding cassette (ABC) proteins family, are involved in sterol uptake. Simultaneous deletion of *AUS1* and *PDR11* results in inability to import exogenous sterols, however it is not clear whether sterols are actual substrates of these pumps. Interestingly, while in prokaryotes ABC proteins are widely involved in uptake of nutrients in eukaryotes they are almost exclusively responsible for pumping substrates out of the cell. Putative sterol importers Pdr11 and Aus1 are two of few eukaryotic exceptions implicated in transport with opposite direction: towards the site of ATP hydrolysis. Pdr11 and Aus1 therefore provide an especially attractive model to study the molecular basis of the transport mechanism of ABC transporters.

We constructed versions of Aus1 and Pdr11 with mutated critical residues in conserved sequences. In addition, we established functional assay to study sterol import utilizing fluorescent sterol probe – dehydroergosterol. Employing this assay and mutational analysis we will link the primary structure to the function of Aus1 and Pdr11.

In order to identify substrate(s) and to study mechanism of action of putative sterol importers, we have overproduced and isolated His tagged Aus1 and Pdr11. Purified proteins will be reconstituted in proteoliposomes and substrate transport assays will be performed.

As indicated above, close homologues of Aus1 and Pdr11 in yeast (e.g. Pdr5 or Snq2) are responsible for detoxification of a wide range of substrates including antifungals, lipophilic dyes, steroids as well as many other chemically unrelated compounds, so called pleiotropic drug resistance (PDR). The effect of Aus1 and Pdr11 on the PDR phenotype will be discussed.

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## P 28) INVESTIGATION OF DETERGENTS AT GABA TRANSPORTERS

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The high resolution crystal structures of the neurotransmitter sodium symporter homolog LeuT known so far are all based on crystals grown from solutions containing *n*-octyl-$\beta$-D-glucopyranoside (OG) as detergent [1]. In the original structure of LeuT solved by Gouaux and colleagues the extracellular vestibule comprising a second substrate binding site (S2) is occupied by OG [2]. Regarding functional aspects of LeuT OG could be shown to compete with substrate at the S2 site and to disrupt sodium coupled transport [2]. In contrast to OG *n*-dodecyl-$\beta$-D-maltopyranoside (DDM) the detergent employed to reconstitute the purified LeuT protein for functional investigations is devoid of inhibitory effects on substrate transport [2].

Considering these results we characterized the inhibitory potency of several detergents including OG as well as DDM in [$^3$H]GABA uptake assays for mGAT1-mGAT4 performed with HEK 293 cell lines stably expressing the individual transporter subtypes. Short term cytotoxic effects possibly affecting cell viability under the incubation conditions of GABA uptake were studied in non transfected HEK 293 cells by determination of cellular ATP. Furthermore, the test compounds were studied in GAT1 binding assays employing NO 711 as a native marker.

Interestingly, OG did not reveal significant inhibitory potency at mGAT1-4 in concentrations up to 1 mM whereas DDM could be shown to be a non selective inhibitor of uptake with potencies ($IC_{50}$ GABA uptake, $K_i$ NO 711 binding) below 100 µM.

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## P 29) Single molecule recognition force spectroscopy (SMRFS) on SERT expressing living cells

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Atomic force microscopy (AFM) is a powerful method to investigate interactions between a ligand on the AFM tip and complementary receptor molecules on a sample surface. The possibility to measure under physiological conditions enables the probing of bioligands on living cells. Due to the enormous diversity of biomolecules a “library” of crosslinkers had to be developed to link all these different molecules of interest to the AFM tip [1]. We established a reliable synthetic pathway for crosslinker molecules yielding fully functional molecules bound to the AFM tips, converting it into a biosensor, which can be used in single molecule recognition force spectroscopy (SMRFS).

These biosensors can now be used to investigate the interaction between a ligand (MFZ) and receptors (SERT) on cellsurfaces. Different SMRFM applications on living cells using these crosslinkers were implemented in our laboratory and are briefly discussed [2, 3].

One further aim is to investigate the geometrical assembely and clustering of receptors on cellsurfaces. The use of a two-armed or a four-armed linker carrying two or four identical ligands, could give insights in the spatial assembly of cell receptors deciphering it´s oligomeric state with SMRFM.

These applications show the great potential of SMRFM to study binding kinetics and the supramolecular assembly of receptors on living cells under physiological conditions.

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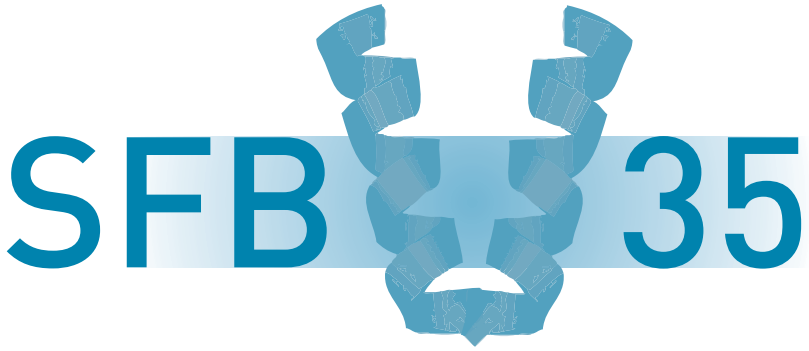
# IMPRESSUM

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