

SFB-Symposium

11

TRANSMEMBRANE TRANSPORTERS IN HEALTH AND DISEASE

hNET (89) NGGGARIPYTLF...LIAGMP...
hSERT (112) NGGGARLPYTIM...IFGGIF...
GAT-1 (77) NGGGARIPYFLT...IFAGVF...
T-1 (77) NGGGARIPYFLT...IFAGVF...
AT-2 (65) NGGGARIPYLIF...FTCGIF...
AT-3 (83) NGGGARIPYVVF...ICCGIF...
LYT-1 (119) NGGGARFPYFIM...IFCGIF...
GLYT-2 (224) NGGGARIPYLMM...ALAGLF...
hTAUT (74) NGGGARIPYFIF...FGSGLF...
hCRET (85) NGGGVRIIPYVLI...LVGGIF...
hPROT (70) NGGGARVPYFLM...AICGIF...

B
hDAT (318) VCFSLVGRVLIAFSS
hNET (315) IFFSLVAGRVLIAFAS
hSERT (333) IFFSLVGRVLLAFAS
hGAT-1 (292) IFFSYVGLVSLIALGS
rGAT-1 (292) IFFSYVGLVSLIALGS

SEPTEMBER 8. – 9. 2011

GREAT LECTURE HALL

INSTITUTE OF PHARMACOLOGY

SCIENTIFIC SESSIONS AND POSTERSESSION

- Jacob Andersen, University of Copenhagen, Denmark
- Richard Callaghan, University of Oxford, United Kingdom
- Peter Chiba, Medical University of Vienna, Austria
- Niels Christian Danbolt, University of Oslo, Norway
- Aurelio Galli, Vanderbilt University Medical Center, Nashville, USA
- Ulrik Gether, University of Copenhagen, Denmark
- Baruch Kanner, Hebrew University of Jerusalem, Israel
- Haley Melikian, University of Massachusetts Medical School, Worcester, USA
- Manuel Palacin, University of Barcelona, Spain
- Lukas Pezawas, Medical University of Vienna, Austria
- Hanne Poulsen, University of Aarhus, Denmark
- Walter Sandtner, Medical University of Vienna, Austria
- Sander Smits, Heinrich Heine University Düsseldorf, Germany
- Bruno Stieger, University Hospital Zurich, Switzerland
- Tetsuya Terasaki, Tohoku University, Sendai, Japan
- Michael Trauner, Medical University of Vienna, Austria

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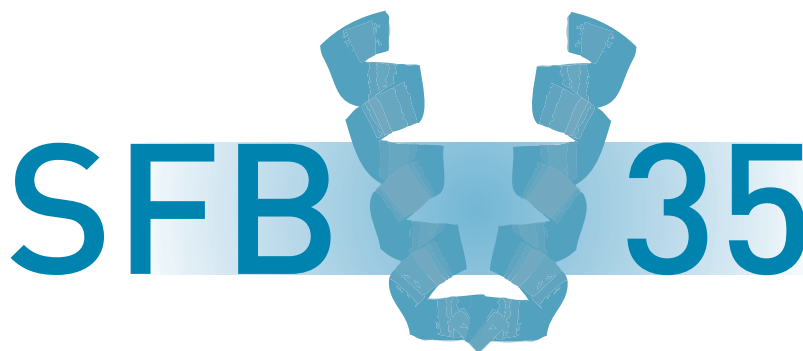
The 4th SFB35 Symposium 2011 gratefully acknowledges the support of the following sponsors:



4TH SFB35–SYMPOSIUM 2011, VIENNA

TABLE OF CONTENTS:

PROGRAMME – OVERVIEW	4
POSTERS (SHORT):	7
PLENARY LECTURES:	13
SESSIONS:	17
Session 1: Trafficking of Neurotransmitter Transporters	17
Session 2: The Structural Basis of Transport	19
Session 3: Serotonin Transporters: from structure to clinics	22
Session 4: ABC-transporters heavy traffic in the liver	25
POSTERS:	29
IMPRESSUM	87



PROGRAMME – OVERVIEW

4TH SFB35–SYMPOSIUM 2011, VIENNA

Thursday, 8th of September

- 07:45 **Registration**
- 08:30 Opening Remarks
- 08:40 **Session 1: Trafficking of Neurotransmitter Transporters**
(Chair: Heinz Bönisch – University of Bonn, Germany)
- 08:45 Speaker 1: **Ulrik Gether** (University of Copenhagen, Denmark)
The dopamine transporter: PICK1-ing new functions of C-terminal PDZ-domain interactions
- 09:15 Speaker 2: **Haley Melikian** (UMASS Medical School Worcester, USA)
Dopamine Transporter Dancing Partners: Rin GTPase takes the stage
- 09:45 Coffee/Tea break
- 10:15 **Plenary lecture 1:**
(Chair: Oliver Langer – Medical University of Vienna, Austria)
- 10:20 Plenary Speaker: **Tetsuya Terasaki** (Tohoku University, Sendai, Japan)
Quantitative targeted absolute proteomics of membrane transporters in the normal and disease
- 11:20 **Plenary lecture 2:**
(Chair: Donald Miller –University of Manitoba, Winnipeg, Canada)
- 11:25 Plenary Speaker: **Richard Callaghan** (Merton College - University of Oxford, United Kingdom)
Multi-drug efflux by p-glycoprotein: why has this protein not been stopped yet?
- 12:25 **Lunch**

13:40 **Session 2: The Structural Basis of Transport**
(Chair: Arne Schousboe – University of Copenhagen, Denmark)

13:45 Speaker 1: **Manuel Palacin** (University of Barcelona, Spain)
Molecular basis of substrate-induced permeation by an amino acid antiporter

14:15 Speaker 2: **Baruch Kanner** (Hebrew University of Jerusalem, Israel)
Mechanism of sodium-coupled neurotransmitter transport in the brain

14:45 Speaker 3: **Niels Christian Danbolt** (University of Oslo, Norway)
GABA and glutamate transporters around synapses: numbers matter

15:15 **Postersession & Coffee/Tea**

17:10 **Session 3: Serotonin Transporters: from structure to clinics**
(Chair: Gary Rudnick – Yale University, USA)

17:15 Speaker 1: **Walter Sandtner** (Medical University of Vienna, Austria)
The conducting state of the human serotonin transporter is entered via an inward facing conformation - a unifying concept of serotonin transporter associated currents

17:45 Speaker 2: **Jacob Andersen** (University of Copenhagen, Denmark)
Molecular determinants for selective recognition of antidepressants in the human serotonin and norepinephrine transporters

18:15 Speaker 3: **Lukas Pezawas** (Medical University of Vienna, Austria)
Neural activity of the anterior cingulate is responsible for remission maintenance in depression and correlated with platelet 5-HT re-uptake velocity

19:30 **Heurigen Restaurant "10er Marie" - Ottakringer Straße**



Ottakringer Str. 222-224, A-1160
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Public transport:
Please use tram No. 37, 38, 40, 41, 42 to "Schottentor". Change to underground U2 (purple line) until "Rathaus" station, then change to tram #2 to "Ottakringer Strasse / Erdbrustgasse" and get off at tram station "Johannes-Krawarik-Gasse" and walk the few meters to the Heurigen Restaurant "10er Marie".

Friday, 9th of September

- 08:30 **Registration**
- 08:55 **Session 4: ABC-transporters heavy traffic in the liver**
(Chair: Thomas Stockner – Medical University of Vienna, Austria)
- 09:00 Speaker 1: **Peter Chiba** (Medical University of Vienna, Austria)
Interaction of ABC-transporters with small molecules and their potential role as pharmacological chaperones
- 09:30 Speaker 2: **Sander Smits** (Heinrich Heine University Düsseldorf, Germany)
Analyzing the haemolysin secretion machinery of E. coli
- 10:00 Speaker 3: **Bruno Stieger** (University Hospital Zurich, Switzerland)
Canalicular Microdomains and Formation of Primary Bile in Rat Hepatocytes
- 10:30 Speaker 4: **Michael Trauner** (Medical University of Vienna, Austria)
Role of canalicular phospholipid export pump (ABCB4) for pathogenesis and therapy of cholestatic liver diseases
- 11:00 Coffee/Tea break
- 11:25 **Plenary lecture 3:**
(Chair: Gerhard F. Ecker – Department of Medicinal Chemistry, Vienna)
- 11:30 Plenary Speaker: **Hanne Poulsen** (University of Aarhus, Denmark)
Structure and Function of the Na,K-ATPase - Tail or Swift Leak
- 12:30 **Lunch & Postersession & Coffee/Tea**
- 14:25 **Plenary lecture 4:**
(Chair: Claus Juul Loland – University of Copenhagen, Denmark)
- 14:30 Plenary Speaker: **Aurelio Galli** (Vanderbilt University Medical Center, Vanderbilt, USA)
Syntaxin: Teaching an old Dog New Tricks
- 15:30 **End of meeting**

POSTERS (SHORT):

Thursday, 8th of September

- P 1) Neural dynamic interplay between amygdala and anterior cingulate cortex in remitted Major Depressive Disorder**
BARTOVA Lucie, HARTINGER Beate, SCHARINGER Christian, DIERS Kersten, HUF Wolfgang, PAIL Gerald, KASESS Christian, KONSTANTINIDIS Anastasios, ESTERBAUER Harald, SITTE Harald H., WINDISCHBERGER Christian, KASPER Siegfried, MOSER Ewald, BROCKE Burkhard, PEZAWAS Lukas
- P 2) Protein interactions and regulation of the ABC transporter MRP4 (ABCC4)**
Gabriele Jedlitschky, Marie-Luise Kromrey, Yvonne Schaletzki, Sebastian Zang, Susanne Bröderdorf, Igor Mosyagin and Heyo K. Kroemer
- P 3) Dicysteine Crosslinking in the human Serotonin Transporter**
Henriette Bjerregaard, Kasper Severinsen, Pella Söderhielm, Anders Skov Kristensen, Ove Wiborg & Steffen Sinning
- P 4) GABA REVERSE TRANSPORT BY THE NEURONAL COTRANSPORTER GAT1: INFLUENCE OF INTERNAL CHLORIDE DEPLETION**
Elena Bossi, Simone Bertram, Francesca Cherubino, Michela Castagna, and Antonio Peres
- P 5) Subcellular distribution and sorting of the orphan carrier SLC10A4 in neuronal cell lines**
M. Moncada, S. Burger, S. Schmidt, J. Geyer
- P 6) Virtual screening against Alzheimer's acetylcholinesterase: Suggested workflow for compound prioritization using 3D pharmacophore, docking & 2D QSAR analysis**
N. Chitranshi, P.K. Tripathi & P.K. Seth
- P 7) Comparison of the substrate pattern of the SLC10 carriers NTCP, ASBT, and SOAT**
Döring B, Grosser G, Geyer J
- P 8) Adaptation of microdialysis techniques to study brain penetration of drugs and substrate - inhibitor interactions of ABC transporters at the blood-brain barrier in mice.**
Erdő F., P. Trampus, M. Sike, P. Molnár, P. Krajcsi and I. Sziráki,
- P 9) Kinetic evaluation of substrate binding to the Leucine Transporter (LeuT); the apparent two-substrate model is a consequence of slow kinetics and non-equilibrated protein-ligand complexes**
Anja Bjerregaard Christiansen, Pernille Noer, Steffen Sinning
- P 10) Identification and analysis of critical amino acids in the transmembrane 2 of human OATP1B1**
Nan Li, Weifang Hong, Guangyu Lin, Hanping Lu, Mei Hong

- P 11) Interactions of fluorone dyes with the sodium pump observed by fluorescence methods**
Marika Janovská, and Martin Kubala
- P 12) Modulation of UCP1-mediated proton transport by phosphoinositides.**
Olga Jovanovic, Cheng-Kui Qu, Elena E. Pohl
- P 13) Benzochalcones as selective inhibitors of BCRP**
Kapil Juvale, Michael Wiese
- P 14) Application of post-docking molecular dynamics simulations to evaluate binding modes of propafenone analogues in P-gp**
Klepsch F, Vosmeer CR, Stockner T, Chiba P, Geerke DP, Ecker GF
- P 15) Beyond the Crystals: Spectroscopic Experiments and MD Simulations Reveal Na⁺/K⁺-ATPase Cytoplasmic Headpiece Dynamics and Electrostatic Surface Potential Changes Induced by Ligand Binding**
Martin Kubala
- P 16) Key Residues Controlling Conformational Transitions in the Serotonin Transporter**
Pernille Thornild Møller, Kasper Severinsen, Heidi Koldsø, Gary Rudnick, Birgit Schiøtt, Ove Wiborg & Steffen Sinning
- P 17) Subgenual Anterior Cingulate Cortex Mediates Functional Relation Between Emotional and Cognitive Regions in the Human Brain**
Pail Gerald, Huf Wolfgang, Kalcher Klaudius, Boubela Roland, Hartinger Beate, Kasess Christian, Moser Ewald, Windischberger Christian, Kasper Siegfried, Pezawas Lukas
- P 18) NMDA receptor activation downregulates KCC2 in a calpain-dependent manner**
Martin Puskarjov, Peter Blaesse, Faraz Ahmad, Kai Kaila
- P 19) Platelet serotonin uptake predicts cingulate cortex activation**
SCHARINGER Christian, KASESS Christian, UZELAC Zeljko, KALCHER Klaudius, HUF Wolfgang, RABL Ulrich, HASLACHER Helmut^f, ESTERBAUER Harald, KASPER Siegfried, MOSER Ewald, SITTE Harald H., PEZAWAS Lukas
- P 20) Computational estimation of biliary excretion of drugs using transporters**
Sharifi M, Gardener I, Ghafourian T
- P 21) Molecular determinants of a novel inhibitor of the betaine-GABA transporter**
Jonas Skovgaard Petersen
- P 22) Amphetamine- and cocaine-sensitisation in Calmodulin kinase II alpha knock-out mice – preliminary results of a pilot study**
Thomas Steinkellner, Birgit Eisenrauch, Lisa Konrad, Oliver Kudlacek, Michael Freissmuth, Ype Elgersma, Harald H. Sitte, Daniela Pollak

- P 23) TRUNCATIONS IN THE AMINO TAIL REVEAL A REGION KEY TO SUPPORTING AMPHETAMINE-INDUCED EFFLUX BY THE SEROTONIN TRANSPORTER**
Sucic S., Kern C., Vidovic D., Sarker S., Kudlacek O., Sitte H.H. and Freissmuth M.
- P 24) MS-Binding Assays – An Efficient Alternative to Radioligand Binding Applied to the Human Serotonin Transporter**
Marielle Hess, Georg Höfner, Klaus T. Wanner
- P 25) Improvement of transmembrane sequence coverage of P-glycoprotein using pepsin and combinations of chymotrypsin with mass compatible detergents**
Svenja Wieschrath, Sabine Metzger, Michael Wiese
- P 26) Linking Antidepressants' Drug Targets to Side Effects**
ZAUNER Miriam, MICHL Johanna, SITTE Harald, ECKER Gerhard F., PEZAWAS Lukas
- P 27) Binding strength and dynamics of MFZ2-12 interaction with the serotonin transporter on living cells by single molecule force spectroscopy**
Linda Wildling, Christian Rankl, Thomas Haselgrübler, Hermann J. Gruber, Marion Holy, Amy Hauck Newman, Mu-Fa Zou, Rong Zhu, Michael Freissmuth, Harald H. Sitte and Peter Hinterdorfer
- P 28) ABCG2/BCRP probe substrate for regulatory studies – in vitro evaluation**
Ildikó Makai¹, Zsolt Fekete², Zsuzsa Rajnai², Emese Kis², Erzsébet Beéry¹, Krisztina Herédi-Szabó², Márton Jani¹, Péter Krajcsi
Late abstract - abstract body can be found on the last page
- P 29) Concept of dual solute translocation paths of ABCB1 allows studying the role of individual transmembrane domain residues in solute interaction**
Zahida Parveen, Thomas Stockner, Diethart Schmid, Martin Kraupp, Gerhard F. Ecker and Peter Chiba
Late abstract - abstract body can be found on the last page

Friday, 9th of September

- P 1) Working memory function and associated neural activation in remitted Major Depressive Disorder**
BARTOVA Lucie, DIERS Kersten, SCHARINGER Christian, RABL Ulrich, HUF Wolfgang, STROBEL Alexander, MOSER Ewald, KASPER Siegfried, BROCKE Burkhard, PEZAWAS Lukas
- P 2) A database with mutagenetic data extracted from the literature for comparative analysis of mutations in ABC proteins**
Gergely Gyimesi, David Borsodi, Balazs Sarkadi, Tamas Hegedus
- P 3) Frog Oocytes to Unveil the Structure and Supramolecular Organization of Human Transport Proteins**
Marc J. Bergeron, Rajendra Boggavarapu, Marcel Meury, Zöhre Ucurum, Luc Caron, Paul Isenring, Matthias A. Hediger and Dimitrios Fotiadis
- P 4) Highly Consistent Resting State Networks revealed by exploratory Analysis of the Functional Connectomes Database using massively parallelized FENICA**
BOUBELA Roland N., HUF Wolfgang, KALCHER Klaudius, SCHÖPF Veronik, SCHARINGER Christian, PAIL Gerald, FILZMOSEER Peter, KASPER Siegfried, MOSER Ewald, PEZAWAS Lukas, WINDISCHBERGER Christian
- P 5) THE HIGHLY CONSERVED GLYCINE TRIPLET PRESENT IN THE FIRST EXTRACELLULAR LOOP OF KAAT1 IS INVOLVED IN CATION INTERACTION**
Matteo Giovanola, Francesca D'antoni, Massimo Santacroce, Stefania A. Mari, Elena Bossi, Vellea F. Sacchi and Michela Castagna
- P 6) BODIPY-DYES AS LIGANDS FOR GAT**
Markus Daerr, Klaus T. Wanner
- P 7) Molecular Determinants for Ecstasy Analogue Selectivity**
Ebisse Chalchissa Wamiand Steffen Sinning
- P 8) Modeling the dynamics of the human serotonin transporter**
Julie Grouleff, Heidi Koldsø and Birgit Schiøtt
- P 9) Scintillation proximity to characterize the ligand binding properties of detergent-solubilized transport proteins**
Daniel Harder & Dimitrios Fotiadis
- P 10) Interaction of the sodium potassium pump with the fluorescent dyes using time-resolved fluorescence spectroscopy**
Miroslav Huličiak, Marika Janovská and Martin Kubala
- P 11) Effect of quantitative inhibition of P-glycoprotein at the blood-brain barrier in rats and humans measured with (R)-[11C]verapamil and positron emission tomography**
Martin Bauer, Markus Zeitlinger, Rudolf Karch, Peter Matznelle1, Johann Stanek, Claudia Kuntner, Markus Müller, Oliver Langer

- P 12) Molecular Dynamics - Assisted Comparative Modeling and Docking Studies of the Gamma-Aminobutyric Acid Transporter**
A. Jurik, B. Zdrzil, R. Reicherstorfer, H.H. Sitte, G.F. Ecker
- P 13) Methodological investigations on fixed and random effect modeling in dynamic causal modelling**
Kasess Christian, Stephan Klaas E., Weissenbacher Andreas, Moser Ewald, Pezawas Lukas, Windischberger Christian
- P 14) Characterising the interaction between the COPII component SEC24C and the human serotonin transporter**
Florian Koban, Michael Freissmuth and Sonja Sucic
- P 15) X-linked Adrenoleukodystrophy: targeting of the ABC-transporter ALDP to peroxisomes**
Markus Kunze, Christoph Wiesinger, Johannes Berger
- P 16) Wistar Zagreb-5HT rat: a rodent model of constitutive dysregulation of serotonin transporter**
Gordana Mokrovic and Lipa Cicin-Sain
- P 17) Characterization of the role of individual sodium binding sites in the human serotonin transporter.**
P. R. NOER, K. SEVERINSEN, K. A. VINBERG, O. WIBORG, S. SINNING;
- P 18) UCP2 up-regulation due to immune cell activation.**
Elena E. Pohl, Anne Rupprecht, Anja U. Bräuer, Carmen Infante-Duarte, Alina Smorodchenko
- P 19) COMT Val 158 allele heterozygosity abolishes the impact of life stress on hippocampal volume**
RABL Ulrich, HUF Wolfgang, BOUBELA Roland, BERGER Andreas, HOFMAIER Tina, ESTERBAUER Harald, KASPER Siegfried, WINDISCHBERGER Christian, MOSER Ewald, PEZAWAS Lukas
- P 20) Oxalate's precursor glyoxylate increases the expression of sulfate-anion-transporter-1, sat-1, in HepG2 cells**
N Schnedler, AR Asif, G Burckhardt, BC Burckhardt
- P 21) MOLECULAR MODELLING OF GAMMA-AMINO BUTYRIC ACID TRANSPORTER FUNCTION**
Á. Simon, Á. Bencsura J. Kardos
- P 22) Two approaches towards facilitating crystallization of the peptide transporter YdgR**
Bettina Spitzenberger, Elisabeth Malle and Peggy Stolt- Bergner
- P 23) Which conformation does P-glycoprotein prefer within the membrane?**
Thomas Stockner, Yaprak Dönmez, Harald H. Sitte and Peter Chiba
- P 24) Putative yeast sterol importers, Aus1 and Pdr11.**
Martin Valachovic, Cornelia Klein and Karl Kuchler

