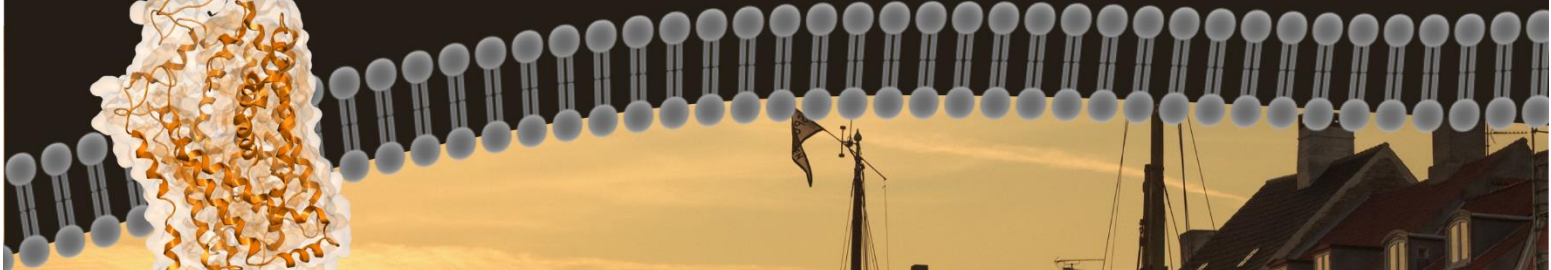
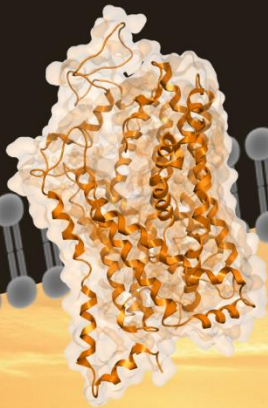




International Transmembrane
Transporter Society

ITTS SYMPOSIUM | 2022 ⁷⁻¹⁰ June Copenhagen

Catching Transport in motion



Preface

Welcome to the 2nd International Transmembrane Transporter Society (ITTS) meeting “*Catching Transport in Motion*” in wonderful Copenhagen on June 7-10, 2022.

We are delighted to present top speakers from some of the best laboratories worldwide working with transport proteins.

The program will include 4 keynote presentations, 11 panel sessions, a young scientist session and two poster sessions (poster format W115 x H185 cm). Additionally, there will be social activities including a fascinating sightseeing boat trip and a tasty farewell dinner (included in your registration).

Great thanks to our sponsors the University of Copenhagen (Denmark), the journal Society for Basic & Clinical Pharmacology & Toxicology, the Carlsberg Foundation (Denmark), The Stiles-Nicholson Brain Institute (Florida Atlantic University). The Brain in Flux, Nanion Technologies and Frontiers in Physiology.

Best regards,

The Local Organizing Committee

Prof. Stine F. Pedersen

Prof. Petrine Wellendorph

Prof. Ulrik Gether

Prof. Claus J. Loland (chair)

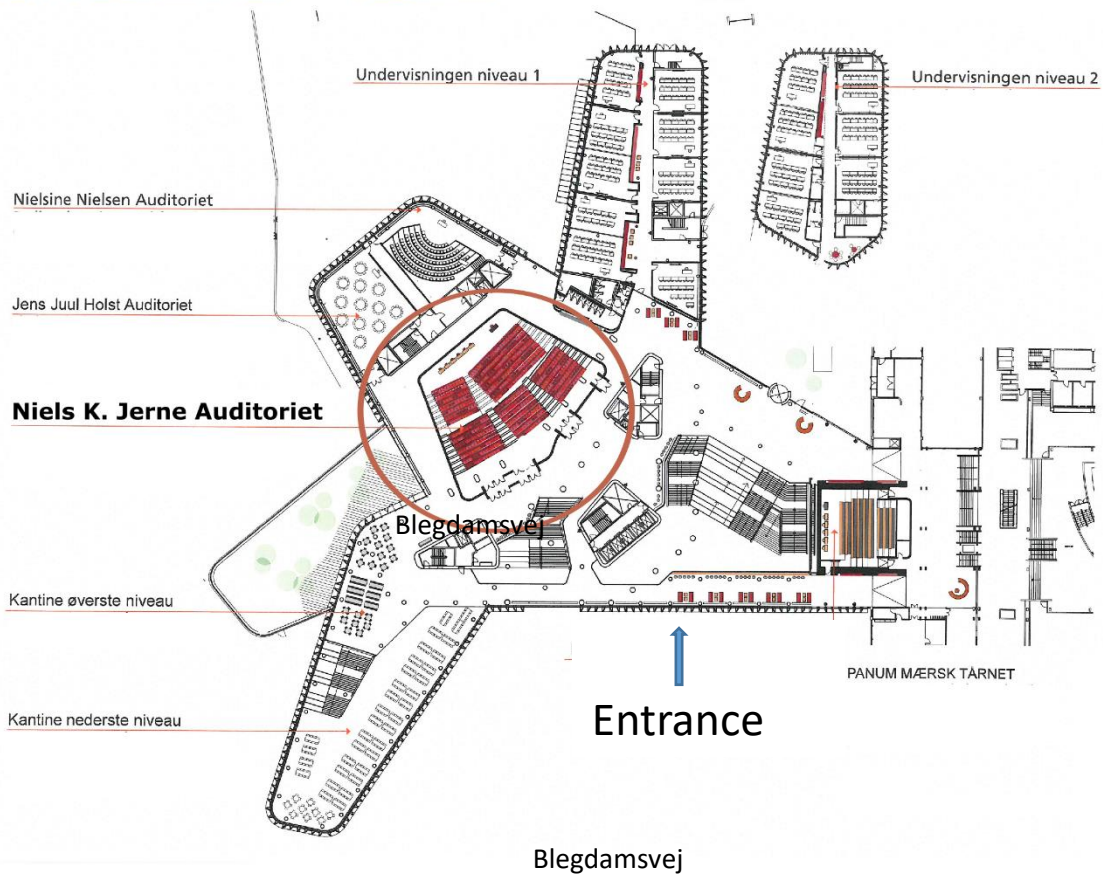
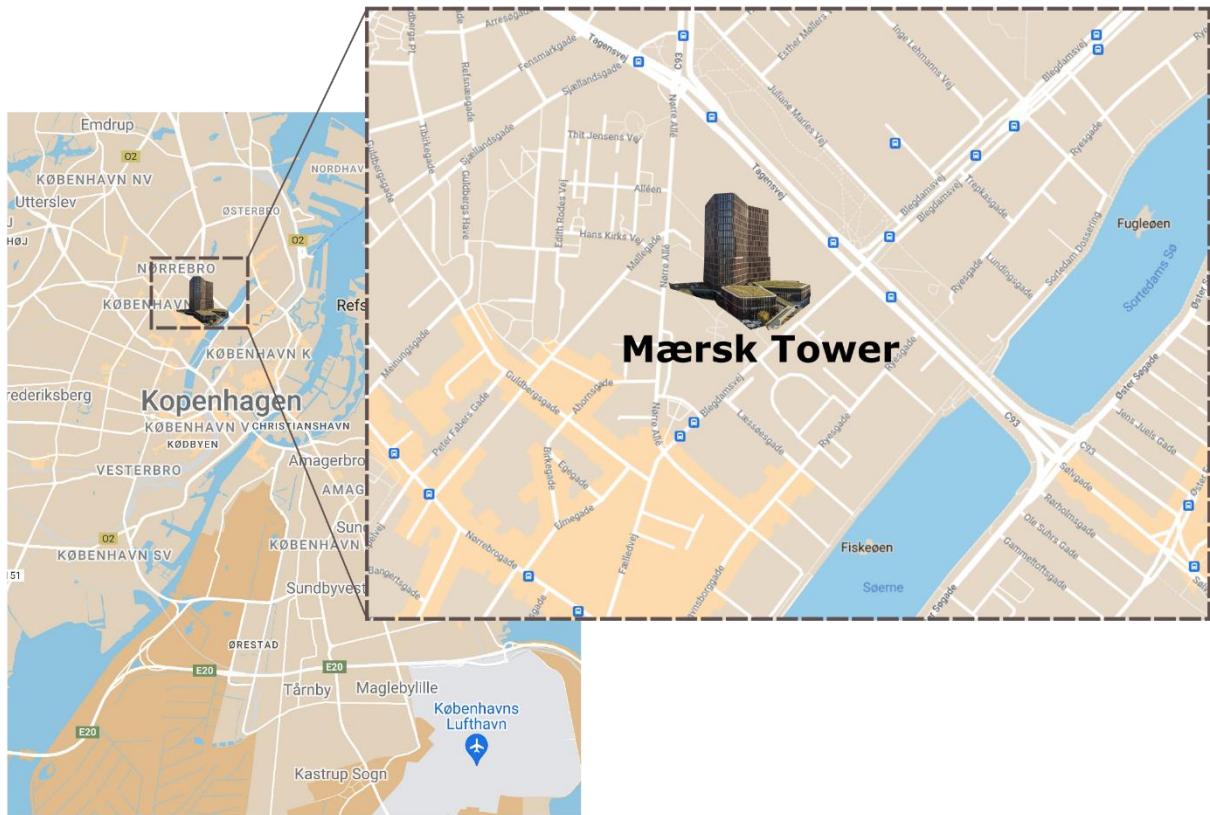