P 25) Molecular dynamics simulations propose a structural water involved in the binding of imipramine to SERT

Weissensteiner R, Sarker S, Zdrazil B, Sitte H.H, Freissmuth M, Sucic S, Ecker G.F

P 26) Multidrug Resistance associated Protein in vitro assay

Gloria Wissel, Heidi Kidron, Henri Xhaard and Arto Urtti

P 27) The betaine-GABA transporter (BGT1, slc6a12) is unlikely to control of GABA receptor activation

Y. Zhou, S. Holmseth, T. Holen, N. C. Danbolt.

P 28) ABC transporters of Fasciola hepatica as putative drug targets

Oliver Kudlacek, Ali El-Kasaby, Zahida Parveen, Thomas Stockner, Peter Chiba and Michael Freissmuth

PLENARY LECTURES:

PL 1) Tetsuya Terasaki

Quantitative Targeted Absolute Proteomics of Membrane Transporters in the Normal and Disease

Tetsuya Terasaki, Yasuo Uchida, Sumio Ohtsuki
Graduate School of Pharmaceutical Science, Tohoku University, Sendai, JAPAN

The purpose of the present study was to validate a hypothesis that an in vivo BBB transporter activity is reconstructed by a pharmacokinetic model using an in vitro intrinsic transporter activity per transporter protein and a transporter protein concentration of the brain capillary endothelial cells (B-cap) in any animal species at any diseased state. Brain-to-plasma concentration ratios (K_p brain) and its ratios between wild-type and *mdr1a/1b* (-/-) mice (K_p brain ratio) were obtained from literature (risperidone, indinavir, paclitaxel, verapamil, loratadine and diazepam) or by intravenous constant infusion studies (quinidine, loperamide, digoxin, dexamethasone and vinblastine). In vitro P-gp transport activities were determined by a transcellular transport studies using a monolayers of mouse P-gp transfected LLC-PK1 (L-*mdr1a*) and parental cells. P-gp protein concentration were determined for the mouse brain capillaries and the mouse P-gp/*mdr1a* transfected LLC-PK1 cell line by the LC-MS/MS Shotgun protein quantification method reported previously (1). For all substrates, the reconstructed K_p brain ratios were within 1.6-fold of observed values, supporting our hypothesis (2). In order to expand the study for the animal that could not be obtained for a transporter gene knockout, K_p ,brain was used to validate the hypothesis. The unbound plasma binding fraction (f_u ,plasma) and the unbound brain tissue binding fraction (f_u ,brain) were determined by in vitro studies and the K_p ,brain were reconstructed for 11 drugs. Interestingly, those of reconstructed K_p ,brain were close to those of observed values (within 3 folds) (2). Moreover, the study was expanded for the reconstruction of *Cynomolgus* monkey brain drug distribution. The reconstructed K_p , brain values were well coincided with those of observed for indinavir, quinidine, loperamide, paclitaxel, diazepam and verapamil (within 3 folds). The study was also expanded for the diseased model such as pentylenetetrazole (PTZ) treated mouse and phenytoin (PHT) treated mouse. The lower in vivo K_p ,brain in the diseased model were fairly reconstructed from in vitro studies, suggesting the up-regulation of *mdr1a* protein in the diseased mouse play a determinant for the diseased effect on the brain drug distribution.

Recently, we have reported the BBB transporter protein concentration in human (3, 4), therefore, we are ready to predict in vivo drug distribution in human when the intrinsic transport activity per protein in human would be determined. Pharmacoproteomics (PPx) based research (5) is useful approach for the understanding of transport function and its relevance to the drug distribution in the body.

1. *Pharm. Res.*, **25**: 1469-1483 (2008).
2. *J. Pharmacol. Exp. Ther.*, accepted (2011)
3. *J. Neurochem.*, **117**: 333-345 (2011).
4. *Mol. Pharm.*, 2011 Jun 27. <http://pubs.acs.org/doi/pdf/10.1021/mp200129p>
5. *J. Pharm. Sci.*, 2011 May 10, doi: 10.1002/jps.22612.

PL 2)

Richard Callaghan

Multi-drug efflux by P-glycoprotein; why has this protein not been stopped yet?

Richard Callaghan, Nuffield Department of Clinical Laboratory Sciences, University of Oxford, UK

Several ATP-Binding Cassette (ABC) transporters confer resistance to chemotherapy used in the treatment of cancer, bacterial infections and numerous parasitic infections. These proteins confer resistance by preventing the attainment of sufficient intracellular concentrations of cytotoxic drugs through active efflux. This efflux based resistance mechanism is simple, yet highly effective and a widely used strategy. The efflux pumps share the ability to bind, and translocate, a large number of functionally and chemically unrelated drugs. Consequently, the transporters have been collectively grouped as multidrug efflux pumps and share a common structural organisation. Each fully functional efflux pump contains two transmembrane domains (TMD) and two cytosolic nucleotide binding domains (NBD). The TMDs constitute the drug recognition sites and the translocation pathway through the membrane, whilst the NBDs provide energy for translocation (against large concentration gradients) from ATP hydrolysis.

P-glycoprotein (P-gp or ABCB1) is the archetypal multidrug efflux pump from the ABC family and has been established to confer resistance in numerous solid and blood-borne cancers. Given its prevalence and burden to chemotherapy, this protein has been the subject of intensive investigation for over three decades. Its astonishing ability to interact/translocate over 200 compounds has been suggested as a biological enigma. The research in our laboratories has a central objective to provide a molecular mechanism of drug translocation by P-gp. We have utilised biochemical, pharmacological and biophysical approaches to reach this objective and focussed on three specific areas:

How does P-gp bind so many compounds? Convention dictates that substrate binding requires high affinity, directional and selective chemical interactions with the protein. Should this be upheld by P-gp it may require the presence of multiple drug binding sites. Alternatively, the protein may have evolved a binding site capable of mediating the translocation of substrates without the requirement of specific interactions. We have adopted a pharmacological strategy to explore the nature of multi-drug binding by P-gp and two related drug efflux pumps. P-gp does indeed interact with substrates/inhibitors with high affinity and at multiple pharmacologically distinct sites. Moreover, the sites form a complex allosteric communication network.

What is the mechanism coupling drug binding with energy provision? P-gp is able to hydrolyse nucleotide in the absence of any bound substrate and was initially suggested to be an uncoupled transporter. However, the presence of drugs increases the rate of hydrolysis several-fold in a manner suggestive of coupling between domains. Furthermore, there are numerous two-way communication pathways between the drug binding sites and NBDs. We have detailed the involvement and nature of these pathways during the translocation process.

What topographical alterations occur during drug translocation? Structural and biophysical approaches have demonstrated large conformational changes within the TMDs in response to events at the NBDs. It is also clear that TM helices 6 and 12 are intimately involved in propagating this inter-domain coupling. We have demonstrated a number of local topographical changes in TM6/12 and that the two helices mediate their effects in a drug specific manner, indicating multiple communication routes.

Data from these research investigations have been assimilated into a potential translocation mechanism for P-gp, which may form a template for multidrug transport by ABC proteins.

PL 3)

Hanne Poulsen

Structure and Function of the Na,K-ATPase - Tail or Swift Leak

Hanne Poulsen(1), Himanshu Khandelia(2), J. Preben Morth(3), Maike Bublitz(1), Ole G. Mouritsen(2), Jan Egebjerg(4) & Poul Nissen(1)

(1) PUMPKIN ? Centre for Membrane Pumps in Cells and Disease, Aarhus University, Aarhus C, Denmark

(2) MEMPHYS ? Center for Biomembrane Physics, University of Southern Denmark, Odense, Denmark

(3) University of Oslo, Norway

(4) H. Lundbeck A/S, Copenhagen, Denmark

The sodium pump is essential for all animal life and is the target of ancient drugs and potent toxins. Its basic function is to pump three sodium ions out of the cell and take up two potassium ions, which creates chemical and electrical gradients across the plasma membrane. The gradients are utilized to drive numerous cellular processes, not least in the brain, where they provide the energy for many aspects of neuronal communication.

The sodium pump belongs to the family of P-type ATPases. At present, the crystal structures have been solved for four types of P-type ATPases, including the sodium pump, and they reveal a similar overall structure of the intracellular ATPase machinery that couples to the transmembrane ion transporting helices. The structures furthermore shine light on the important structural differences between functional states and between different pumps.

We have used electrophysiology to characterize the sodium pump functionally. From these studies, we have proposed that release the three sodium ions depends on a proton piston, and that the proton compensates for the vacant site in the pump when the two potassium ions are bound. The proton piston is controlled by the C-terminal region of the catalytic Na,K-ATPase subunit, and if the C-terminal structure is disturbed by mutations, they can cause the human diseases hemiplegic migraine with aura or rapid-onset dystonia parkinsonism. Currently, we are examining the requirements for the C-terminal structure further.

PL 4)

Aurelio Galli

Syntaxin: Teaching an old Dog New Tricks

E. CARTIER¹, P. HAMILTON¹, C.S. KARAM², Y. ZHANG², U. GETHER³, J. A. JAVITCH², P. G. ULERY-REYNOLDS⁴, H. MATTHIES¹, A. GALLI¹

¹*Mol. Physiol. and Biophysics, Vanderbilt Univ., Nashville, TN;*

²*Psychiatry and Pharmacol., Col. of Physician and Surgeons, Columbia Univ., New York, NY
Pharmacology,*

³*Panum Institute, Univ. of Copenhagen, Copenhagen, DK;;*

⁴*Neurol. and Psychiatry, Univ. of Texas, SW Med. Ctr., Dallas, TX*

Stx1 is phosphorylated at its N-terminus (Ser14) by Casein Kinase 2 (CK2). However, the functional role of Stx1 phosphorylation in neurotransmitter transporter function and AMPH-induced DA efflux is unknown. Our results suggest that AMPH stimulates Stx1 phosphorylation and this phosphorylation promotes Stx1/DAT association. Here, we examine whether these molecular events are required for AMPH-induced DA efflux. Consistent with this hypothesis, inhibition of CK2 strongly reduces Stx1 phosphorylation and DA efflux. Furthermore, mutation of Stx1 Ser14 to Ala prevents AMPH-induced Stx1 phosphorylation, inducing a decrease in DA efflux.

Based on data showing that both CK2 and Stx1 localize to presynaptic sites, we developed an in vivo model system for mechanistic examination of the role of phosphorylated Stx1 in AMPH action. We established two behavioral assays for DAT-mediated effects of AMPH in *Drosophila melanogaster*. In fly adult males, deletion of DAT or knockdown of CK2 in DA neurons, inhibits AMPH-induced grooming. In contrast, overexpression of CK2 in DA neurons enhances AMPH-induced behaviors. Furthermore, knockdown of CK2 selectively in DA neurons abolishes AMPH-induced DAT-mediated hyperlocomotion of fly larvae.

Here, we aimed to understand how DAT function, including AMPH-induced DA efflux and associated behaviors, is modulated by DAT interaction with phosphorylated Stx1 by CK2, together with post-translational modifications of DAT and other DAT-associated proteins.

SESSIONS:

Session 1: Trafficking of Neurotransmitter Transporters

Speaker 1: Ulrik Gether

The dopamine transporter: PICK1-ing new functions of C-terminal PDZ domain interactions

Ulrik Gether, Laboratory for Molecular Neuropharmacology, Lundbeck Foundation Center for Biomembranes in Nanomedicine, Department of Neuroscience and Pharmacology, University of Copenhagen, Copenhagen, Denmark,

The dopamine transporter (DAT) belongs to the family of Neurotransmitter:Sodium:Symporters (NSS family). DAT mediates rapid reuptake of dopamine from the synaptic cleft and is the principle target for widely abused psychostimulants, such as cocaine and amphetamines. A major goal of our research is to understand the molecular and cellular mechanisms governing the activity and availability of the DAT and related transporters in the presynaptic membrane and how these processes are influenced by drugs targeting DAT. We hypothesize that DAT is part of a highly complex multi-protein network within the presynaptic compartment controlling critical aspects of DAT function including targeting and distribution. PDZ (PSD-95/Discs-large/ZO-1 homology) domain proteins orchestrate the assembly of large multi-protein complexes in specific cellular locations and DAT contains at its extreme C-terminus a PDZ domain binding sequence known to interact with the PDZ domain protein PICK1 (protein interacting with C kinase-1). To assess in vivo the physiological significance of C-terminal PDZ domain interactions for DAT function we generated using homologous recombination techniques a novel DAT knock-in mouse strain with a modified C-terminus. In the mutant mice, the C-terminal PDZ target sequence (-LLV) was substituted for three alanines (-AAA) resulting in abolished PDZ domain interactions. The DAT-AAA mice were characterized by dramatic loss of DAT expression in the synaptic terminals in the striatum. Western blotting analysis, radioligand binding experiments and synaptosomal [3H]-dopamine uptake experiments suggested altogether that ~10% functional transporter was present in DAT-AAA mice as compared to wild type. The dramatic loss of synaptic DAT in the DAT-AAA mice caused locomotor hyperactivity and an impaired response to amphetamine challenge. Midbrain dopaminergic cultures demonstrated that DAT-AAA is fully glycosylated and not retained within the ER. By use of recently developed fluorescently tagged cocaine analogues we analyzed by confocal live imaging the trafficking properties of the transporter in DAT-AAA midbrain neurons. Our data suggested a marked relative increase in constitutive internalization of DAT-AAA as compared to the wild type, implying impaired surface stability at the presynaptic membrane possibly attributable to loss of PDZ-domain mediated scaffolding of DAT. Our observations were substantiated by the analysis of another DAT knock-in mouse in which PDZ domain interactions were disrupted by adding an extra alanine to the C-terminus of the transporter (DAT+Ala). A corresponding phenotype was, however, not found in PICK1 knock-out mice suggesting that yet unknown PDZ domain proteins play an essential role in governing presynaptic distribution of DAT in vivo.

Speaker 2: Haley Melikian

Dopamine Transporter Dancing Partners: Rin GTPase takes the stage

Haley E. Melikian

Dopamine (DA) homeostasis in the brain impacts multiple physiological and pathological processes, including movement control, mood and addiction. Extracellular DA availability is tightly controlled by Na⁺/Cl⁻-dependent presynaptic reuptake, facilitated by the plasma membrane dopamine transporter (DAT). DAT is also the primary target for both therapeutic and addictive substances, such as methylphenidate (Ritalin), bupropion (Wellbutrin, Zyban), cocaine and amphetamines, all of which potently inhibit DAT activity. A wealth of evidence has demonstrated that DAT dynamically traffics to and from the cell surface and that PKC activation and amphetamine exposure acutely modulate DAT surface expression. Multiple molecular mechanisms have been implicated in basal and regulated DAT trafficking. We recently reported that DAT carboxy terminal residues 587-590 (FREKLAYAIA) encode a PKC-sensitive endocytic regulatory region. Using this domain as bait in a yeast two-hybrid screen, we identified the neuronal GTPase Rin (Rit2) as a DAT-interacting protein. Coimmunoprecipitation, FRET and in vitro pulldown assays demonstrate that Rin directly interacts with DAT, but not the neuronal GABA or serotonin transporters. Co-localization studies reveal that DAT/Rin interactions occur primarily in lipid raft microdomains. PKC activation increases DAT/Rin interactions, and mutations that increase DAT endocytosis also increase DAT/Rin interactions. Internalization studies using Rin GTPase mutants and shRNA-mediated Rin knockdown demonstrate that Rin is required for PKC-stimulated DAT internalization. These studies implicate Rin in membrane trafficking and suggest that the endocytic machinery facilitating regulated DAT internalization utilizes Rin and its downstream signaling partners.

Session 2: The Structural Basis of Transport

Speaker 1: Manuel Palacín

Molecular basis of substrate-induced permeation by an amino acid antiporter

Manuel Palacín

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Transporters of the amino acid, polyamine and organocation (APC) superfamily play essential roles in cell redox balance, cancer and aminoacidurias. The bacterial L-arginine/agmatine antiporter, AdiC, is the main APC structural paradigm and shares the “5+5 inverted repeat” fold found in other families like the Na⁺-coupled neurotransmitter transporters. The available AdiC crystal structures capture two states of its transport cycle: the open-to-out apo and the outward-facing Arg⁺-bound occluded. However, the role of Arg⁺ during the transition between these two states remains unknown. Recently, we reported the crystal structure at 3.0 Å resolution of the Arg⁺-bound AdiC N101A mutant in the open-to-out conformation, completing the picture of the major conformational states during the transport cycle of the “5+5 inverted repeat” fold-transporters (1). The N101A structure is an intermediate state between the previous known AdiC conformations. The Arg⁺-guanidinium group in the current structure presents high mobility and delocalization, hampering substrate occlusion and resulting in a low translocation rate. Further analysis supports that proper coordination of this group with residues Asn101 and Trp293 is required to transit to the occluded state, providing the first clues on the molecular mechanism of substrate-induced fit in a “5+5 inverted repeat” fold-transporter. The pseudo-symmetry found between repeats in AdiC, and in all fold-related transporters, restrains the conformational changes, in particular the transmembrane helices rearrangements, which occur during the transport cycle. In AdiC these movements take place away from the dimer interface, explaining the independent functioning of each subunit.

(1) Kowalczyk L, Ratera M, Paladino A, Bartoccioni P, Errasti-Murugarren E, Valencia E, Portella G, Bial S, Zorzano A, Fita I, Orozco M, Carpena X, Vázquez-Ibar JL, Palacín M. (2011) Molecular basis of substrate-induced permeation by an amino acid antiporter. *Proc Natl Acad Sci U S A*. 108:3935-40.

Speaker 2: Baruch Kanner

Mechanism of Sodium-Coupled Neurotransmitter Transport in the Brain.

Baruch Kanner

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Neurotransmitter transporters have a critical role in regulating neurotransmission and are targets for psychostimulants, anti-depressants and other drugs. We have studied the mechanism of prototypes of each of the two families of neurotransmitter transporters: the Neurotransmitter:sodium symporters (NSS) and the Glutamate transporters. Both of these classes power the accumulation of the neurotransmitter against huge concentration gradients by cotransport with sodium, thereby utilizing the electrochemical sodium ion gradient present across the plasma membrane of the cell. In addition to sodium, other ions are employed as well, namely chloride in the NSS and potassium in the Glutamate transporters. Insights from structural studies of bacterial homologues (LeuT for NSS and GltPh for the Glutamate transporters) coupled with functional studies by our group and by others of their counterparts from the brain, have shown that these two classes of transporters have developed distinct mechanisms to render their binding pocket sequentially accessible to the outside and the inside of the cell: rotation of "bundle" helices relative to a fixed domain in the membrane for the NSS and an "elevator" mechanism for the Glutamate transporters, in which the so-called transport domain moves vertically by around 18Å relative to the "trimerization" domain. Due to the lower resolution of the GltPh structure as compared to that of LeuT, there still is uncertainty on the sodium binding sites in the Glutamate transporters. Functional studies, to examine if some of the conserved acidic amino acid residues are likely to play a role in cation binding in the Glutamate transporters, will be presented.

Speaker 3: Niels Christian Danbolt

GABA and glutamate transporters around synapses: numbers matter

Niels Chr. Danbolt, University of Oslo, Oslo, Norway.

The major inhibitory and the major excitatory neurotransmitters in the mammalian brain are, respectively, gamma-aminobutyric acid (GABA) and glutamate. These transmitters activate receptors at the cell surface and are inactivated by cellular uptake catalyzed by transporter proteins. The mammalian genome contains four genes encoding GABA transporters (GAT1, slc1a1; GAT2, slc6a13; GAT3, slc6a11; BGT1, slc6a12) and five genes encoding glutamate transporters (EAAT1-5). Mice deficient in EAAT1, EAAT2, GAT1 have phenotypes supporting the notion that these transporters are important for transmitter removal. In contrast, the physiological roles of EAAT4 and EAAT5 appear to be related to their chloride conductance rather than their transport functions. The roles of BGT1, GAT2 and EAAT3 remain elusive. To localize these transporters by immunocytochemistry it is necessary to compare labeling of tissue from wild-type and knockout littermate pairs processed in parallel in order to distinguish between background labeling and labeling representing the transporters. We show that EAAT3 is present in cell bodies and dendrites of most if not all hippocampal neurons. It is neither in glial cells nor in axon-terminals. The tissue content of EAAT3 is about 100 times lower than that of EAAT2. If all EAAT3 transporters are at the surface, the average EAAT3 density would be approximately 90 transporters per square micrometer. The levels of BGT1 in the brain were even lower, 2-3 orders of magnitude lower than those of GAT1. Most of the tiny amounts of BGT1 present in the brain are concentrated in the leptomeninges, while brain parenchyma itself is virtually BGT1 deficient. BGT1 is neither detected in brain endothelium nor in ependymal cells. The highest BGT1 expression levels (mRNA and protein) are found in hepatocytes and in the renal medulla. The low densities of EAAT3 and BGT1 suggest that these transporters play metabolic roles and that they are not involved in rapid clearance of neurotransmitter released during synaptic transmission.