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Conference Venue



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Program

ITTS Symposium Copenhagen, June 7 - 10 2022

'Catching Transport in Motion'

Tuesday

09:00-10:15

Registration w/coffee

10:15-10:25

Opening v. Claus Loland

10:25-12:30

Session 1. ***New kids on the monoamine transporter block - Implications for treatment of psychiatric and substance use disorders***

Chair: Lynette Daws, Univ. of Texas (US), co-chair: Sonja Sucic, Medical Univ. of Vienna (AT)

Prof. **Lynette Daws**, Univ. of Texas (US)

Title: [Unfaithful transporters: Insights into mechanisms of action of therapeutic and abused drugs](#)

Prof. **Harald H. Sitte**, Medical Univ. of Vienna (AT)

Title: [Impact of plasma membrane constituents on transporters responsible for monoamine clearance](#)

Asst. Prof. **T. Lee Gilman**, Kent State Univ (US)

Title: [Pharmacologically unmasking the functional contribution of PMAT to monoamine uptake](#)

Post doc. **Felix P. Mayer**, Florida Atlantic Univ. (US)

Title: [Dopamine transporters out of control: Anomalous dopamine efflux and a potential remedy via manipulation of kappa opioid signaling](#)

Assoc. Prof. **Ali Salahpour**, Univ. of Toronto (CA)

Title: [Structure activity relationship of DAT pharmacological chaperones](#)

12:30-13:30

Lunch

13:30-14:15

Keynote Speaker: **Christine Ziegler**, Univ. of Regensburg "A new role of osmoregulated BCC secondary transporters in antibiotic-resistant pathogens"

14:15-14:35

Coffee break

14:35-16:15

Session 2. ***Structural and functional dynamics of transporters***

Chair: Ute Hellmich, Friedrich Schiller University Jena (DE)

Assoc. Prof. **Oded Lewinson**, Technion (IL)

Title: [Conformational dynamics of ABC transporters: From single molecules to in vivo studies](#)

Prof. **Enrica Bordignon**, University of Geneva (CH)

Title: [Dynamics of ABC transporters in cellular membranes: a milestone achievable with EPR](#)