Session 3: Serotonin Transporters: from structure to clinics

Speaker 1: Walter Sandtner

THE CONDUCTING STATE OF THE HUMAN SEROTONIN TRANSPORTER IS ENTERED VIA AN INWARD FACING CONFORMATION - A UNIFYING CONCEPT OF SEROTONIN TRANSPORTER ASSOCIATED CURRENTS

Klaus Schicker¹, Zeljko Uzelac¹, Joan Gesmonde², Simon Bulling¹, Thomas Stockner¹, Michael Freissmuth¹, Stefan Boehm¹, Gary Rudnick², Harald H Sitte¹ and Walter Sandtner¹

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Serotonin (5-HT) uptake by the human serotonin transporter (hSERT) is driven by ion gradients. The stoichiometry of transported 5-HT and ions is predicted to result in electroneutral charge movement. However, hSERT mediates a current when challenged with 5-HT. This discrepancy can be accounted for by an uncoupled ion flux. Here we investigated the mechanistic basis of the uncoupled currents and its relation to the conformational cycle of hSERT. Our observations support the conclusion that the conducting state underlying the uncoupled ion flux is entered from the inward-facing state of the transporter with K⁺ bound. We identified conditions associated with accumulation of the transporter in the inward facing conformation. Manipulations that increased the abundance of inward facing states resulted in enhanced steady-state currents. We present a comprehensive kinetic model of the transport cycle, which recapitulates salient features of the recorded currents. This study provides a framework for exploring transporter-associated currents.

Speaker 2: Jacob Andersen

Molecular determinants for selective recognition of antidepressants in the human serotonin and norepinephrine transporters

Jacob Andersen

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Inhibitors of the serotonin and norepinephrine transporters (SERT and NET, respectively) are widely used in the treatment of major depressive disorder. Although SERT/NET selectivity is a key determinant for the therapeutic properties of these drugs, the molecular determinants defining SERT/NET selectivity are poorly understood. In the present study, the structural basis for selectivity of the SERT selective inhibitor citalopram and the structurally closely related NET selective inhibitor talopram is delineated. A systematic structure-activity relationship study allowed identification of the substituents that control activity and selectivity towards SERT and NET and revealed a common pattern showing that SERT and NET have opposite preference for the stereochemical configuration of these inhibitors. Mutational analysis of non-conserved SERT/NET residues within the central substrate binding site (denoted the S1 site) was performed to determine the molecular basis for inhibitor selectivity. Changing only five residues in NET to the complementary residues in SERT transferred a SERT-like affinity profile for citalopram into NET, showing that the selectivity of this inhibitor is determined by amino acid differences in the central S1 binding site of the transporters. In contrast, the activity of the NET selective inhibitor talopram was largely unaffected by any mutations within the S1 site of SERT and NET as well as in the outer vestibule of NET, suggesting that citalopram and talopram bind to distinct sites on SERT and NET. Together, these findings provide important insight into the molecular basis for SERT/NET selectivity of antidepressants, which can be used to guide rational development of novel transporter inhibitors with fine-tuned transporter selectivity.

Speaker 3: Lukas Pezawas

NEURAL ACTIVITY OF THE ANTERIOR CINGULATE IS RESPONSIBLE FOR REMISSION MAINTAINANCE IN DEPRESSION AND CORRELATED WITH PLATELET 5-HT RE-UPTAKE VELOCITY

Lukas Pezawas, Christian Scharinger, Christian Kassess, Beate Hartinger, Ulrich Rabl, Tina Hofmaier, Lucie Bartova, Wolfgang Huf, Klaudius Kalcher, Kersten Diers, Zeljko Uzelac, Gerald Pail, Thomas Perkmann, Andreas Meyer-Lindenberg, Siegfried Kasper, Michael Freissmuth, Matthaeus Willeit, Rupert Lanzenberger, Christian Windischberger, Harald Esterbauer, Burkhard Brocke, Ewald Moser, Harald H Sittte

INTRODUCTION: The clinical course of Major Depressive Disorder (MDD) is characterized by waxing and waning of major depressive episodes (MDEs). While striking evidence hints towards alterations within a neural circuitry encompassing the amygdala and the anterior cingulate cortex (ACC), neural mechanisms contributing to remission once treatment has been discontinued are far less investigated. Specifically, the subgenual ACC (sACC) has been associated with unpleasant emotion processing, depression, and serotonin transporter (5-HTT) re-uptake inhibition. However, it remains obscure whether 5-HTT function directly translates into altered sACC activity under physiological conditions. Here, we will present results of two studies addressing these scientific questions. Firstly, we investigated neural adaptation mechanisms in a rare sample of remitted drug-free depressive (rMDD) patients and secondly, we studied neural 5-HTT distribution within the cingulate cortex in healthy controls and tested, if correlates of platelet 5-HT uptake velocity (V_{max}) converge at regions of maximal 5-HTT concentration.

METHODS: 1. We performed a magnetic resonance imaging (MRI) study obtaining functional and structural MRI measures in 38 drug-free rMDD patients and 38 gender-matched healthy controls. 2. We performed another MRI study obtaining PET ($n=8$), and functional as well as structural MRI measures, V_{max} and 5-HTT genotypes in 48 healthy controls. MRI data were collected using a 3T Siemens TIM Trio Scanner, structural images were acquired using a 3D MPRAGE, functional data were obtained via a phase corrected blipped gradient echo, single shot echo planar imaging sequence. Subjects underwent a functional block-design matching paradigm comprising visual unpleasant emotional stimuli. [^{11}C]DASB has been used as PET tracer. Platelet 5-HT uptake was assessed by using a dilution technique with unlabeled 5-HT to reveal V_{max} and K_m values. Detailed information on methods being used will be presented during the lecture.

RESULTS: 1. rMDD patients showed significant bilateral activation decreases of the amygdalae and sACC. Brain systems level measures demonstrated increased functional and structural connectivity between the amygdala and sACC in patients and have been correlated with clinical course information. 2. We report a significant coupling between platelet V_{max} and neural activity of the sACC, a region also showing maximal 5-HTT availability within the cingulate cortex in our PET study. Furthermore, we demonstrate that 5-HTTLPR cannot sufficiently explain this linkage.

CONCLUSIONS

Our findings suggest that remission of MDEs in MDD constitutes an active process of neural system stabilization by compensating for a dysfunctional amygdala-sACC circuitry. Furthermore, our study results indicate that neural system stabilization in MDD can only be achieved by an inhibition of amygdalar activity beyond the level observed in healthy subjects, thereby implicating that remission cannot be interpreted as *restitutio ad integrum* from a neurobiological perspective. Findings of our second study expand the understanding of serotonergic neurotransmission by relating *in vitro* measures of extracellular serotonin concentration to *in vivo* human brain function, thereby supporting the idea of a systems biology approach in the search of diagnostic and treatment-response markers for depression.

Session 4: ABC-transporters heavy traffic in the liver

Speaker 1: Peter Chiba

Interaction of ABC-transporters with small molecules and their potential role as pharmacological chaperones

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The majority of drugs used in the treatment of human disease are small molecular weight compounds. Their biological action relies on the ability of specific interaction with macromolecular targets, many of which are integral membrane proteins. Presently less than 1,300 unique chemical entities are contained in more than 21,000 approved drugs. At least 14 human ABC-transporters are associated with genetic diseases and several of them contribute to drug resistance by preventing pharmacological compounds from reaching their cellular target structures. Missense mutations in ABC-transporters frequently cause aberrant folding, which is sensed by the ER-quality control system to ensure that incorrectly folded structures are not processed along the secretory pathway. The free energy optimum of a protein allowing efficient folding, but also sufficient functional flexibility, is narrow. Small molecule folding correctors termed pharmacological chaperones (PCs) are able to reestablish the delicate energetic balance. Most of these PCs share binding sites with physiological effector molecules and are thus classified as active-site compounds. Nonsynonymous single-nucleotide polymorphisms in at least 10 different ABC-transporters of the A, B, C and G subfamilies have been shown to interfere with correct folding and trafficking. Examples of diseases with altered trafficking behavior are intrahepatic cholestasis (ABCB11), cystic fibrosis (ABCC7) and gout (ABCG2). In 2007 the first pharmacological chaperone has been approved for human use. We employed a biology oriented synthesis approach to improve our understanding of the interaction principle of small molecules with active sites of ABC transporters. Small molecules were functionalized in pharmacophore-neutral positions to contain benzophenones or trifluoromethyl-aryl-diazirines as otherwise stable precursors of photon-induced carbonyl-biradical or carbene intermediates. Introduction of these chemical functionalities allow formation of a covalent bond between target transporters and small molecules upon irradiation at 340-360nm. Subsequent proteolytic degradation of the protein and ligand-directed purification allowed identification of ligand modified component peptide fragments by MALDI-TOF-mass spectrometry. ESI-tandem mass spectrometry experiments are presently ongoing. Insights into the dual interaction mode of active site ligands with the paradigm ABC-transporter ABCB1 (P-glycoprotein) will be discussed along with the design principle for photoligands and chemical capture tools. Successful use of selected tool compounds in photolabeling and rescue experiments of ER-retained ABCB1 mutants will be demonstrated.

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Speaker 2: Sander Smits

Analyzing the haemolysin secretion machinery of *E. coli*

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In Gram-negative bacteria, Type 1 secretion systems (T1SS) export their cognate substrates in a single step directly from the cytosol to the extracellular medium without the formation of periplasmic substrate intermediates. T1SS facilitate the secretion of structurally and functionally distinct proteins, which include pore-forming toxins like haemolysin A (HlyA), hemophores (HasA), adenylate cyclases, lipases, proteases and surface layer proteins. HlyA is a member of the repeats in toxin (RTX) protein family as it contains glycine rich peptide repeats in the C-terminal domain.

One of the best-studied T1SS is the haemolysin (Hly) secretion system of *Escherichia coli*. The Hly translocator consists of the inner membrane protein HlyB, which is an ATP binding cassette (ABC) transporter, the outer membrane protein TolC and the membrane fusion protein HlyD anchored in the inner membrane. The interaction of the substrate HlyA with HlyB and HlyD triggers recruitment of TolC, thereby creating a continuous, but transient channel-tunnel from the cytosol directly into the extracellular medium

Here, I will summarize our recent progress concerning the molecular analysis of the HlyA secretion machinery, in particular the ABC transporter HlyB and present a model that tries to covers various aspects of recognition and secretion in this T1SS.

Speaker 3: Bruno Stieger

Canalicular Microdomains and Formation of Primary Bile in Rat Hepatocytes

Bruno Stieger, Division of Clinical Pharmacology and Toxicology, University Hospital, 8091 Zurich, Switzerland

Body detoxification and bile formation are key functions of the liver. Bile is mainly composed of bile salts and phospholipids, which form mixed micelles. They act as acceptors for poorly water soluble substances destined for biliary excretion, e.g. cholesterol. In addition, bile contains organic anions, e.g. glutathione or metabolites of endogenous substances and of drugs. Bile is formed by hepatocytes, which take up bile salts and other cholephilic substances from the portal blood plasma and secrete them across the canalicular plasma membrane into bile. This occurs against steep concentration gradients and involves several ATP-binding cassette (ABC) transporters. Biliary lipid secretion requires the bile salt export pump BSEP, which exports bile salts from hepatocytes. Due to their detergent properties, bile salts release from the outer hemileaflet of the canalicular plasma membrane phosphatidylcholine, which is replaced in the outer leaflet by the multidrug resistance protein MDR2. Release of cholesterol from the canalicular membrane into bile is facilitated by ABCG5/ABCG8. Total bile salt concentration in bile can exceed 50 mM. Consequently, the canalicular membrane needs protective elements against the detergent action of bile salts. We have demonstrated that the rat canalicular plasma membrane contains two types of microdomains or lipid rafts, which are enriched in cholesterol, sphingolipids and the raft marker alkaline phosphatase: Triton X-100 and Lubrol WX microdomains. Caveolin-1 partitions into Lubrol rafts, but barely into Triton rafts, while reggie-1 and -2 partition into Triton rafts and partially into Lubrol rafts. ABC-transporters, with the exception of minor amounts of Mrp2 and Mdr1, are absent from Triton rafts, but partition to variable extents into Lubrol rafts. Hence, Lubrol rafts contain the machinery necessary for canalicular bile formation and might represent a protective element of the canalicular membrane and the starting point for canalicular lipid secretion. Subsequently, we were able to demonstrate that bile salts above their critical micellar concentration induce canalicular microdomains with a similar composition as Lubrol rafts.

ABC-transporters, e.g. Bsep or Abcg2 or Mdr1 have been shown to be modulated by plasma membrane cholesterol content, whereby their activity positively correlates with cholesterol content. We have confirmed and extended these results: Transport activity of BSEP is stimulated by an increased concentration of cholesterol, predominantly by increasing the v_{max} . This stimulation occurs for the 444V and 444A variants, the latter variant being a susceptibility factor for drug-induced liver injury and cholestasis of pregnancy. BSEP does not display cooperativity irrespective of cholesterol and substrate concentration. Also MRP2 is stimulated by increasing cholesterol concentrations. In contrast, MRP2 exhibits cooperativity, which depends on the membrane cholesterol content in conjunction with the molecular weight of the substrate. This mild cooperativity is only observed with substrates of relatively low molecular weight. We conclude that the binding pocket of BSEP may be much narrower than the binding pocket of MRP2, which may explain the difference in the substrate specificity of the two transporters.

Speaker 4: Michael Trauner

Role of canalicular phospholipid export pump (ABCB4) for pathogenesis and therapy of cholestatic liver diseases

Michael Trauner, Division of Gastroenterology and Hepatology, Department of Internal Medicine III, Medical University of Vienna, email: michael.trauner@meduniwien.ac.at

Hepatobiliary ATP-binding cassette (ABC) transporters are responsible for biliary excretion of various endo- and xenobiotics including bile salts, cholesterol and phospholipids. Hereditary and acquired alterations in the expression and function of these transport systems result in cholestasis with subsequent intrahepatic and systemic retention of potentially toxic biliary constituents. Chronic bile duct diseases (cholangiopathies), such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) present a major clinical challenge and medical treatment options (preventing liver transplantation) are urgently needed. Disturbances in the delicate balance between bile salts, phospholipids and cholesterol can cause and/or aggravate bile duct injury as exemplified in *Abcb4*^{-/-} mice lacking the canalicular phospholipid export pump (ABCB4) and hereditary forms of cholestasis due to ABCB4 deficiency ranging from neonatal cholestasis to adulthood ductopenia. Genetic variants of ABCB4 may contribute to the course of cholangiopathies such as PBC and PSC.

Ursodeoxycholic acid (UDCA), the standard treatment of PBC stimulates ABCB4 but is of limited efficacy in the treatment of PSC. Fibrates also enhance ABCB4 expression and may be effective in non-responders to UDCA. The *Abcb4*^{-/-} mouse model provides the unique opportunity to study novel treatment options (e.g. norUDCA) for chronic cholangiopathies. norUDCA is a side chain-shortened C23 homologue of UDCA and is more resistant to conjugation with taurine or glycine than UDCA. Unconjugated norUDCA undergoes absorption by cholangiocytes, returns to the liver and is resecreted into bile. Such cholehepatic shunting leads to a bicarbonate-rich hypercholerisis and also results in improved targeting to the injured bile duct epithelium. As such, norUDCA (but not “conventional” UDCA) reverses sclerosing cholangitis in the *Abcb4*^{-/-}-cholangiopathy model. Recent studies have adressed the exciting therapeutic opportunities of novel ligands for bile acid receptors (FXR, TGR5) and could identify stimulation of bicarbonate-rich choleresis as potential common therapeutic mechanism. These effects make these novel bile acid derivatives attractive candidate drug for the treatment of cholangiopathies such as PSC and PBC.

POSTERS:

Thursday, 8th of September

P 1) Neural dynamic interplay between amygdala and anterior cingulate cortex in remitted Major Depressive Disorder

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Objectives: The natural course of Major Depressive Disorder (MDD) is characterized by alternating phases of illness and symptom remission. While previous neuroimaging studies demonstrated increased activation within a neural circuitry encompassing the amygdala and the anterior cingulate cortex (ACC) as well as decreased functional coupling between both regions during acute MDD, limited information is available on how the brain is actually managing to maintain stable remission.

Methods: Within a multi-center, cross-sectional functional and structural magnetic resonance imaging (MRI) study, we investigated the neural dynamic interplay between amygdala and ACC in 38 adult drug-free remitted MDD (rMDD) patients and 38 adult gender-matched healthy volunteers without any psychiatric life-time diagnosis. All participants underwent a functional paradigm in block-design fashion comprising one emotional (angry/fearful faces and fearful/threatening scenes) and one neutral control (simple shapes) matching task.

Results: Our analyses of local brain measures indicated significantly attenuated amygdala reactivity as well as a border-line significant decrease of subgenual ACC (sACC) activation in rMDD patients in comparison to healthy subjects. On a brain systems level, we detected a significant increase of functional and structural connectivity between amygdala and sACC in rMDD patients as compared to controls. Moreover, we performed behavioral correlation analyses in order to explore possible effects of clinical course characteristics of rMDD patients as well as neuroticism, which significantly increased in the rMDD group. Interestingly, we found significant correlations between amygdala-sACC functional connectivity and cumulative number of depressive months as well as significant correlations between amygdala-anterior midcingulate cortex (aMCC) functional connectivity and neuroticism in the rMDD group as compared to the control group.

Conclusions: Our findings constitute opposing alterations in neural circuits known to be affected during acute MDD and point towards an active neural adaptive process that predominantly counterbalances dysfunctional amygdala-ACC circuitry during remission.

P 2) Protein interactions and regulation of the ABC transporter MRP4 (ABCC4)

Gabriele Jedlitschky, Marie-Luise Kromrey, Yvonne Schaletzki, Sebastian Zang, Susanne Bröderdorf, Igor Mosyagin and Heyo K. Kroemer

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Background

The ABC transporter MRP4 (ABCC4) transports many drugs as well as a number of endogenous signalling molecules. Besides its unique substrate specificity MRP4 distinguishes itself from other ABCC subfamily members by its cell type-dependent membrane localization. MRP4 may localize to either apical or basolateral plasma membranes in polarized cells or to intracellular storage compartments as the delta-granules of platelets¹. Disturbance of the correct localization as observed in patients with delta-storage pool deficiencies may result in impaired function². It has been recognized that protein-protein interactions are important for transporter localization and function. We therefore investigated protein interactions of MRP4 especially in platelets as well as its regulation on the transcriptional as well as post-transcriptional level.

Methods and results

We screened for possible adaptor proteins binding to the COOH-terminus of MRP4 containing a PDZ-interaction motif in platelets and in kidney-derived epithelial cells. The approach included a pull-down system with a glutathione S-transferase/MRP4 fusion protein and with a synthetic peptide consisting of the COOH-terminal MRP4 sequence coupled to a sepharose matrix as well as co-staining in immunofluorescence microscopy. Candidates identified as possible interaction partners of MRP4 in platelets include a heat shock protein and a PDZ domain-containing adaptor protein, which was so far described to be mainly expressed in neurons. Furthermore, MRP4 was found to be regulated by protein kinase C activation resulting in an internalization of the transporter.

Conclusions

MRP4 directly interacts with several adaptor proteins which may be essential for its correct localization and function and is also regulated by protein kinase C.

1. Jedlitschky et al.: The nucleotide transporter MRP4 (ABCC4) is highly expressed in human platelets and present in dense granules, indicating a role in mediator storage. *Blood*. 2004; 104:3603.

2. Jedlitschky et al.: Role of MRP4 (ABCC4) in Platelet Adenine Nucleotide-Storage: Evidence from Patients with delta-Storage Pool Deficiencies. *Am. J. Pathol.* 2010; 176:1097.

P 3) Dicysteine Crosslinking in the human Serotonin Transporter

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The human serotonin transporter (hSERT) actively regulates the concentration of serotonin in the synaptic cleft by mediating reuptake of serotonin. hSERT is a molecular target for drugs used to treat affective disorders such as depression.

Conformational changes of hSERT occur during substrate and ligand binding and during translocation of serotonin. During the transport cycle, hSERT shifts from an outward facing conformation to an occluded state ending in an inward facing conformation.

A bound ligand induces a given conformation on hSERT and vice versa; the conformation of hSERT has profound implications on ligand characteristics.

The interplay between ligand binding, transport, and hSERT conformation is not fully understood. This interplay can be studied by analyzing the reactivity of introduced cysteines directed by the structure of LeuT and our homology model of hSERT. Furthermore, the effect of locked conformations established by cysteine crosslinking on ligand binding kinetics can reveal important aspects about the impact of protein conformations on ligand binding.

We here characterize a battery of introduced extracellular cysteines and their impact on transport activity. We also show how cysteine crosslinking can be used to manipulate transporter conformation and how this affects ligand binding.

P 4) GABA REVERSE TRANSPORT BY THE NEURONAL COTRANSPORTER GAT1: INFLUENCE OF INTERNAL CHLORIDE DEPLETION

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Neurotransmitters are normally taken up in neurons and glial cells through the transporter proteins using sodium and chloride electrochemical gradient. These neurotransmitter transporters (NSS) are essential for the correct cellular communication. Leakage of neurotransmitters in the extracellular fluid is involved in pathological and/or abnormal conditions of the central nervous system. Elevated extracellular levels of glutamate cause excitotoxicity, while higher than normal GABA concentrations have been hypothesized to concur in causing epileptic seizures. The neurotransmitter cotransporters can work in a reverse transport mode, as demonstrated for example in the GABA transporter GAT1, or in the dopamine transporter DAT1, in which reversal of the operation is promoted by the presence of amphetamines.

We have undertaken experiments aimed to investigate the role of internal chloride on the efficiency of reverse neurotransmitter transport by the neuronal transporter GAT1. Taking advantage of the possibility of co-expressing other transporters in the *Xenopus* oocytes, we also explored the possibility that physiologically regulated internal chloride changes might influence the reverse transport process

Briefly, reverse GABA transport was induced by intracellular GABA injection in *Xenopus* oocytes heterologously expressing the rat neuronal transporter rGAT1. By measurement of outward transport currents in voltage-clamp, as well as by [³H]GABA efflux determinations, reverse transport was estimated. Experiments confirm that when a high concentration of GABA is present in the cytoplasm GAT1 can operate in reverse mode. The reverse transport is affected by the internal concentrations of the two ions, Na⁺ and Cl⁻, known to be involved in the forward transport cycle. To examine the role of intracellular sodium its concentration must be increase and for intracellular chloride, the concentration must be reduced. Rising concentration of Na⁺ by injection of Na solution, enhanced the ratio of outward to inward transport current. The depletion of internal Cl⁻ was achieved by the co-expression of ion cotransporter KCC2 (K⁺-Cl⁻ co-transporter) and by overnight exposure to hypotonic Cl⁻-free extracellular solution. The efficacy of treatments was verify by testing, in the presence of the ionophore ionomycin, the reversal potential (E_{rev}) of the Ca²⁺-induced Cl⁻ current endogenously present in the *Xenopus* oocytes. The reduction of Cl⁻ concentration induced a significant decrease in the relative reverse transport current. This result may confer an interesting physiological relevance to the role of intracellular chloride, in fact suggests that reverse GABA transport may be regulated by KCC2 activity during early neuronal development or in injury and epilepsy, similarly to GABA receptors.

In addition, any other conditions favouring higher intracellular chloride levels, such as under-expression of chloride exporters, defects in osmoregulation or in ionic homeostasis, may be considered potentially relevant in affecting the balance between forward and reverse mode of neurotransmitter transport.

P 5) Subcellular distribution and sorting of the orphan carrier SLC10A4 in neuronal cell lines

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SLC10A4 belongs to the solute carrier family SLC10 whose founding members are 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 identified as a transporter for sulfoconjugated steroid hormones and was referred to as sodium-dependent organic anion transporter (SOAT; SLC10A6) [2]. These three well-characterized transporters are typically expressed at the plasma membrane and their transport is strictly sodium-dependent. In 2004, a further new member of this carrier family, SLC10A4, was cloned and showed specific expression in cholinergic and monoaminergic neurons and innervations [3]. Recently, immunoprecipitation and electron microscopy proved the expression of SLC10A4 in synaptic vesicles, rather than in the plasma membrane [4]. The SLC10A4 protein consists of 437 amino acids, exhibits a seven transmembrane domain topology with an N_{exo}/C_{cyt} orientation and shows 30 % sequence identity to NTCP. But in contrast to NTCP, SLC10A4 has a clear vesicular expression pattern as demonstrated in the catecholaminergic CAD mouse cell line, mouse neuroblastoma NS20Y cells, human SH-SY5Y cells and stably transfected SLC10A4-HEK293 cells. As the most prominent structural difference between NTCP and SLC10A4 is represented by an additional N-terminal domain of about 60 amino acids in the SLC10A4 protein, we speculated that this domain might be responsible for the vesicular sorting of SLC10A4. Therefore, we generated a SLC10A4-NTCP chimera in which this SLC10A4-domain was added to the N-terminus of NTCP (SLC10A4-NTCP). This construct, together with NTCP and SLC10A4, was transfected into CAD cells. Protein sorting was analyzed by immunofluorescence and functional activity was assessed by transport assay with [³H]taurocholate (a prototypical substrate of NTCP). Interestingly, neither membrane sorting, nor taurocholate transport activity were affected for the SLC10A4-NTCP construct in comparison with NTCP, meaning that the N-terminal domain of SLC10A4 is not involved in the vesicular sorting of the SLC10A4 protein. Further chimeric constructs between NTCP and SLC10A4 are currently under investigation focusing on the C-terminus of the proteins in order to further clarify the vesicular sorting of SLC10A4.

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P 6) Virtual screening against Alzheimer's acetylcholinesterase: Suggested workflow for compound prioritization using 3D pharmacophore, docking & 2D QSAR analysis

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In this study, we suggest a new workflow for the identification and prioritization of potential compounds targeted against Alzheimer's disease acetylcholinesterase, an important neurotransmitter catalysing enzyme and a validated target for the development of anti- Alzheimer agents. First, we have performed an integrated pharmacophore and structure-based virtual screening using Maybridge small molecule database; subsequently molecular docking patterns from known actives to the receptor were applied for scoring and ranking the virtual screening hits.

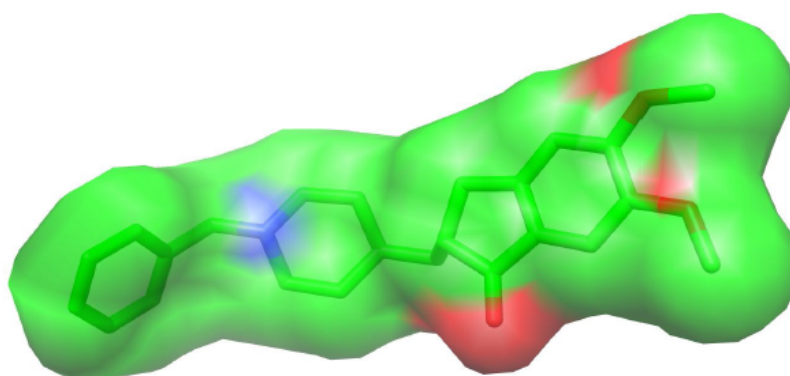


Figure 1 3D View. Energy-minimized three dimensional (3D) structure and molecular surface representation of E20 (Donezipil) taken from PDB 1EVE.

Using this approach we screened ~61,000 Maybridge compounds and 14 compounds were prioritized as promising virtual screening hits. Though using a dock scoring and quantitative structure activity approach, the results were not biased toward the chemical classes of the known actives and the proposed compounds were structurally diverse with low molecular weights and structural complexities. Our results suggest that structure-based virtual screening Figure 1 3D View.

Energy minimized three dimensional (3D) structure and molecular surface representation of E20 (Donezipil) taken from PDB 1EVE. coupled with the molecular docking and 2D QSAR should be a useful to a medicinal chemist or combinatorial chemist to pick up the new molecular starting points for medicinal chemistry optimization for the design of novel acetylcholinesterase inhibitors for Alzheimer's disease.

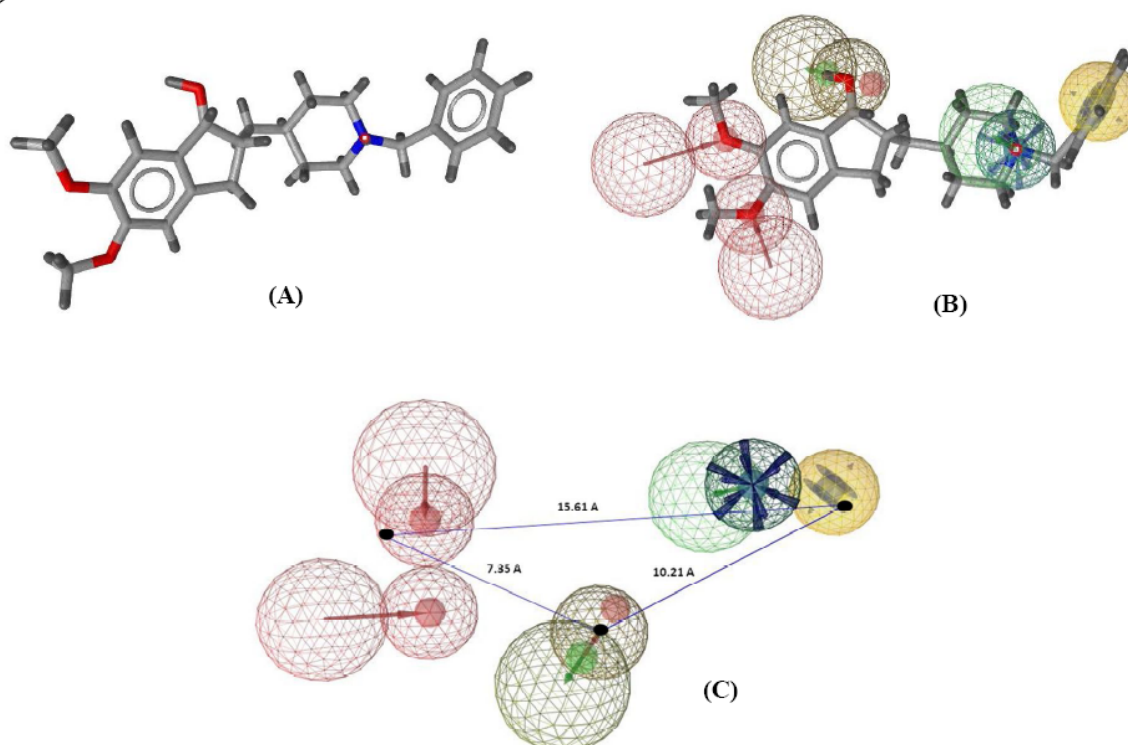


Figure 2 (A) 3D structure of Acetylcholinesterase inhibitor Donezipil from PDB: 1EVE (B) Pharmacophore of its abstract molecule (C) Pharmacophore features are one hydrophobic aromatic, one ring aromatic, two hydrogen-bond donor, three hydrogen-bond donor, one positive ionizable and five excluded volumes

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P 7) Comparison of the substrate pattern of the SLC10 carriers NTCP, ASBT, and SOAT

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The solute carrier family SLC10 comprising seven members formerly was known as the family of “sodium bile acid cotransporters”. The founding member of this family, the Na⁺/taurocholate cotransporting polypeptide (NTCP; SLC10A1) shows 35% sequence identity to the apical sodium-dependent bile acid transporter (ASBT; SLC10A2) and 33% to a novel member of the SLC10 carrier family, the sodium-dependent organic anion transporter (SOAT; SLC10A6). However, within the SLC10 carrier family the highest sequence identity of 42% exists between SOAT and ASBT [1]. While NTCP is mainly expressed in the liver and transports bile acids, sulfoconjugated bile acids, and sulfoconjugated steroid hormones, ASBT is expressed in the brush border membrane of ileocytes, in the apical domain of the proximal tubules in the kidney, and the apical membrane of cholangiocytes in the liver. Its substrate pattern is restricted to bile acids. Both transporters are important factors for the maintenance of the enterohepatic circulation of bile acids between the liver and the gut. SOAT has been shown to transport sulfoconjugated steroid hormones in hormone-responsive tissues such as testis, placenta and mammary gland [2].

Because NTCP, ASBT, and SOAT show high phylogenetic relationship, but clear differences in the substrate pattern, we directly compared the substrate specificities and kinetic parameters of these carriers in stably transfected HEK293 cells. In contrast to NTCP and ASBT, cholic acid, taurocholic acid, deoxycholic acid, glycochenodeoxycholic acid, glycodeoxycholic acid, glyoursodeoxycholic acid, taurodeoxycholic acid, taurochenodeoxycholic acid, tauroursodeoxycholic acid, and ursodeoxycholic acid were not transported by SOAT. SOAT and NTCP, but not ASBT, showed transport activity for the sulfoconjugated steroid hormones dehydroepiandrosterone sulfate, estrone 3-sulfate, pregnenolone sulfate with comparable transport kinetics. Interestingly, tauroolithocholic acid was transported by all three carriers. Affinity of this substrate was in the order ASBT>>NTCP>SOAT with Km values of $5.9 \pm 1.8 \mu\text{M}$, $18.4 \mu\text{M} \pm 2.3$, and $19.3 \pm 6.8 \mu\text{M}$, respectively.

In conclusion, gene expression and functional properties are in general quite different between the SLC10 carriers NTCP, ASBT, and SOAT. On the one hand these carriers have high structural homology and close phylogenetic relationship (in particular ASBT and SOAT). Identification of a common substrate for all three carriers will be helpful to further localize the substrate binding domains for bile acids and sulfoconjugated steroid hormones in the respective carriers.

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P 8) Adaptation of microdialysis techniques to study brain penetration of drugs and substrate - inhibitor interactions of ABC transporters at the blood-brain barrier in mice.

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Dual- and triple-probe microdialysis (MD) techniques have been applied at SOLVO to provide evidence that quinidine (QND) is a good probe-substrate to study ABCB1 function at the BBB in rats (1).

The aim of this study was to adapt the dual-probe microdialysis approach for simultaneous assessment of test drug distribution in blood and brain of anesthetized mice. Our further objective was to set up a validated MD-system to detect ABCB1 substrate/inhibitor interactions at the BBB using QND and PSC-833 in anesthetized and non-anesthetized mice.

Results: Four sets of experiments were run to study brain penetration of QND and ABCB1 substrate/inhibitor interactions in anesthetized mice. Data are shown in Table 1. The AUC_{brain}/AUC_{blood} was much lower in control animals than the ratio in animals treated with the combination of PSC-833 and QND. It is clear that inhibition of P-gp by PSC-833 increases the brain penetration of QND not only in rats (1) but in mice, too. Similarly to the anesthetized mice, PSC-833 increased the brain penetration of QND in awake mice, too using both single or dual-probe approach. MD experimentation in awake animals allows longer sample collection period, and even provides possibility for repeated drug administration.

Table 1: Pharmacokinetic parameters of unbound QND in brain and blood dialysate samples from anesthetized mice.

Treatment groups (mg/kg; i.p.)	Brain		Blood		Brain/Blood	
	AUC (pmol/ml*min)	C _{max} (pmol/ml)	AUC (pmol/ml*min)	C _{max} (pmol/ml)	AUC	C _{max}
QND (20)	3560±663	24±3.	76844±12409	434±54	0.05±0.01	0.06±0.01
PSC-833 (10) & QND (20)	15078±4000 ⁺	78±18 ⁺	89614±4959	519±28	0.17±0.05 ⁺	0.15±0.04 ⁺
QND (40)	11052±1841 ^{**}	62±9 ^{**}	158216±14787 ^{**}	889±86 ^{***}	0.07±0.01	0.07±0.01
PSC-833 (10) & QND (40)	25076±7178	150±26 ⁺	136371±19172	747±132	0.18±0.04 ⁺	0.22±0.04 ^{**}

Values are means ± SEM, n = 4-6. ^{**}, ^{***} Significant differences between treatment groups of QND 20 mg/kg vs. QND 40 mg/kg (p < 0.01 and p < 0.001, respectively). ⁺, ^{**} Significant differences between QND treated vs. PSC-833& QND treated groups (p < 0.05 and p < 0.01, respectively).

Methodology: Anesthetized mice: Vascular probes (Microbiotech, Sweden) were implanted into the jugular vein of NMRI male mice under chloral hydrate anesthesia. Then the animals were placed in a stereotaxic frame for implanting a brain probe in the frontal cortex (FC) and for running experiments. Sampling (perfusion rate: 0.5 μ L/min) was performed between 120 min before and 300 min after dosing the mouse intraperitoneally (i.p) with QND or with the combination of QND and PSC-833. Experimentation with non-anesthetized mice: It starts with implantation of a guide cannula into the FC followed a recovery period. One day prior to an MD experiment brain probes (CMA/7) were inserted in the tissue of FC under light anesthesia and the animals were placed into an MD-system. In dual-probe studies vascular probes were also implanted and the animals were staying in a movement-responsive animal system during the experiments. Determination of QND in dialysate samples: QND levels were measured by HPLC with fluorescence detection using excitation wavelength of 248 nm and emission wavelength of 460 nm.

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P 9) Kinetic evaluation of substrate binding to the Leucine Transporter (LeuT); the apparent two-substrate model is a consequence of slow kinetics and non-equilibrated protein-ligand complexes

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The Leucine Transporter serves as a structural and functional model for neurotransmitter transporters. Recently, a model has been proposed based on binding experiments using the Scintillation Proximity Assay (SPA) where simultaneous occupation of two substrate sites is required for transport. Such a two-substrate model has eluded detection in numerous functional studies on mammalian neurotransmitter transporters.

We here show that the apparent two-substrate model for LeuT proposed by others is a byproduct of very slow binding kinetics and SPA experiments that were not allowed to reach to equilibrium. By detailed analysis of substrate binding to and dissociation from LeuT and secondary site mutants of LeuT we can determine kinetic parameters that are fully consistent with a one-substrate model and does not support the proposed two-substrate model.

P 10) Identification and analysis of critical amino acids in the transmembrane 2 of human OATP1B1

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The organic anion-transporting polypeptides (OATPs, gene symbol SLCO) are a family of transporters that mediate sodium-independent transport of wide spectrum of structurally independent compounds. Substrates of OATPs are mainly amphipathic organic molecules including bile salts, hormones and their conjugates, toxins and different drugs. Besides these charged compounds, they also transport uncharged drugs such as glycosides digoxin and ouabain. Because of their broad substrate specificity, wide tissue distribution and the involvement of drug-drug interaction, OATPs have been extensively recognized as key determinants of absorption, distribution, metabolism and excretion (ADME) of various drugs, xenobiotics and toxins. As an important structure in membrane proteins, transmembrane domains (TM) have been found to be crucial for properly targeting the protein to cell membrane as well as carrying out transport functions in transporters. In the present study, we used site-directed mutagenesis and alanine-scanning to study amino acids within TM2 of OATP1B1 and so far identified a row of 4 amino acids within this transmembrane domain are important for its transport activity. Further study showed that these amino acids affect OATP1B1 transport activity in different ways.

P 11) Interactions of fluorone dyes with the sodium pump observed by fluorescence methods

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Na^+/K^+ -ATPase (sodium pump) is the protein responsible for the active transport of Na^+ and K^+ ions across the plasma membranes of most higher eukaryotes. The Na^+ and K^+ gradients are required for maintaining membrane potentials, cell volume and secondary active transport of other solutes, e. g. , the transcellular transport processes in intestine, glands and kidney. Modulation in the activity of the Na^+/K^+ ATPase, by drugs or intrinsic factors has been reported to affect a variety of physiological phenomena.

Dyes, or similar molecules, are commonly used in medicine as diagnostic, analytic or „photochemotherapy“ tools. They are also commonly used in food industry. Eosin Y, a fluorescein analogue, can attach to Na^+/K^+ -ATPase and its fluorescence behaviour is very sensitive to conformation changes of the enzyme¹. Eosin Y, fluorescein and erythrosine belongs to the family of „fluorone dyes“, and they are often used as drug components, which don't have to be focused on Na^+/K^+ -ATPase regulation (virostatic drugs). We expect that all of these commonly used dyes can interact with Na^+/K^+ -ATPase, based on structure similarity with eosin, and can cause its inhibition. Because of this fact, there can be undesirable sideeffect of medicaments, in the field of pharmacology or diagnoses, or toxic risk for consumers, in the field of food industry.

Our experiment, using a combination of steady-state and time-resolved fluorescence techniques enabled observation of interactions of fluorone dyes with Na^+/K^+ -ATPase. In view of the fact that dyes should occur in cytoplasm, we expect that they will interact with cytoplasm part of the enzyme. Because of this presumption, interaction of fluorescein analogues with Na^+/K^+ -ATPase is tested on two different systems. First, with enzyme isolated from pig brain, secondary, with large cytoplasmic loop of Na^+/K^+ -ATPase located between fourth and fifth transmembrane segment of Na^+/K^+ -ATPase alpha-subunit (C45 loop).

Our results suggest that all studied dyes (rose bengal, erythrosine and eosin Y) with the exception of fluorescein can interact with both used systems. We are able to determinate dissociation constants of these interactions. Based on the enzyme activity measurements, we can describe physiological impact of these interactions.

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P 12) Modulation of UCP1-mediated proton transport by phosphoinositides.

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UCP1, a member of mitochondrial uncoupling protein subfamily, is expressed in brown adipose tissue and plays a major role in thermogenesis in newborn mammals, hibernators and cold adapted rodents. Moreover, the regulation of UCP1 activity is supposed to reduce obesity and to improve insulin sensitivity. Here we study the effect of different acidic phosphoinositides (PIPs) on the activity of the purified and reconstituted proton transporter UCP1. Previously it was shown that PIPs activate potassium and calcium channels. All PIPs (PI(3,5)P₂, PI(5)P or PI(3,4)P₂) used in this work have eight-carbon saturated aliphatic chains (diC₈) and are water soluble. The addition of PIPs to the planar bilayers containing both UCP1 and arachidonic acid led to a significant increase in total membrane conductance (G) in contrast to membranes containing either UCP1 or arachidonic acid or none of both. The observed effect is transient. Its kinetic is compatible with the kinetic of proton release due to phosphoinositide binding to the membrane. To confirm this hypothesis the comparison of short-chain and the long-chain PIPs concerning their absorbance to the membrane is required.

P 13) Benzochalcones as selective inhibitors of BCRP

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Breast Cancer Resistance Protein (BCRP/ABCG2), belongs to the ATP binding cassette family of transport proteins. BCRP has been found to confer multidrug resistance in cancer cells, transporting large molecules with amphiphilic character using energy from ATP hydrolysis. BCRP substrates include the anticancer drugs mitoxantrone, topotecan, irinotecan and its active metabolite, etoposide and flavopiridol. A strategy to overcome resistance due to BCRP overexpression is the investigation of potent and specific BCRP inhibitors.

The aim of current study was to investigate different multi-substituted benzochalcones for their BCRP inhibition. We synthesized several benzochalcones with different substituents on ring B of the chalcone structure. All synthesized compounds were tested by Hoechst 33342 accumulation assay to determine inhibitory activity in MCF7-MX and MDCK cells expressing BCRP. The compounds were also screened for their P-glycoprotein (P-gp) and Multidrug resistance-associated protein 1 (MRP1) inhibitory activity by calcein AM accumulation assay and were found to be selective towards inhibition of BCRP. In general 5',6'-benzochalcones were found to be more potent as compared to 3',4'-benzochalcones. It was observed that there is greater influence of substituents on ring B of benzochalcones and presence of 3,4-dimethoxy substitution on ring B was found to be optimal for BCRP inhibition.

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P 14) Application of post-docking molecular dynamics simulations to evaluate binding modes of propafenone analogues in P-gp

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The membrane-bound ABC-transporter P-glycoprotein (P-gp, ABCB1, MDR1) is responsible for the export of xenotoxic compounds out of the cell. However, due to its chemically diverse substrate profile, overexpression of P-gp is highly related to the acquisition of multidrug resistance (MDR), one major reason for the failure of antibiotic and antitumor chemotherapy. A promising concept for overcoming these obstacles would be the identification of potential P-gp inhibitors. Propafenone analogs are known for their P-gp modulating activity and several *in silico* studies could identify a clear structure-activity relationship between this class of molecules and P-gp [1]. Nevertheless, the absence of structural information of the protein hindered the identification of the concrete binding mode.

Using homology modeling the putative structure of human P-gp in two different conformations, representing the high- and low-affinity state of the protein, has been determined. Additionally, the application of a knowledge-based docking protocol retrieved a small number of possible binding modes, which suggested important interactions on the molecular level [2]. As docking only represents a snapshot of the protein-ligand interaction, the binding modes were further investigated by means of molecular dynamics computer simulations. By including a phospholipid bilayer in the simulations, not only the flexibility of the complex but also the effect of the membrane could be considered in the calculations. Post MD-analysis tools were applied to compare the stability of the system and of the different binding modes.

In agreement with the 'classical' interpretation of the catalytic cycle of P-gp, the MD simulations suggest that propafenone binding poses in the high-affinity state may be more stable than in the low-affinity conformation. The high-affinity state poses were characterized by hydrogen bonds that were either formed directly with the protein or mediated by a water molecule. The functional groups of the ligand contributing to hydrogen bonding have been previously shown to be important for P-gp activity. On the protein side the amino acid residues Tyr307 and Tyr310 were found to be mainly involved in hydrogen bond interactions. The docking poses in the low-affinity structures primarily formed interactions with water molecules in the central pore, which may result in weakened protein-ligand interactions. These findings form the basis for future simulation studies that could help in elucidating the differences in activity between propafenone analogs.

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P 15) Beyond the Crystals: Spectroscopic Experiments and MD Simulations Reveal Na⁺/K⁺-ATPase Cytoplasmic Headpiece Dynamics and Electrostatic Surface Potential Changes Induced by Ligand Binding

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Recently, we can see rapid development of techniques that are capable to show the structure of biomolecules on the atomic resolution. However, it turns out that the static structure itself is not sufficient for the understanding how the biomolecules work. The secret of biomolecules is in their dynamic properties, i.e. in their ability to adopt various conformations and to interact with various ligands.

Crystallographically determined structures of P-type ATPases published during the past decade revealed many secrets of these transmembrane pumps and provided a good basis for further experiments. Despite lower spatial resolution, spectroscopic techniques have several advantages over crystallography, particularly the ability to work with natural ligands (without inhibitors) under physiological conditions and to observe enzyme dynamic [1].

The catalytic cycle of Na⁺/K⁺-ATPase is usually described by the Albers-Post scheme, which postulates that the enzyme adopts two major conformations E1 and E2. We have analyzed the influence of the cytoplasmic ligands (i.e. ATP and/or Mg²⁺) on the conformation of the large cytoplasmic loop connecting the transmembrane helices 4 and 5 (C45) by means of the intrinsic tryptophan fluorescence and MD modeling. Our data revealed that the C45 is found in the closed conformation in the absence of any ligand, in the presence of Mg²⁺ only, or in the simultaneous presence of Mg²⁺ and ATP. Binding of the ATP alone (i.e. in the absence of Mg²⁺) induced open conformation of the C45. Our data are consistent with the model, where ATP binding to the low-affinity site induces conformational change of the cytoplasmic part of the enzyme, traditionally attributed to E2→E1 transition, and subsequent Mg²⁺ binding to the enzyme-ATP complex induces in turn conformational change traditionally attributed to E1→E2 transition [2]. Preliminary experiments with the entire enzyme seem to confirm this hypothesis.

Further, we have introduced an original method for experimental evaluation of the changes in the electrostatic surface potential (ESP). Monitoring of the conformational changes from this point of view complements the abovementioned observations of the changes in the C45 geometry. The ATP-binding induced ESP changes on the reverse side of the C45, and we hypothesize existence of a cytoplasmic channel leading the transported cations toward/from the binding sites in the transmembrane region. Furthermore, our data suggested a mechanism, how the information about the nucleotide binding could be propagated to the distal sites of the enzyme. Especially, our data indicate that the bottom part of the C45 communicates with the short cytoplasmic loop C67, which further transmits the information to the C-terminal transmembrane helices [3].

Acknowledgement: This work was supported by grant of Czech Science Foundation P301/10/0883 and by grant of Czech Ministry of Health NT11071 and by research project MSM 6198959215 from Czech Ministry of Schools, Youth and Sports.

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P 16) Key Residues Controlling Conformational Transitions in the Serotonin Transporter

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The crystal structure of the human serotonin transporter homolog, LeuT, advanced the understanding of ligand and substrate binding to SERT. In addition, it allowed modeling of the large conformational changes that takes place during substrate translocation. The transporter must harbour a mechanism that senses the simultaneous binding of the necessary ions and an appropriate substrate before triggering the conformational changes leading to translocation. However, the molecular basis of the trigger mechanism that controls the equilibrium between the different conformational stages remains elusive. Guided by homology models, we combined different biochemical methods and molecular dynamics simulations to characterize the interplay between three key residues in SERT that control the conformational equilibrium of the transporter and allows the transporter to discriminate between substrates and inhibitors.

P 17) Subgenual Anterior Cingulate Cortex Mediates Functional Relation Between Emotional and Cognitive Regions in the Human Brain

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Introduction: It has long been established that there is an interdependence of emotional and cognitive processes in the human brain. The exact mechanisms, however, have only partially been elucidated. Several influential publications have on the one hand demonstrated significant functional connectivity between amygdala and dorsolateral prefrontal cortex (DLPFC) as well as top down regulatory effects of dorsolateral cortical regions on key emotional regions, in particular the amygdala. The pathways of supposed bottom-up regulation of the amygdala on cortical regions, on the other hand, have not yet been satisfactorily explored. We thus developed a new functional magnetic resonance (fMRI) paradigm inducing experimental variation of the BOLD signal in both amygdala and key cognitive regions, including the DLPFC, allowing to identify mediators of amygdala-DLPFC functional connectivity.

Methods: Subjects underwent a novel cognitive fMRI paradigm comprising two tasks (2-back, labeling) in a blocked fashion with emotional stimuli (positive, negative, neutral valence) presented using pseudorandom interstimulus intervals. Activation of emotional as well as cognitive regions was established using classical general linear modeling following preprocessing of fMRI data. Only then, whole brain mediation analysis on the (simple) connectivity between amygdala and DLPFC was performed using the method published by Baron and Kenny (1986).

Results: Our newly developed paradigm robustly activated bilateral amygdala as well as DLPFC regions. The subgenual anterior cingulate cortex (sACC) was identified as the most prominent mediator of amygdala-DLPFC connectivity.

Conclusions: Preliminary results indicate a positive correlation of sACC with amygdala contrasting a negative correlation between DLPFC and amygdala. The sACC was identified as the most prominent mediator of amygdala-DLPFC connectivity. This region having the highest serotonin transporter density in the human frontal lobes, we further aim to investigate the role of 5-HTTLPR within this circuit affecting both, emotional and cognitive functionality over the forthcoming months.

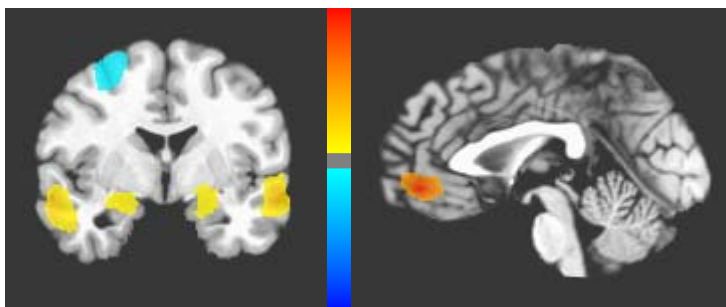


Figure: Illustration demonstrating that the highest coupling of amygdala and DLPFC (left) within the whole brain converge at the sACC (right).

P 18) NMDA receptor activation downregulates KCC2 in a calpain-dependent manner

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Abstract

The neuron-specific K-Cl cotransporter isoform 2 (KCC2) is the main Cl⁻ extruder in central neurons and is responsible for the low intracellular Cl⁻ concentration and the consequent inwardly-directed Cl⁻ electrochemical gradient driving hyperpolarizing GABA_A receptor-mediated Cl⁻ currents (Blaesse et al. 2009). Several studies have shown a marked reduction in KCC2 expression leading to a depolarizing shift in EGABA following epileptiform activity and excitotoxic events. A recent study has shown a link between excitotoxic NMDA receptor (NMDAR) activation and a decrease in functional expression of KCC2 caused by dephosphorylation and decrease in total protein (Lee et al. 2011). Nevertheless, the detailed mechanisms whereby KCC2 is lost upon excessive NMDAR activation remain unknown. Here, we show using whole-cell patch-clamp recordings and quantitative immunoblotting that inhibition of protein synthesis using cycloheximide does not result in rapid loss of KCC2 function or total KCC2 protein. This indicates that the basic turnover of KCC2 is low and that mere cessation of KCC2 gene expression is not sufficient to cause a rapid decrease in KCC2 functionality and protein levels under pathophysiological conditions. However, NMDAR activation-triggered downregulation of KCC2 is sensitive to calpeptin and MDL-28170, inhibitors of the calcium-dependent protease calpain, identifying KCC2 as a novel calpain substrate. The present data point to enhanced calpain-mediated degradation as a mechanism for rapid KCC2 downregulation triggered by NMDAR activation. The role of calpain-mediated KCC2 degradation in in vitro models of seizure activity will be discussed.

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P 19) Platelet serotonin uptake predicts cingulate cortex activation

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Subgenual anterior cingulate cortex (sACC) activation has been associated with emotion processing, major depressive disorder, and serotonin transporter (5-HTT) re-uptake inhibition. However, there is no evidence available today whether 5-HTT function directly translates into altered sACC activity under physiological conditions. Hence, we measured maximal serotonin uptake velocity (Vmax) in blood platelets, a proxy for neural 5-HTT function, as well as blood-oxygen-level dependent (BOLD) signaling in forty-eight healthy subjects.

Our results indicate a significant coupling between platelet Vmax and neural activity of the sACC ($p=0.038$, $t=-3.77$, $r=-0.45$), a region also showing maximal 5-HTT availability within the cingulate cortex in our study. Furthermore, we analyzed whether this correlation is mediated by genetic variation within the 5-HTT gene and demonstrate that 5-HTTLPR, a functional promoter polymorphism within the 5-HTT gene, cannot sufficiently explain this linkage.

Our findings expand the understanding of serotonergic neurotransmission by relating in vitro measures of extracellular serotonin concentration to in vivo human brain function. Thereby, our study results support the idea of a systems biology approach in the search of diagnostic and treatment-response markers for major depressive disorder.

P 20) Computational estimation of biliary excretion of drugs using transporters

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The liver is the important site of biliary excretion of many drugs and compounds. Organic anion transporting polypeptides (OATPS/SLCO), organic anion transporters (OATs) and organic cation transporters (OCTs) are hepatic uptake transport proteins in liver and p-glycoprotein, MDR3, BSEP, MRP2 and BCRP are highly expressed in hepatic efflux transporter [1]. High biliary excretion of a number of compounds has been linked to their high affinity to specific transporters, indicating their role in drug excretion via the bile. For example biliary excretion of mitoxantrone is linked to BCRP and P-gp activity [2], and biliary excretion of β -lactam antibiotics is associated with multidrug resistance-associated protein 2 (MRP2) [3].

Biliary excretion is one of the main elimination pathways for drugs and/or their metabolites. The aim of this study is investigating the structural determinants of biliary excretion and further, the role of P-gp, Mrp2 and BCRP in this elimination route. In this study, a diverse dataset of 244 compounds was used which mainly consisted of the dataset published by Morris et al [4] but also included some additional compounds from other sources. The data consisted of the percentage of dose excreted into bile as intact compound measured in vivo in rat. An exhaustive literature survey was conducted to identify amongst these compounds the substrates of various transporter types. Molecular modelling followed by molecular descriptor calculations enabled development of linear regression and nonlinear regression tree, random forest, boosted tree and MAR Spline models. The models were compared and validated using a test set consisting 25% of the compounds.

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P 21) Molecular determinants of a novel inhibitor of the betaine-GABA transporter

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Background

γ -Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain. Normal GABAergic neurotransmission is critically dependent on the termination of signaling by removal of GABA from the synaptic cleft and reuptake into synaptic processes by GABA transporters (GATs). Four different subtypes of GATs exist (GAT-1, betaine-GABA transporter-1 (BGT-1), GAT-2, GAT-3), each with a unique subcellular distribution. Inhibition of specific GAT subtypes enhances GABAergic transmission and poses a novel pharmacological strategy for treating disorders associated with GABAergic hypoactivity such as epilepsy, anxiety, neuropathic pain and sleep disorders. The antiepileptic drug Gabitril® (tiagabine) is an inhibitor of GAT-1 and acts to increase the concentration of GABA in the synaptic cleft. Despite the proven therapeutic effects of GAT inhibitors in epilepsy, neuropathic pain and anxiety, Gabitril is the only GAT inhibitor on the market. The fact that the different GAT subtypes have unique subcellular distributions, argues for distinct functional roles. However, the physiological function and therapeutic potential of the non-GAT-1 subtypes remains unclear, as no true selective potent drugs against these transporters are available. The overall aim of this project is to gain molecular and pharmacological insight into GAT subtypes, most notably BGT-1 by identifying and characterizing subtype-selective compounds.

Project objectives

The objective of the project is to investigate the molecular determinants responsible for the binding as well as the mechanism of action of the compound BC-TK-08 (Hit8), a recently identified selective BGT-1 inhibitor (unpublished data). Hit8 is the first true selective inhibitor of BGT-1 and represents a unique tool and lead structure for advancing the understanding of the molecular pharmacology of BGT-1 and its potential role as a drug target. Hit8 is a non-competitive inhibitor of BGT-1 and is expected to bind to an allosteric rather than an orthosteric site on the transporter. However, the exact binding site of Hit8 is unknown. I will try to identify this site by 1) generating functional chimeras between BGT-1 and GAT-3 in order to narrow down relevant binding regions and 2) identify which amino acids participate in the binding using epitope-tagged point-mutated transporters. The second objective can be completed independently by analyzing alignments of the four GATs, based on the reasonable assumption that the subtype selectivity of Hit8 stems from interaction with an allosteric region of low sequence conservation. The chimeras and mutants will be analyzed using the [³H] GABA uptake assay and the FLIPR-Membrane Potential (FMP) assay.

Outcome

Identification of the binding site for Hit8 will provide information about the molecular basis for its selective binding in BGT-1 and direct the design and development of second-generation analogues using structure-based methods.

P 22) Amphetamine- and cocaine-sensitisation in Calmodulin kinase II alpha knock-out mice – preliminary results of a pilot study

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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 α (CaMKII α) can physically bind the DAT C-terminus and phosphorylate N-terminal serines (Fog et al.2006). We could also show that pharmacological inhibition as well as genetic ablation of CaMKII α reduces amphetamine-triggered reverse transport in ex vivo preparations of the mouse striatum (Steinkellner et al., unpublished data).

The DAT is the major target of psychostimulant drugs like amphetamines and cocaine which both lead to increases in extracellular dopamine. These increases are associated with the initiation of drug addiction. Additionally, CaMKII α is known to be crucial for learning and memory and is highly implicated in the acquisition of a drug memory. We thus sought to elucidate the role of the interaction between DAT and CaMKII α using a psychostimulant sensitisation approach to examine whether CaMKII α -deficient mice display addicted behaviour to amphetamine and cocaine. Preliminary results show that CaMKII α knock-out mice respond, unexpectedly, with a significantly higher locomotion to both amphetamine and cocaine after an acute injection, but they show severe deficits in sensitisation after chronic administration of either amphetamine or cocaine.

P 23) TRUNCATIONS IN THE AMINO TAIL REVEAL A REGION KEY TO SUPPORTING AMPHETAMINE-INDUCED EFFLUX BY THE SEROTONIN TRANSPORTER

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The serotonin transporter (SERT) terminates neurotransmission via reuptake of serotonin from the synaptic cleft. Upon stimulation with amphetamines, SERT switches into the outward mode of transport to rapidly release serotonin into the synapse. We have previously shown that truncation of the first 64 residues of the amino terminus of SERT leads to abolition of amphetamine-induced efflux (1). This was comparable to the effects of a single point mutation of a juxtamembrane threonine residue at position 81 (1). In our present study, truncations in the SERT amino terminus were generated to locate the region responsible for the maintenance of amphetamine-induced efflux by the transporter. The truncation mutants were created by removing 22 (delta 22-SERT), 32 (delta 32-SERT) or 42 (delta 42-SERT) residues of the amino tail of hSERT. Additional alanine scanning mutagenesis was also performed within a stretch of amino acid residues 32-42. We first examined the cellular expression pattern for all mutated SERTs by confocal microscopy, which revealed no differences compared to the wild type SERT, i.e. all mutants were normally expressed at the cell surface. Functional analysis of the mutants showed modest changes in their substrate uptake properties (no significant changes in K_m values and a moderate decrease in the V_{max} value only for delta 42-SERT). Similarly, there were no dramatic changes in the K_D and B_{max} values of imipramine or in the K_i values of *p*-chloroamphetamine and ibogaine, determined by radiolabelled binding assays. However, while amphetamine-induced efflux was unimpaired for the delta 22-SERT and to some extent decreased for delta 32-SERT, it was completely abolished for the delta 42-SERT truncation mutant. Hence, our results shed new light on the functional role of the amino terminus and identify the segment encompassing residues 32-42 as a key region in regulating amphetamine-induced efflux by SERT.

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P 24) MS-Binding Assays – An Efficient Alternative to Radioligand Binding Applied to the Human Serotonin Transporter

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MS-binding assays have the potential to be a valuable and versatile tool in the drug discovery process. They offer all advantages of conventional and widely accepted radioligand binding assays without drawbacks such as the need for labelling or the handling of hazardous radioactive material.^[1]

The aim of the present study was to apply this concept to the human serotonin transporter (hSERT) – the primary target for drugs used in the treatment of emotional disorders such as depression. Binding experiments based on a HEK293 cell line stably expressing hSERT and (S)-Fluoxetine as native marker were established in a 96 well plate format in analogy to conventional radioligand binding assays. In contrast to the latter, however, (S)-Fluoxetine bound to hSERT was quantified after liberation from its binding site by LC-ESI-MS/MS.

The LC-ESI-MS/MS method developed for this purpose (API 5000, Fluoxetine (m/z) 310 → 148, internal standard [²H₅]-Fluoxetine (m/z) 315 → 153) enabled rapid and reliable quantification of (S)-Fluoxetine from 3 nM down to 50 pM (LLOQ) within 60 s per sample (chromatographic cycle time). A validation of the established LC-ESI-MS/MS method with respect to linearity, intra- and inter-batch accuracy and precision showed that the requirements according to the FDA guidance for bioanalytical methods were met.^[2]

In this way, K_d - and B_{max} -values for (S)-Fluoxetine as well as K_i -values for a series of serotonin reuptake inhibitors could be determined in saturation and competition experiments, respectively. Furthermore, saturation and competition experiments employing (R)-Fluoxetine as non labelled marker could be performed and analyzed under the conditions established for the (S)-enantiomer. All results were found to be in good accord with literature data obtained in radioligand binding experiments.

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P 25) Improvement of transmembrane sequence coverage of P-glycoprotein using pepsin and combinations of chymotrypsin with mass compatible detergents

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The ABC transporter P-glycoprotein is a key player in xenobiotic and drug transport as well as in cancer multidrug resistance, by mediating the active outward efflux of structurally highly diverse compounds. In order to explain this polyspecific behaviour, photoaffinity labeling via photoreactive substrates/inhibitors followed by protease digestion and identification of labelled peptide fragments with electrospray ionization mass spectrometry can be applied. A prerequisite for successful photolabeling experiments is a preferably complete sequence coverage of P-glycoprotein. Unfortunately the transmembrane regions of the protein in particular have proven to be difficult to approach for proteases. As the transmembrane domains of P-glycoprotein are considered to contain the drug-binding sites, it is important to increase the sequence coverage of these regions. To achieve this aim, purified human P-glycoprotein reconstituted into proteoliposomes was digested in-solution with the protease pepsin alone and combinations of chymotrypsin with a mass compatible anionic or zwitterionic acid labile surfactant, respectively. In doing so, optimization with regard to digestion time, detergent concentration and digestion buffer was accomplished. Both digestion strategies led to an improvement in transmembrane sequence coverage of the protein compared to digestion with trypsin alone or chymotrypsin without addition of detergent.

P 26) Linking Antidepressants' Drug Targets to Side Effects

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Antidepressant agents not only bind to one primary target, they also have effects on a variable number of other receptors within the whole body. These secondary targets may cause additional beneficial or undesirable side effects. Side effects are a common cause for the intolerance and discontinuation of the antidepressant therapy. Furthermore secondary effects influence the choice of the substance. Thus it is important to comprehend the relationship between receptors antidepressants bind to, and their side effects.

The aim of this study is to statistically link the specific receptor binding profile of antidepressants to their side effects.

Therefore we retrieved the binding affinities measured as K_i value from the PDSP Database (<http://pdsp.med.unc.edu>) for 21 antidepressants and 17 receptors including SERT (serotonin transporter), NET (norepinephrine transporter), DAT (dopamine transporter) and several serotonin, norepinephrine, muscarine, histamine, and dopamine receptors. Besides we searched the Cochrane Database of Systemic Reviews for the incidence of following side effects: agitation and anxiety, anorexia, constipation, diarrhea, dizziness, dry mouth, fatigue, gastrointestinal symptoms, headache, hypotension, insomnia, nausea and vomiting, sexual problems, sleepiness, and suicide attempts. Data was extracted from 6 reviews containing altogether 280 randomized, double-blind studies investigating approximately 33.000 patients.

For the statistical analysis we built two matrices, one X matrix containing the K_i values, and the Y matrix consisting of the incidence of side effects. These two data matrices were related via PLS (partial least square) regression. For every side effect we received positive or negative coefficients for every receptor indicating a positive or negative correlation.

Our data suggests that our PLS regression model indicates a high correlation between K_i values and side effect frequencies and is suitable to link adverse events to specific receptor systems.

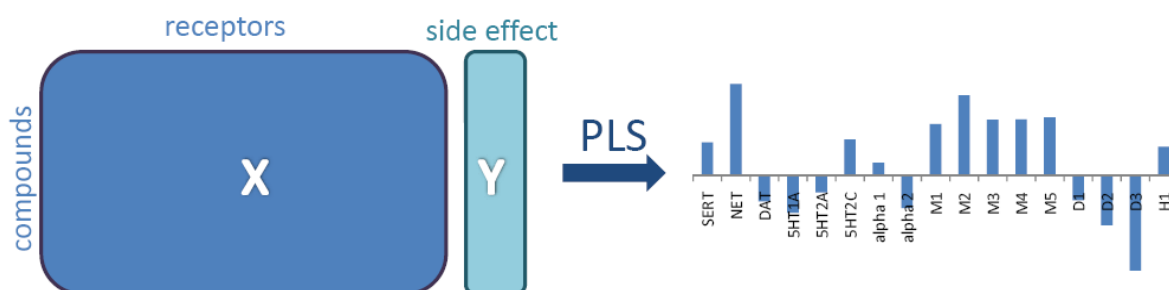


Figure: Schematic diagram of the PLS model showing the positive or negative correlation (columns on the right) for each receptor (X) with one side effect (Y).

P 27) Binding strength and dynamics of MFZ2-12 interaction with the serotonin transporter on living cells by single molecule force spectroscopy

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The serotonin transporter (SERT) terminates neurotransmission by removing serotonin from the synaptic cleft. In addition, it is the site of action of antidepressants (which block the transporter) and of amphetamines (which induce substrate efflux). The forces involved in binding to and blocking of the transporter are unknown. Here, we used atomic force microscopy (AFM) to probe single molecular interactions between the serotonin transporter and MFZ2-12 (a potent cocaine analog) in living CHOK1 cells. For the AFM measurements MFZ2-12 was immobilized on AFM tips by using a heterobifunctional crosslinker. By varying the pulling velocity in force distance cycles drug/transporter complexes were ruptured at different force loadings allowing for mapping of the interaction energy landscape. We derived chemical rate constants from these recordings and compared them with those inferred from inhibition of transport and ligand binding: k_{off} values were in good agreement with those derived from uptake experiments; in contrast, the k_{on} values were scaled down when determined by AFM. Our observations provide new insights into the energy landscape of the interaction between SERT and inhibitors. They also emphasize the unique potential of AFM to study plasma membrane transporters in living cells at the single molecule level.

Friday, 9th of September

P 1) Working memory function and associated neural activation in remitted Major Depressive Disorder

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Objectives

Patients suffering from Major Depressive Disorder (MDD) do not only exhibit emotional symptoms such as depressed mood, anhedonia or anxiety, but experience also cognitive impairment. While previous neuroimaging studies have supported this clinical observation by demonstrating alterations within cognitive brain circuits in patients with concurrent major depressive episodes, imaging data are lacking investigating the functionality of cognitive brain circuitries in remitted MDD (rMDD) patients.

Methods

We have initiated a multi-center, cross-sectional functional magnetic resonance imaging (fMRI) study with the goal to investigate if working memory (WM) function and associated neural activation differ between rMDD patients and healthy subjects. 71 adult healthy volunteers without any psychiatric life-time diagnosis and 69 adult drug-free gender-matched rMDD patients underwent a functional n-back paradigm comprising 2-back and 0-back conditions while fMRI data was acquired.

Results

Our results indicated greater activation of the bilateral prefrontal, bilateral parietal, and anterior cingulate cortex in the 2-back condition versus the 0-back condition, which was significantly increased in rMDD patients as compared to healthy subjects. On a brain systems level, we detected elevated functional connectivity within this WM network in rMDD patients in comparison to controls. Regarding accuracy and reaction time performance data we did not find any differences between the rMDD and the control group.

Conclusions

Our results point towards increased activation as well as increased functional connectivity within this WM network in rMDD patients, which is in agreement with previous findings suggesting greater WM related activation during acute MDD. In summary, our data may indicate a compensatory mechanism to maintain normal levels of cognitive function during stable remission and may correspond to our observations of elevated functional connectivity between amygdala and anterior cingulate cortex in rMDD patients suggesting a neural adaptive mechanism within emotional circuits that predominantly counterbalances neural state known to be affected in acute MDD.

P 2) A database with mutagenetic data extracted from the literature for comparative analysis of mutations in ABC proteins

Gergely Gyimesi, David Borsodi, Balazs Sarkadi, Tamas Hegedus

ABC (ATP Binding Cassette) transporters with altered function are responsible for numerous human diseases. To overcome the pathological phenomena and plan strategies to modulate their function, the mechanism of the action of ABC transporters are studied extensively. The major way to learn about the mechanism of ABC transporters is designing and studying mutant constructs. Selecting the position and amino acid for the mutagenetic experiment raises trivial questions, such as whether or not a particular position has been mutated by others, what type of amino acid has been used to replace the original amino acid, what the environment of that position in the 3D structure is. To be able to answer these questions, we set up a database with interactive tools. First, features (e.g. sequence, topology, location of conserved regions) of ABC proteins were inserted into the database. Then, references to mutations were extracted from papers investigating ABC proteins, using automatic mining approaches. Because of the limitation of mutation-mining tools, the hits were verified manually. A web interface to the database makes the interactive mapping of the mutated positions onto the structure of the given protein possible, employing homology models where necessary. In addition, our tool is able to identify and visualize mutations at homologous positions in various ABC proteins based on sequence alignments. Since the currently available protein databases hold almost exclusively disease associated mutations, our database is unique because it stores mutagenetic data utilized in in vitro experiments. Our database of collected mutations and the corresponding web application give a unique insight into literature data and will advance the design of experimental studies to help to unravel the working mechanism of this protein family.

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P 3) Frog Oocytes to Unveil the Structure and Supramolecular Organization of Human Transport Proteins

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The major hurdle for structural studies of membrane proteins is obtaining micro- to milligram amounts of good quality protein. To overcome this problem several expression systems, from bacteria to mammalian cells have been used. Unfortunately, every expression system has its own disadvantages making it a necessity to develop improved heterologous expression systems.

In this work we used *Xenopus laevis* oocytes to express recombinant mammalian transport proteins for their subsequent purification, and biochemical and structural characterization. One channel and one solute carrier protein (SLC) were used as model proteins. Target proteins were cloned in a pol1 derived oocyte specific expression vector which provides an N-terminal decahistidine-tag for the convenient affinity purification by metal affinity chromatography. Five transport systems were purified in microgram quantities using this method: aquaporin-1 (AQP1), glutamate transporter 1 (EAAC1 or SLCA1), peptide transporter 1 (PEPT1 or SLC15A), sodium-glucose-cotransporter 1 (SGLT1 or SLC5A) from human and potassium-chloride-cotransporter 4 (KCC4 or SLC12A7) from mouse. Negative stain transmission electron microscopy (TEM) and single particle analysis (SPA) of purified AQP1 and KCC4 indicated homogenous distribution and the expected oligomeric states. Importantly, we also could grow 2D crystals from purified recombinant AQP1 expressed in *Xenopus laevis* oocytes, paving the way for future structural analyses of mammalian membrane proteins by crystallography techniques. Especially for TEM and SPA analyses, relatively low (30-60 µg/ml) concentrations of protein are needed. This implies that the method presented here is highly attractive for structural studies of recombinant membrane proteins and their complexes by TEM and SPA.

P 4) Highly Consistent Resting State Networks revealed by exploratory Analysis of the Functional Connectomes Database using massively parallelized FENICA

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Introduction

Since the seminal report by Biswal et al. (1995), low-frequency spontaneous fluctuation in blood oxygen level dependent (BOLD) signal have consistently been found, and the associated networks are referred to as resting state networks (Fox and Raichle, 2007). With the increasing availability of large-scale datasets – such as the Functional Connectomes Dataset (Biswal et al., 2010), a collection of (to date) about 1500 subjects from 38 released independent datasets – and of more powerful computing hardware exploratory procedures are becoming increasingly relevant to push further the frontiers of neuroimaging. We applied Fully Exploratory Network ICA (FENICA), developed by Schöpf et al. (2010), to 300 subjects sampled out of the Functional Connectomes Database, taking into consideration the datasets originating from Berlin, Cambridge, Cleveland, ICBM, Leipzig, and Newark.

Methods

The raw images as provided by the Functional Connectomes project were blurred with 8mm FWHM isotropic Gaussian kernels, corrected for motion, and filtered using a bandpass in the range of 0.1 to 0.01 Hz (Weissenbacher et al., 2009). Independent component analysis (ICA) was calculated with the FSL tool MELODIC (<http://www.fmrib.ox.ac.uk/fsl>), the number of components was estimated using the LAP criterion. Subsequently, images were registered to an EPI template in MNI space and resampled to 3mm isotropic grid using AFNI. After these preprocessing steps, the FENICA algorithm itself was applied to the set of all individual subjects' component maps using a spatial correlation coefficient threshold of 0.9 for the elimination of redundant components.

In order to process the large amount of data, we parallelized computationally demanding parts of the algorithm using the snowfall package (Knaus, 2010) for higher-level parallelization in R (www.r-project.org) and OpenMP (<http://openmp.org>) for lower-level parallelization in C.

Results

In total, we found 18 consistent independent components. Among these, 12 were identified as gray matter components, and the remaining 6 components were classified as white matter, CSF, and artifact signals.

Conclusions

To our knowledge, this is the first neuroimaging study employing a fully exploratory approach to analyze a number of subjects in the hundreds, the only other study performing a large scale analysis of the Functional Connectomes Database being the study of Biswal et al. (2010), who used a seed-based (as opposed to fully exploratory) analysis method.

While earlier research on resting-state networks has found a varying number of different networks (van den Heuvel et al., 2010) depending on the sample size of the study and possibly other study-specific parameters like scanner hardware or protocol used, a pooled analysis of a large number of individual subject data from multiple centers allows to perform a synthesis of available evidence on resting-state networks. This results in more consistent components as well as in the identification of effects too subtle to be detected or too similar to be distinguished from one another in the usual fMRI studies with sample sizes of about 30 subjects or less. In these, 8 to 10 (Damoiseaux et al., 2006) different resting state network are usually reported. The high sensitivity due to the large sample size of this study allowed us to distinguish 12 independent components possibly relating to different networks, including a differentiation of the typical default mode network in two components.

As massive parallelization was key to performing this large-scale exploratory analysis, and certainly will become more important in the future of this and other datasets, it will be published in an R package to allow for re-analysis and further analyses by the neuroimaging community.

P 5) THE HIGHLY CONSERVED GLYCINE TRIPLET PRESENT IN THE FIRST EXTRACELLULAR LOOP OF KAAT1 IS INVOLVED IN CATION INTERACTION

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KAAT1 is a member of the NSS/SLC6 family of solute transporters expressed in the midgut and in salivary glands of *Manduca sexta* larvae. It realizes the cotransport of neutral amino acids with a peculiar cation selectivity being activated by Na⁺, Li⁺ and K⁺ (Castagna et al., P.N.A.S.: 1998; Ins. Mol. Biol.: 2009; Bossi et al., J. of Physiol.: 1999). As more than 80% of the family members, KAAT1 shows a stretch of three glycines (G85-G87) located at the first extracellular loop (EL1).

According to the crystal structure of LeuT, the protein model of the family (Yamashita et al., Nature: 2005), this glycine triplet is located close to the access of the permeation pathway of the transporter but its functional role has not been fully understood yet.

The aim of this work has been investigating the role of this sequence by alanine and cysteine scanning methods and expression in *Xenopus laevis* oocytes.

The surface expression level, measured by a chemiluminescence based approach, was reduced for G85A and G87A mutants but was unaffected for G86A mutant. Nevertheless, all these mutants presented altered uptake activity and transport associated currents in the presence of all the cations that can be exploited by the wt. G85A and G86A mutants in particular showed also reduced uncoupled currents in Na⁺ and Li⁺ compared with wt, whereas G87A mutant showed increased uncoupled fluxes in the presence of all the cations. The switch of the entire triplet toward the N terminus of the protein generated a mutant unable to reach the membrane whereas the shift in the opposite direction produced a protein that was localized at the plasma membrane but showed a deep reduction in transport activity. Cross-linking studies performed by the treatment of cysteine mutants with the oxidative complex Cu(II)(1,10-phenanthroline)₃ showed that a covalent blockage of position 87 causes a significant reduction of amino acid uptake. The effect was specific and revertible by DTT treatment and, interestingly, Na⁺ protected G87C mutant from oxidation, probably inducing a movement of the EL1 that increases the distance between the two cysteine partners of the disulfide bond. Our data indicate that the conserved glycine triplet in KAAT1 provides EL1 with the structural flexibility required for the initial steps of cation interaction with protein.

P 6) BODIPY-DYES AS LIGANDS FOR GAT

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γ -aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the central nervous system (CNS) addressing three different kinds of receptors: GABAA, GABAB, and GABAC. The neuronal signal is terminated by reuptake of GABA from the synaptic cleft by GABA-Transporters (GAT) [1].

The analysis of ligand binding is usually performed by using radio ligand assays which suffer from certain disadvantages like high costs, health risks, and contaminated waste. Fluorescence binding assays on the other side reduce the health risks and are highly sensitive. Moreover, fluorescence markers can also be used with techniques like FRET or LRET which allow to monitor conformational changes in single protein molecules as well as in living cells [2].

Fluorescence dyes of the difluoro-boraindacene (BODIPY) family are characterized by high photostability, large extinction coefficients, and contain high quantum yields. Furthermore, they are characterized by sharp absorption bands (FWHM 25-35 nm) and long excited singlet-state lifetimes (1 to 10 ns) [3].

Due to the above mentioned importance of fluorescence markers and the outstanding spectroscopic properties of BODIPYs, we initiated a project aimed at the development of fluorescence ligands for the different subtypes of GAT. The design of these ligands followed the general structure activity relationships for GABA transporter inhibitors. According to that, GAT inhibitors consist of a cyclic amine or a cyclic amino acid like nipecotic acid and a "diaryl-moiety" connected by a linker of variable length originating from the amino nitrogen [4]. With the core structure of BODIPY derivatives being of similar size and polarity as the aforementioned "diarylmoieties", it was envisaged that substitution of "diaryl-moieties" by BODIPY units might lead to the desired fluorescent GAT inhibitors.

For the construction of the target compounds, we are synthesizing various BODIPY derivatives exhibiting an alkyl chain as spacer which carries a terminal halide or tosyl ester function for nucleophilic substitution reactions. These BODIPY derivatives are subjected to nucleophilic substitution with different cyclic amines. The resulting compounds are evaluated for their biological activity as GABA uptake inhibitors.

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P 7) Molecular Determinants for Ecstasy Analogue Selectivity

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The human Serotonin transporter (hSERT) belongs to a family of sodium/chloride-dependent transporters and it is responsible for the reuptake of serotonin (5-HT) from the synaptic cleft back into serotonergic nerve terminals. hSERT is the primary target for the widely used 5-HT reuptake inhibitors such as antidepressants as well as 3,4-methylenedioxymethamphetamine (MDMA), also known as ecstasy. MDMA belongs to a widely abused class of drugs, the amphetamines that predominantly cause release of serotonin and other neurotransmitters into the synapses via reversal of their respective transporters. In order to understand the interaction of MDMA analogues with its primary molecular target we undertook a combined mutational and structure-activity study.

Mutations were introduced into the substrate binding site of the hSERT and the effects of these mutations were studied by functional assays. We identified hSERT residues that play a crucial role in the recognition of MDMA analogues and their selectivity. Results from the uptake assay suggest that the potency of MDMA analogues, e.g. MDA and PMA are increased in the mutants Y95F, A173M, A173L compared to hSERT wild type. Our data provides experimental support for an orientation of MDMA and analogues within the central binding site of hSERT.

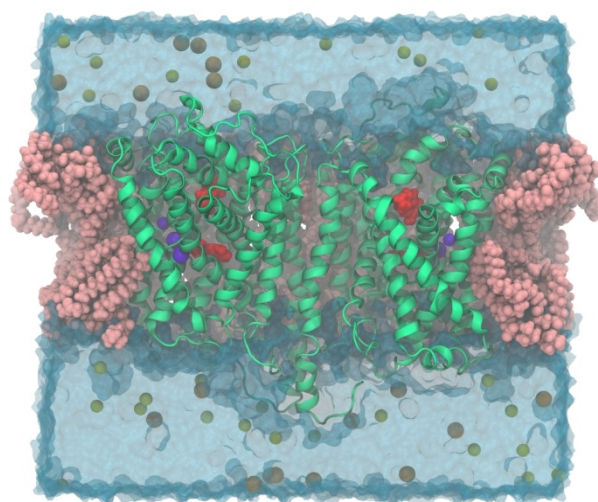
P 8) Modeling the dynamics of the human serotonin transporter

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The human serotonin transporter (hSERT) is a member of the neurotransmitter sodium symporters (NNS) family and is responsible for the re-uptake of serotonin from the synaptic cleft. The re-uptake terminates the action of serotonin in the nerve synapse and is driven by the co-transport of sodium and chloride ions. Imbalance of serotonin concentration is linked to several diseases such as depression, obsessive-compulsive disorder and ADHD and the transporter is thus an important pharmacological target.¹ No experimental 3D structure of hSERT has yet been determined, but the crystal structure of a homologous bacterial leucine transporter² (LeuT) has provided us with the possibility to construct a homology model of hSERT. This model allows us to investigate the interactions present in hSERT as well as dynamics of the transporter. Several classical non-biased molecular dynamics (MD) simulations of hSERT have been performed with the transporter as a dimer embedded in a POPC lipid bilayer surrounded by an aqueous environment. A total of 250 ns of MD simulations have been performed on the hSERT dimer with serotonin bound in the central binding site. To probe the possibility of an allosteric binding site in the extracellular part of the transporter, simulations of the hSERT dimer with serotonin bound in both the central binding site and in the extracellular cavity have been carried out. Furthermore the binding of extracellular serotonin to the transporter has been studied through biased MD simulations.



¹Torres *et al.* (2003) *Nature Reviews Neuroscience*, **4**, 13-25.

²Yamashita *et al.* (2005) *Nature*, **437**, 215-223.

P 9) Scintillation proximity to characterize the ligand binding properties of detergent-solubilized transport proteins

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The scintillation proximity assay is a simple and rapid radioligand binding assay. Its application for membrane transporters allows assessing substrate binding properties, e.g. substrate specificity and kinetics, of purified, detergent-solubilized transporters [1]. The experimental set-up represents an isolated system that permits the study of one specific transporter type, e.g. without interference from other transporters. No functional reconstitution of the target protein into liposomes is necessary to acquire its ligand binding properties. The procedure allows a relatively high sample throughput due to 96-well format and simple handling without the need of washing or separation steps. Basically, all components are mixed in one well and the signal is measured directly after incubation. Therefore, it is excellent for the characterization of mutant transporters and screening of adequate detergents for purification, i.e. mild detergents that do not disrupt ligand binding, and subsequent crystallization for structure determination. We present functional data of the L-arginine/agmatine exchanger AdiC [2] and the glucose transporter IICB [3] from the bacterium *Escherichia coli*, which were used to establish and validate the scintillation proximity assay in our laboratory.

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P 10) Interaction of the sodium potassium pump with the fluorescent dyes using time-resolved fluorescence spectroscopy

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The Na⁺/K⁺-ATPase is an integral protein of the plasma membrane that is responsible for maintaining ionic homeostasis in animal cells. This member of P-type ATPases pumps three sodium ions out and two potassium ions into the cell for each ATP molecule and maintains their electrochemical potential gradients, required for electrical excitability and transport of other ions, nutrients, and neurotransmitters, as well as for regulation of cell volume and intracellular pH. Change of activity of this enzyme can influence these mechanisms and can potentially result in variety of diseases. Therefore chemicals, which interact with this protein and can change its activity, deserve particular attention.

Erythrosine is a fluorescent dye, which is primary used for food coloring. Eosin, fluorescein and rose bengal, which are used in medicine for better imaging of cells, are also fluorescent.

Measurement of fluorescence decay using the TCSPC method was used to monitor interaction between sodium potassium pump and these fluorescent dyes.

We discovered, that the lifetimes of eosin, erythrosine and rose bengal were longer than lifetimes of free dyes, clearly indicating that the interaction occurs. Moreover, the eosin fluorescence decay seems to be sensitive also to the conformational state of the protein. On the other hand, we found no alteration of the fluorescence decay of fluorescein in the presence of the enzyme compared to free dye, and therefore we can see no evidence for interaction.

Acknowledgement: This work was supported by grant of Czech Science Foundation P301/10/0883 and by grant of Czech Ministry of Health NT11071.

P 11) Effect of quantitative inhibition of P-glycoprotein at the blood-brain barrier in rats and humans measured with (R)-[11C]verapamil and positron emission tomography

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Background:

The adenosine triphosphate (ATP) binding cassette (ABC) transporter P-glycoprotein (Pgp, ABCB1) acts as a gatekeeper at the level of the vascular endothelium of the blood-brain barrier (BBB) preventing brain uptake of a wide range of lipophilic molecules by active ATP-driven efflux transport. Pharmacological inhibition of Pgp might provide a useful strategy for increasing brain uptake and thereby therapeutic efficacy of drugs targeted at the central nervous system. Aim of this study was to study the effect of the potent third-generation Pgp inhibitor tariquidar on cerebral Pgp in rats and humans by assessing brain uptake of the model Pgp substrate drug (R)-[11C]verapamil by means of positron emission tomography (PET) imaging.

Materials and methods:

(R)-[11C]verapamil PET scans (rats: 60 min, humans: 40 min) and arterial blood sampling were performed in female Sprague-Dawley rats and healthy male human subjects at baseline and at 2 h (rats) or 1 h (humans) after infusion of tariquidar at doses of 1.5, 2.6, 3.75, 7.5, 15 and 30 mg/kg body weight (n=2 per dose group) or 2, 3, 4, 6 and 8 mg/kg (n=3 per dose group), respectively. Brain uptake of radioactivity was quantified in terms of the volume of distribution (VT) obtained from kinetic modelling using a 2-tissue-4-rate constant compartment model. Tariquidar plasma levels at the end of the PET scan were determined by LC/MS. Sigmoidal dose-response curves were fitted to the data using the Hill-equation.

Results:

Tariquidar was well tolerated in rats and human study subjects. Administration of tariquidar at different doses exerted no effect on peripheral metabolism and plasma protein binding of (R)-[11C]verapamil. Baseline brain radioactivity uptake (VT) was 1.27 ± 0.15 in rats and 0.66 ± 0.12 in humans. Tariquidar plasma levels at the end of the PET scan ranged from 230-7800 ng/ml in rats and from 281-1241 ng/ml in humans. Both in rats and humans, saturation of brain radioactivity uptake was observed at tariquidar plasma concentrations >1000 ng/ml with half-maximum effect concentrations of 544 ± 32 ng/ml in rats and 561 ± 24 ng/ml in humans. The maximum increase in brain radioactivity uptake as compared to baseline scans (without tariquidar administration) was 11.0-fold in rats and 2.7-fold in humans.

Conclusion:

This is to the best of our knowledge the first study where quantitative inhibition of Pgp at the human BBB is reported. Tariquidar was found to be equipotent in rats and humans to inhibit cerebral Pgp. However, there were pronounced species differences with respect to maximum increases in drug brain distribution following quantitative Pgp inhibition. The exact reasons for these differences are unknown but might be related to species differences in Pgp expression/function or differences in physiological parameters such as cerebral blood flow.

Funding:

The research leading to these results has received funding from the Austrian Science Fund (FWF) project "Transmembrane Transporters in Health and Disease" (SFB F35) and from the European Community's Seventh Framework Program under grant agreement no. 201380 (Euripides).

P 12) Molecular Dynamics - Assisted Comparative Modeling and Docking Studies of the Gamma-Aminobutyric Acid Transporter

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Several diseases of the central nervous system are associated with the transport system responsible for the reuptake of gamma-aminobutyric acid (GABA) out of the synaptic cleft. As experimentally resolved 3D structures of the four GABA-Transporter (GAT) subtypes have not yet become available, the molecular basis of drug binding is not fully understood¹, although fine-tuning of which GAT subtypes to be addressed is considered to be desirable towards minimizing side effects of drug therapy.

Based on crystal structures of the prokaryotic amino acid transporter LeuT in different opening states, homology models of GAT1-3 and BGT-1 were constructed. To optimize their geometry as well as to prove their stability, the models underwent an extensive molecular dynamics (MD) simulation process, ensuring full equilibration of the system.

A selection of known GAT substrates and inhibitors was docked into the relaxed structures in order to gain insight into their respective binding behavior. Preliminary results showed the consistency between occluded-state docking poses of small ligands taken from literature references and experimental data even concerning stereochemical features. MD simulations of hGAT-1 in the open-to-out state in complex with the docked inhibitor tiagabine nicely demonstrated its potency to stabilize the transporter, thus disrupting the transport cycle.

Similar setups in models of the other subtypes will elucidate the structural basis of their respective different binding behavior.

We acknowledge financial support provided by the Austrian Science Fund, grant F03502.

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P 13) Methodological investigations on fixed and random effect modeling in dynamic causal modelling

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Introduction: Methods for assessing brain connectivity are in general divided into two groups: functional connectivity and effective connectivity. Effective, in contrast to functional connectivity, aims at identification of direction or causality of a connection. A promising method for computing effective connectivity is dynamic causal modeling (DCM), where strengths of a priori defined connections are estimated using differential equations. Currently, most studies that employ DCM use random-effects (RFX) analysis to make group inferences, applying a second-level frequentist test to subjects' parameter estimates. In some instances, however, fixed-effects (FFX) analysis can be more appropriate. This work aimed at investigating differences between these estimation methods.

Methods: In a first step, data from 8 healthy subjects (mean age 26 years, 4 male) were analyzed to find effective connectivity patterns between SMA and M1 during motor and motor imagery tasks. From 28 candidate models, the best was determined by comparing the Bayes factors of the models (Kasess et al., Neuroimage, 2008). In a second step, the model thus identified was used to simulate new datasets to explore different methods of group-level analysis in DCM, comparing the commonly used random effects methods to three fixed effect methods: (multivariate) Bayesian parameter averaging (BPA), univariate Bayesian parameter averaging, and temporal averaging. Data sets were simulated assuming either a homogeneous large population (N=60) with constant connectivities across subjects or a heterogeneous population with varying parameters.

Results: Simulation showed that results were mostly comparable, but BPA yields best results for data with signal to noise ratio below 1, TA showed advantages at lower SNR but is limited in its applicability. BPA and PVWA can yield non-intuitive results when only considering posterior means in high SNR data.

Discussion: Results of different FFX methods enable Bayesian inference by analysis of a group posterior density while classic RFX analysis which allows only for testing the null hypothesis, a potentially very useful advantage of FFX models. Still, it is worth noting that the classic RFX procedure employed by most current DCM group studies showed robust performance, both for homogenous and heterogeneous groups.

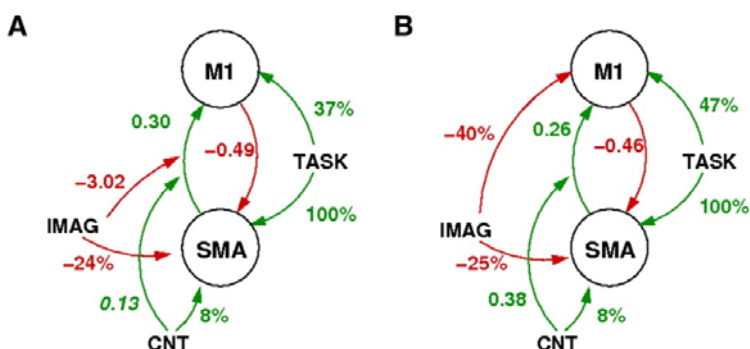


Figure: Best DCM model for motor imagery task (IMAG) with (A) and without (B) allowing modulatory influence of IMAG on supplementary motor area (SMA) influence on primary motor cortex (M1).

P 14) Characterising the interaction between the COPII component SEC24C and the human serotonin transporter

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The serotonin transporter (SERT) belongs to the SLC6 family of neurotransmitter transporters, which mediate reuptake of previously released neurotransmitters from the synapse. Mutation of C-terminus residues R1607-608 to alanine results in intracellular retention of SERT [1]. We subsequently showed that SERT depends on the COPII component SEC24C for its ER export and proposed R1607-608 as a putative interaction site on SERT for SEC24 proteins [2]. The aim of our current study is to characterise the nature of ER export of monoamine transporters. Using siRNAs to knock down SEC24 isoforms A-D in HeLa cells, we screened a series of double- and truncation mutants generated along the C-terminus of SERT. HeLa cells were transfected with Sec24 siRNAs and after 48h with YFP-tagged transporter plasmids. Functional effects of SEC24A-D knockdowns were determined by substrate uptake assays. Export of the IK(609,610)AA-SERT mutant was not sensitive to knockdown of Sec24C. Remarkably, the closely related transporters, for dopamine (DAT) and noradrenaline (NET), rely on Sec24D, and not C, for their ER export [2]. Accordingly, we replaced K610 by a tyrosine residue (Y) to switch the SERT-export motif to a NET/DAT-motif. The resulting K610Y-SERT mutant was sensitive to the knockdown of SEC24D more than SEC24C. These observations predicted that SLC6 family members with a K-residue at the pertinent position ought to be clients of Sec24C. This prediction was verified by examining mGAT4. The data imply that residue K610 and the equivalent residues in other transporters specifies which SEC24 paralogue is recruited for ER export. These export signals work independently, because a concatemer of SERT and GAT-1 is affected by both, depletion of SEC24C and SEC24D.

This work was supported by SFB35.

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P 15) X-linked Adrenoleukodystrophy: targeting of the ABC-transporter ALDP to peroxisomes

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The neurodegenerative disease X-linked adrenoleukodystrophy (X-ALD) is caused by mutations in ABCD1 encoding the peroxisomal ABC-transporter protein ALDP. The transport of such peroxisomal membrane proteins (PMPs) is mediated by the cooperation of a peroxisomal targeting signal for membrane proteins (mPTS) and the protein PEX19. The largest part of this soluble protein is responsible for its interaction with each of the known PMPs, whereas a smaller domain can specifically interact with the integral membrane protein PEX3. Thus, PEX19 may serve as a link between newly synthesized PMPs and the peroxisomal surface.

Our investigation of the transport process of ALDP revealed that a previously described PEX19-binding domain is not necessary for peroxisomal targeting, although the sequence is sufficient to target a reporter protein to peroxisomes. This suggested another independent mPTS, which was identified by a detailed deletion analysis of an ALDP-EGFP fusion protein. The amino acid sequence of the second mPTS of ALDP appeared very similar to the original mPTS, but its interaction with PEX19 is much weaker. Upon deletion of both mPTSs ALDP-EGFP was found in mitochondria indicating that no further mPTS can be expected. ALDP-EGFP was also found in mitochondria when the protein was expressed in human fibroblasts lacking peroxisomal structures, suggesting that a mitochondrial targeting signal is encoded in the N-terminus of ALDP. Interestingly, we found an additional PEX19-binding site within the N-terminus of ALDP that lacks targeting information. This site overlaps with the mitochondrial targeting signal and may repress it.

Thus, our data suggest that the transport of the ABC-transporter protein ALDP to peroxisomes is a complex process guided by two independent targeting signals and an additional PEX19 binding site. Moreover, we demonstrate that mPTSs can compete with mitochondrial targeting signals but are overruled by signal peptides.

P 16) Wistar Zagreb-5HT rat: a rodent model of constitutive dysregulation of serotonin transporter

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Serotonin transporter (5HTT), a monoamine carrier protein, regulates serotonergic signalling via reuptake of serotonin from synaptic cleft into pre-synaptic neuron. Changes in 5HTT signalling are associated with a variety of behaviors and behavior-related disorders which are effectively treated with selective serotonin reuptake inhibitors (SSRIs). Beside in the brain, structurally identical 5HT transporter protein is expressed in peripheral tissues, including blood platelets. Platelets acquire their own serotonergic system similar to serotonergic neurons, but without synthetic enzyme, and could be used for studying some aspects of brain 5HT mechanisms.

In our laboratory, the extreme phenotype approach was used to develop, by use of selective breeding, two diverse rat sublimes (Cicin-Sain and Jernej, 2010). Platelet serotonin level and velocity of platelet serotonin uptake, which is the main determinant of serotonin level, were applied as selection criteria. Sublimes are termed high- and low-5HT rats and constitute original genetic model, Wistar-Zagreb 5HT rats (WZ-5HT rats). Animals from 5HT-sublimes are characterized by constitutional up-regulation / down-regulation of platelet 5HTT activity and consequently altered peripheral serotonin homeostasis, as evidenced from diverse studies. One of the findings, the decrease in 5HT uptake after systemic administration of SSRIs, which was more pronounced in high-5HT animals, suggests that differences in central pharmacodynamic response to SSRI could also be expected between sublimes. Indeed, animals from high-5HT subline had slightly increased [3H]-citalopram binding sites in several brain regions, and the increase of hippocampal extracellular 5HT after challenge with citalopram was significantly higher also in this subline. On the molecular level, trend for higher 5HTT gene expression in high-5HT animals was observed, but without significance. Notwithstanding, various behavioral studies indicate differences in brain serotonergic signalling between 5HT-sublimes.

Summary of our neurochemical, molecular and behavioral studies on sublimes of Wistar-Zagreb 5HT rat will be presented, focusing to the possibility that our model may represent a useful tool in serotonin transporter research, at both central and peripheral level.

Cicin-Sain L, Jernej B (2010) Wistar-Zagreb 5HT rats: a rodent model with constitutional upregulation/downregulation of serotonin transporter. In: *Experimental Models of Serotonin Transporter Research* (Kalueff A, LaPorte J, eds), pp 214-244: Cambridge University Press.

P 17) Characterization of the role of individual sodium binding sites in the human serotonin transporter.

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The transport process by which the serotonin transporter moves substrate against its chemical gradient is driven by counter- and co-transport of ions. The human serotonin transporter (hSERT) utilizes the sodium gradient to drive this movement of serotonin across the membrane. The alternating access model provides a model of the transport process where simultaneous binding of sodium, chloride and serotonin elicits translocation. Nevertheless, little is known about the sodium binding sites and their exact individual roles during binding and translocation of substrate. The LeuTAA structure and our homology model of hSERT, have identified two sodium binding sites, Na1 and Na2. However, several studies show that serotonin transport is associated with co-transport of only one sodium ion. The role of and the coupling between the two sodium binding sites are not well understood. We have characterized the role of the individual sodium binding sites in the molecular mechanism of the sodium driven transport.

We have systematically mutated the sodium-coordinating residues in the two sodium binding sites and characterized the effect of these changes on transporter function and kinetics, using radiotracer uptake assay and radio ligand binding assays. We have applied the substituted cysteine accessibility method (SCAM) to evaluate possible shifts in the conformational states as a result of the mutations in the sodium sites and their interplay with different cations and ligands. Based on our results we have established a model for the role of the two sodium binding sites in the transport process.

P 18) UCP2 up-regulation due to immune cell activation.

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Uncoupling protein 2 (UCP2) is an inner mitochondrial membrane protein, which transports protons in the presence of fatty acids, similarly to UCP1. Although the protein was discovered 1997, its function is still under debate. One reason for this is the ambiguity of UCP2 expression among different tissues and cell types, caused by a discrepancy between mRNA and protein level. Here, we re-evaluated the protein expression pattern among various tissues of young, mature and adult mice, using anti-UCP2 antibody, which we priorly evaluated with recombinant UCP2 and UCP2 knock-out mice. Protein levels were compared with RNA levels in the same tissues. Our results demonstrate that the protein is mainly expressed in lymphatic organs and immune cells and is not detectable in neurons. We found that the highest levels of UCP2 expression in T-cells coincides with the highest activation state of these cells. Re-stimulation of T-cells leads to a further increase of UCP2 protein level. Both this expression pattern and UCP2 up-regulation after T-cells stimulation imply that UCP2 has a major role in the modulation of the immune response.

P 19) COMT Val 158 allele heterozygosity abolishes the impact of life stress on hippocampal volume

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The interplay between environmental factors such as stress and genetic variants is likely a core mechanism determining susceptibility to psychiatric disorders. However, despite the enormous social and economic burden associated with these disorders, mechanisms underlying vulnerability or resilience are inadequately understood. Stress-related psychiatric illness is thought to result from an inhibition of hippocampal neuroplasticity by cortisol, which is the consequence of hypothalamus-pituitary-adrenal (HPA) axis hyperactivity. Accordingly, gene variants that modulate HPA axis regulation may determine the risk for psychiatric disorders in stress-exposed individuals. Val158Met, a common functional polymorphism in the gene encoding the dopamine catabolizing enzyme Catechol-O-methyltransferase (COMT), is a strong candidate for such an interaction: While the Met allele ('worriers') has been associated with poor emotional regulation and exaggerated hypothalamus-pituitary-adrenal (HPA) axis responses, the Val allele ('warriors') increases stress resilience, though at the cost of decreased cognitive flexibility.

To investigate the possible interaction between COMT genotype and chronic mild stress on an intermediate phenotype level, we acquired structural MRI data and analyzed Val158Met genotypes in 95 healthy participants. Further, lifetime stress exposure has been measured using the Life Events Questionnaire and subcortical structures including hippocampal volumes have been automatically extracted from the MRI data using Freesurfer.

While there was only a borderline significant correlation between age adjusted life stress and average hippocampal volume for the whole group ($r = -0.17$, $p = 0.1$), testing the genotype groups separately, significant, but opposing correlations were found. While average hippocampal volume decreased as a function of life stress in the Met carrier group ($r = -0.26$, $p = 0.02$), we found hippocampal volumes to positively correlate with life stress in the Val/Val group ($r = 0.73$, $p < 0.01$). Finally, we tested this interaction in a robust linear regression model revealing that Met allele carrier status and age adjusted life events interactively impact on hippocampal volumes ($p < 0.001$). While reduced hippocampal volume, which is an intermediate phenotype of several psychiatric disorders, is generally assumed to be stress-related, our study suggests that this relationship can even be reversed in individuals that are more resilient regarding their genetic endowment. Further, our finding implicates dopamine signaling to be involved in HPA axis regulation. Interestingly, COMT effects are assumed to be of primary importance in the dopamine transporter-lacking prefrontal cortex, which is implicated in HPA axis regulation by recent animal and neuroimaging studies. Accordingly, studies addressing the mechanisms by which Val158Met impacts HPA axis regulation may offer mechanistic insights into the intersection between the dopaminergic and the stress system.

P 20) Oxalate's precursor glyoxylate increases the expression of sulfate-anion-transporter-1, sat-1, in HepG2 cells

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Hyperoxaluria is a major public health problem causing nephrolithiasis. About every tenth of the European and North-American population suffers from kidney stones during its lifetime. The main component of renal stones is calcium oxalate. Therefore, oxalate transport in the body is of particular interest. Oxalate is ingested from food, aliments rich in oxalate are cacao, spinach, or rhubarb. Different members of the solute carrier (SLC) 26 transporter family localized in the gastrointestinal tract are mediating oxalate uptake. Endogenous oxalate synthesis from its precursors glycine, hydroxyproline, glyoxylate and glycolate mainly takes place in the liver. Oxalate is released from the liver in exchange for sulfate by sulfate-anion-transporter 1, sat-1. In the kidneys, oxalate is freely filtered in the glomerulus and transported bidirectionally in the proximal tubule, sat-1 is mediating oxalate's passage through the basolateral membrane. Recent findings showed that sat-1 is also capable to transport oxalate's precursor glyoxylate.

To examine the impact of sulfate and different oxalate precursors on sat-1 expression, we used HepG2 cells, an established model for oxalate metabolism. HepG2 cells were incubated in media containing sulfate, oxalate or its precursors glycine, hydroxyproline, glyoxylate, and glycolate for four days. To determine time and concentration dependency of sat-1 mRNA expression, cells were incubated for three hours to six days in 1 mM glyoxylate and for four days in 0,1 to 2 mM glyoxylate. Changes in endogenous sat-1 mRNA and protein expression were examined using real-time PCR and Western blotting. HepG2 cell proteins were separated on a 2D-gel, compared densitometrically, and regulated proteins were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to detect changes in HepG2 cell proteome after glyoxylate incubation.

Endogenous sat-1 mRNA expression in HepG2 cells was upregulated by glyoxylate but not by sulfate, glycine, hydroxyproline, oxalate, or glycolate. Upregulation of sat-1 mRNA expression was mediated by glyoxylate in a concentration dependent manner, the expression reaches a maximum at day four of incubation. After treating the cells with glyoxylate for four days, also sat-1 protein expression increased. Glyoxylate's impact on HepG2 proteome was analysed in 2D-gelelectrophoresis. The regulated proteins mainly belong to protein synthesis or oxidative stress pathways.

An increased expression of sat-1 protein indicates that glyoxylate may be responsible for the elevated oxalate release from hepatocytes observed in hyperoxaluria. Glyoxylate's degradation product oxalate probably causes oxidative stress that leads to decreased protein synthesis.

P 21) MOLECULAR MODELLING OF GAMMA-AMINO BUTYRIC ACID TRANSPORTER FUNCTION

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Gamma-aminobutyric acid (GABA) is involved in the regulation of neuronal activity. It is released in the synapse and serves as the major inhibitory neurotransmitter in the brain. The excess amount of GABA is taken up by GABA transporter subtypes, which belong to the Na⁺ and Cl⁻ ion-dependent solute carrier family (SLC6).

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, homology models of human GATs (hGATs) were built up [1,2,3,4] on the basis of the structure of the bacterial orthologue Leu transporter in the occluded-state [5]. Docking, molecular dynamics (MD) and functional data indicate, that amino acids participating in substrate binding of neuronal (hGAT-1) and glial (hGAT-2, hGAT-3) subtypes are different. By contrast, substrate binding crevices of hGAT-2 and hGAT-3 cannot be distinguished, avoiding sensible prediction of efficient substrate inhibitors. Therefore, a comparative analysis of hGAT-2 and hGAT-3 sequences were performed which revealed substantial differences localized at the putative dimer interface.

MD calculations and stabilization center (SC) analysis were performed to identify residues taking part in large scale molecular motions during transport. Even a 16ns MD revealed the existence of a GABA-Na⁺ complex in the binding crevices of hGAT subtypes while the SC analysis highlighted the importance of residues stabilizing the Na⁺(2) site in the occluded state [6].

MD simulation of the full length transporter in an implicit membrane environment in combination with the growing amount of information obtained from the crystal structures of related bacterial transporters in various conformational states open the way to fully understand the transport mechanism, which serve better the design of more efficient, subtype-selective transport inhibitors in the future.

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P 22) Two approaches towards facilitating crystallization of the peptide transporter YdgR

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Structural and functional studies on transmembrane transporters are crucial to understanding mechanisms of substrate translocation. We have begun structural studies on a prokaryotic member of the proton oligopeptide transporter (PTR) family of symporters. This transporter family takes up di-/tri-peptides, as well as many drugs and peptidomimetics. Membrane protein crystallization is inherently difficult. We are using two strategies to overcome this problem.

First, improvement of thermostability could increase the probability of yielding crystals of membrane proteins. Random mutation and testing for thermostability of the target protein were performed in a high throughput manner to identify mutants that are more thermostable and therefore may have a higher propensity to crystallize. We have identified 2 mutants with increased thermostability of at least 3°C and are currently testing double and triple mutants. Still initial screening goes on.

Second, it has been shown that co-crystallization of Fab fragments with membrane proteins gave rise to well diffracting crystals. Due to the detergent micelle, membrane proteins have restricted accessible areas that are able to form crystal contacts. In complex with mFab fragments, these areas are increased supporting the formation of crystal contacts. Therefore we have produced a conformationally specific monoclonal antibody in hybridoma cells that serve as a tool to improve crystallization.

P 23) Which conformation does P-glycoprotein prefer within the membrane?

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The human genome contains 48 members of the ABC protein family. These proteins utilize ATP binding and hydrolysis for their function. The first structure of an ABC exporter was from *Staphylococcus aureus* [1] and showed a twisted architecture. The same fold was observed in MsbA, mouse P-glycoprotein and the human mitochondrial ABCB10 transporter. Although ABC exporters have now been crystallized in several conformations, uncertainty remained with respect to the physiological conformation, because they seem not to be fully compatible with all biochemical evidence, which might be a consequence of the crystallization procedure or conditions. We focus on the multidrug resistance transporter P-glycoprotein (P-gp, ABCB1), which is expressed at the blood-brain-barrier, in intestine, kidney, liver and macrophages. The function of ABCB1 is to prevent xenotoxic compounds from penetrating into the body or reaching the brain.

We applied homology modeling and MD simulations to determine the equilibrium conformation of the membrane inserted transporter. An initial ABCB1 model, built using the Sav1866 template [1], was in compliance with most, but not all experimental data. Deviations were observed in the central pore, where biochemical evidence indicated a smaller distance between the two sides of the wing-like helical bundles of the transmembrane region. Including cysteine cross-linking informations into the model building process allowed us to create a model [2] that showed a consistently better agreement with biochemical data [3]. MD simulations were then used to probe for the equilibrium conformation of ABCB1 in the membrane environment. We observe the wings to come close and show a helical arrangement as observed in the inward facing mouse ABCB1 and human ABCB10 crystal structures. We observe that the central pore closes while water is expelled from the hydrophobic region, in compliance with experimental observations [3, 4].

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P 24) Putative yeast sterol importers, Aus1 and Pdr11.

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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 lucrative model to study the molecular basis of the transport mechanism of ABC transporters.

In order to identify substrate(s) and to study the mechanism of action of yeast ABC pumps, we have overproduced Aus1 and Pdr11 in yeast. Transport activity and protein properties, like sensitivity to various ABC protein inhibitors are tested in purified plasma membranes with enriched ABC proteins. Similar as in the well-studied yeast PDR pump, Pdr5, we observe strong inhibition of ATPase activity of Aus1 and Pdr11 to oligomycin and vanadate. In addition activity of protein variants with mutated residues in conserved residues of nucleotide binding domains will be discussed.

Furthermore, a 14-Histidine tag is fused to the N-terminus of the proteins to allow their rapid purification by affinity chromatography. Activity of purified proteins (solubilised in detergent or reconstituted in proteoliposomes) will be discussed.

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P 25) Molecular dynamics simulations propose a structural water involved in the binding of imipramine to SERT

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The serotonin transporter (SERT) is a membrane protein, located at the nerve terminals of serotonergic neurons. It is important for the regulation of the neuronal transmission and also involved in various disorders like depression or addiction. Thus, SERT is targeted by therapeutic drugs, such as antidepressants but also by illicit substances, like psychostimulants. How ligands bind exactly to or interact with this monoamine transporter is still a matter of debate.

To get insights into the molecular mechanisms of ligand binding, we investigated SERT interacting with selected tricyclic antidepressants (TCA) by '*in-silico*' (homology modelling, docking, molecular dynamics simulation) and '*in-vitro*' (binding, competition experiments, site directed mutagenesis) methods.

Recently, it has been proposed that tricyclic antidepressants are binding in a similar way to SERT¹. The di-benzazepine ring is located in the changeover of the central binding site and the external vestibule. The di-methylamino-propyl sidechain rises downwards into the central binding site forming a saltbridge between the charged nitrogen and D98 as main interaction. It has been shown, that the mutation Y95F lowers the binding energy of imipramine by $\sim 1.2 \text{ kcal}\cdot\text{mol}^{-1}$, which is in accordance to the loss of a hydrogen bond.

To extend these studies, we performed molecular dynamics simulations of imipramine in the wild type and the mutants of hSERT. The simulation of imipramine in the mutant SERT-I172A showed two stable water molecules in vicinity of Y95 as hypothesized in our previous study. One of these bridges a hydrogen bond between the charged nitrogen and the oxygen of Y95.

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¹Sarker et al., "The high-affinity binding site for tricyclic antidepressants resides in the outer vestibule of the serotonin transporter.", *Mol Pharmacol.*, 2010, 78:1026-1035

P 26) Multidrug Resistance associated Protein in vitro assay

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Multidrug Resistance associated Protein 2 (MRP2) is an efflux transporter protein and a member of the ATP Binding Cassette family, the systematic name is ABCC2 as the gene that expresses it. MRP2 is for example expressed on the apical side in hepatocytes and the endothelial cells of the intestine (Mayer et al., 1995). The physiological role of MRP2 is to transport anionic compounds or conjugates (like glucuronides) from inside to outside of cells ATP dependently (König 1999).

The MRP2 transporter can be stimulated or inhibited by a diversity of drugs. Understanding how they affect this transporter is very important since it has been shown to affect the pharmacokinetic properties of several drugs. These drugs can either be pumped out of the cell or they can inhibit the efflux of other transported drugs (Takano M. et al., 2006). In addition, MRP2 is shown to be overexpressed in some cancer cells causing multidrug resistance (Borst P. et al., 1997).

There are several ways to study efflux proteins in vitro in membrane vesicles. We use inverted vesicles where a probe is pumped inside the vesicle instead of being pumped out, making it more simple to detect. There are different probes, which can be radiolabeled or fluorescent molecules that are substrates for MRP2. We have compared the properties of three commonly used MRP2 probes in this in vitro assay. With these assays it is possible to obtain information on the effect that drugs have on the transported probes, if the transport is stimulated we will have an increase of probe in the vesicle or if inhibited we will have a decrease of it. However, with this indirect assay it is not possible to distinguish if the drug that affects the transporter is by itself transported or not.

P 27) The betaine-GABA transporter (BGT1, slc6a12) is unlikely to control of GABA receptor activation

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Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain and is inactivated by cellular uptake catalyzed by a family GABA transporters (GAT1, GAT2, GAT3 and BGT1). The GAT1 and GAT3 subtypes have been shown to be essential for maintaining low resting levels of GABA. In contrast the roles of the other two are poorly understood. The present study focused on BGT1 by exploring mice lacking the BGT1 gene. These mice appear normal despite lacking both BGT1 mRNA and protein. We confirm that there is BGT1 mRNA in brains of wild-type mice, but find that the levels are several hundred times lower than those of GAT1. Immunocytochemistry and immunoblotting were difficult due to the low levels of the BGT1 protein, and wild-type and knockout littermate pairs had to be processed in parallel to enable distinction between background labeling and true labeling. Further, the tiny amounts of BGT1 present in the brain were concentrated in the leptomeninges, while brain parenchyma itself was virtually BGT1 deficient. BGT1 was neither detected in brain endothelium nor in ependymal cells. Both tanycytes and choroid plexus were unlabeled. The highest BGT1 expression levels (mRNA and protein) were found in hepatocytes and in the renal medulla. Considering that brain BGT1 expression levels were several orders of magnitude lower than those of GAT1, and that both GAT1 and GAT3 have higher affinity for GABA than BGT1, it seems unlikely that BGT1 can play any role in the control of brain GABA receptor activation. The physiological importance of BGT1 is probably related to the methyl-donor role of betaine in the liver and possibly to osmolyte control in the kidney, but also these roles are non-essential under non-challenging rearing conditions. The present study underscores the importance of quantitative data and of using knockout animals as negative controls.

P 28) ABC transporters of *Fasciola hepatica* as putative drug targets

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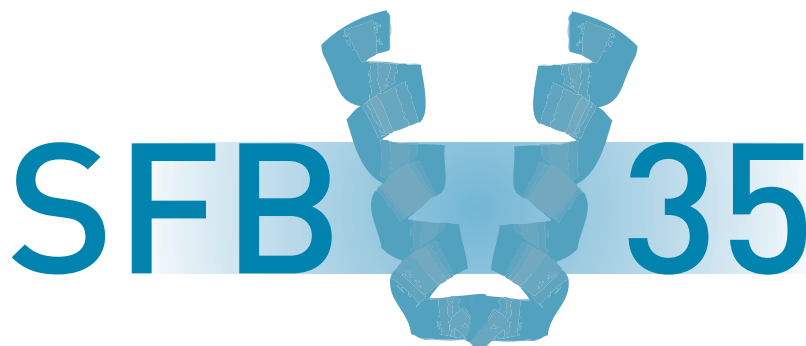
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The liver fluke *Fasciola hepatica* is one of the most important parasites affecting animal health all over the world, causing the so called liver fluke disease (fascioliosis). Although infections of humans are rather rare in most western countries, several million people worldwide are infected by this trematode. Beside its threat to humans the infection of animal stock leads to large financial losses. As vaccinations against this parasite are not available yet, anthelmintic drugs, like triclabendazole, are the treatment of choice. During the last decades more and more flukes resistant to these drugs have been found. One possible mechanism for these resistances seems to be the expression of so called ABC transporters. Inhibitors of P-glycoprotein (ABCB1) can change the status of flukes from resistant to susceptible. Up to date little is known about proteins expressed by the fluke. In addition almost no information on the fluke's genome is available. We therefore try to identify and characterize yet unknown proteins of the fluke to investigate their potency as putative new drug targets.

Starting from a previously published sequence of an ABC transporter of *Fasciola hepatica* we used RACE (rapid amplification of cDNA ends) to generate a full length ABC transporter. Heterologous expression of the protein allows basic analysis of this transporter. Other proteins will be identified by screening a cDNA library, prepared from flukes isolated from the liver of infected cows.

Comparison of the published sequence of a previously identified ABC transporter from *Fasciola hepatica* with other transporters of this family revealed that the sequence was lacking the first six transmembrane regions of the transporter. After cloning of the missing part, it became clear that this transporter is highly homologous not only to ABC transporters of the evolutionary close *Schistosoma mansoni*, but also to transporters of mammals. Expression of this transporter in eucaryotic cell lines should therefore allow testing the transporter's properties, identifying substrates and blockers, and therefore getting a glance on future approaches that can be used to treat fascioliosis in animals and humans.

IMPRESSUM



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Late Abstract

P 28) ABCG2/BCRP probe substrate for regulatory studies – in vitro evaluation

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ABCG2/BCRP is one of only two efflux transporters that made it to the short list of transporters of the International Transporter Consortium (Giacomini 2010). No consensus on the ABCG2/BCRP probe substrate has been reached yet.

The ideal probe substrate would be a drug that works in all in vitro and in vivo assays, therefore it should have a low-to-intermediate passive permeability. It should show an ABCG2/BCRP dependent pharmacokinetics in the clinic therefore it should have a transporter limited absorption and little or no metabolism. It should be commercially available in radiolabelled form or should be fluorescent, and it must be off patent.

Due to the C421A polymorphism that leads to lower ABCG2/BCRP activity clinical data on ABCG2/BCRP dependent pharmacokinetics is available for a couple of potential probe substrates. Although *Bcrp1*^{-/-} mice as well as *Abcg2/Bcrp1* specific inhibitors (e.g. Ko134) exist and are commercially available little preclinical animal data have been published. No correlation studies on potential cellular transport systems (Caco-2 and MDCKII/BCRP) are publicly available either.

This study attempted to characterize specificity as well as ABCG2/BCRP dependent permeability of 5 potential ABCG2/BCRP probes (atorvastatin, chlorothiazide, dantrolene, sulfasalazine and topotecan). Specificity was tested on a panel of membranes overexpressing ABCG2/BCRP, ABCB1/P-gp, ABCC2/MRP2, ABCC4/MRP4 transporters that colocalize in apical membranes in many different tissues / cell types. Permeability experiments were performed on Caco-2 and MDCKII/BCRP cells.

IC₅₀ values of specificity experiments as well as apparent permeability values and efflux ratios will be presented.

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Late Abstract

P 29) Concept of dual solute translocation paths of ABCB1 allows studying the role of individual transmembrane domain residues in solute interaction

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P-glycoprotein (P-gp, ABCB1) is a multi-specific drug efflux pump which plays an important role in pharmacokinetics of systemically administered medicines and resistance to anticancer drugs. Its two transmembrane domains form composite solute translocation paths, which allow movement of structurally diverse compounds across the plasma membrane. The fact that P-gp arose from a homodimeric and rotationally symmetric ancestor by gene duplication made us consider that pseudo-symmetry would have been retained and solute might interact with it in a dual mode. In order to support this hypothesis P-gp was photolabeled with propafenone analogs, purified, digested proteolytically and peptide fragments were identified by high-resolution mass-spectrometry. Labeling was assigned to two regions in the protein by projecting data into homology models. Subsequently, symmetric residue pairs in the putative translocation paths were identified and replaced by site directed mutagenesis. Transport assays corroborated the existence of two pseudosymmetric translocation paths (TPs). While rhodamine123 has a preference to take one path, verapamil, propafenones and vinblastine preferentially use the other. Two major findings ensued from this study: the existence of two solute translocation paths in P-gp as a reflection of evolutionary origin from a homodimeric ancestor and selective but not exclusive use of one of these paths by different P-gp solutes. It had been reported that H-bonding is important for P-gp-solute interactions and tyrosines often represent major small molecule interaction partners. Therefore, we further investigated the role of tyrosine residues (Y307 and Y310) in translocation path 1. The reason for choosing TP1 as compared to TP2 was the lower number of tyrosine residues (2 vs. 6). Transport assays showed a significant decrease in rhodamine123 efflux in the double tyrosine mutant (Y307F.Y310F), as well as the triple mutant Y307F.Y310F.Q132R. This indicates that hydrogen bonds are formed between tyrosines and rhodamine123. In contrast, the interaction with propafenone analogues is not affected. For the first time to our knowledge, the concept of dual translocation paths allowed studying and understanding the role of individual amino acid residues of P-gp for solute interaction. This project is supported by the Austrian Science Fund (SFB35, project part 03509). ZP acknowledges support by the Higher Education Commission Pakistan