Prof. **Dimitrios Stamou**, Univ. of Copenhagen (DK)

Title: [Off-cycle transport states in function and regulation](#)

Assoc. Prof. **Thomas Stockner**, Medical Univ. of Vienna (AT)

Title: [Using MD simulations to investigate transporter dynamics at all atom resolution](#)

16:15-16:35

Break

16:35-18:15

Session 3. **Heteromeric amino acid transporters**

Chair: Manuel Palacín, Univ. de Barcelona (ES)

Prof. **Simon Newstead**, Univ. of Oxford (UK)

Title: [Molecular basis for redox control by the human cystine/glutamate antiporter System xCT](#)

Prof. **Manuel Palacín**, Univ. de Barcelona (ES)

Title: [Structural determinants of substrate affinity and selectivity in HATs](#)

Prof. **Yoshikatsu Kanai**, Osaka Univ. (JP)

Title: [Structural and cellular signaling bases of anti-cancer therapeutics targeting amino acid transporter LAT1/4F2hc](#)

Ph.D student **Lisa Knaus**, Inst. of Science and Technology (AT)

Title: [The role of *Slc7a5* during a critical developmental window of cortical refinement and metabolic adaptation in the brain](#)

18:15-?

Welcome Reception

Wednesday

08:45-08:50

Welcome

08:50-10:30

Session 4. **The neglected SLC6 transporters: Structure and function of the GABA transporter subfamily**

Chair: *Petrine Wellendorph, Univ. of Copenhagen, co-chair: Stefanie Kicking, Univ. of Copenhagen*

Asst. Prof. **Azadeh Shahsavari**, Aarhus Univ. (DK)

Title: [Structural insights into the mechanism of glycine transport and inhibition](#)

Prof. **Klaus T. Wanner**, LMU Munich (DE)

Title: [Identification of new GAT inhibitors by means of MS binding assays in combination with affinity selection mass spectrometry](#)

Prof. **Claire Colas**, Univ of Vienna (AT)

Title: [Structural determinants of binding of the creatine transporter \(CreaT, SLC6A8\) inform the functional annotation of the SLC6 transporters family](#)

Prof. **Petrine Wellendorph**, Univ. of Copenhagen (DK)

Title: [Molecular characterization of the extrasynaptic GABA transporters and their pharmacological potential in ischemic stroke](#)

10:30-10:50

Coffee break

10:50-12:30

Session 5. **Cancer metabolism – shaping metabolic flux through membrane transport**

Chair: *Louise Fets, MRC London Inst. of Medical Sciences (UK)*

Post doc **Ahmad Cluntun**, University of Utah (US)

Title: [Controlling the flow - exploiting pyruvate and lactate transport to treat cardiac hypertrophy](#)

Prof. **Daniel Tennant**, Univ. of Birmingham (UK)

Title: [Source matters; handling of exogenous versus endogenous proline suggests transporters as a selective therapeutic target](#)

Group leader **Susumu Hirabayashi**, MRC London Inst. of Medical Sciences (UK)

Title: [The role of transporters on host-tumour metabolic interactions](#)

Post doc **Emily Barnes**, MRC London Inst. of Medical Sciences (UK)

Title: [Investigating the therapeutic implications of SLC transporters and metabolism in cancer ns](#)

12:30-14:15 Lunch & Poster Session 1

14:15-15:00 Keynote Speaker: **Robert H. Edwards**, UCSF School of Medicine "*The allosteric regulation of synaptic vesicle glutamate transport*"

15:00-15:20 Coffee break

15:20-17:00 Session 6. **Slc38 transporters in neuronal signaling and brain diseases**

Chair: *Farrukh A. Chaudhry, Univ. of Oslo (NO)*

Prof. **Farrukh A. Chaudhry**, Univ. of Oslo (NO)

Title: [Ammonium ion \(NH₄⁺\) perturbs SLC38 glutamine transporter function which underpins neurotoxicity in hyperammonemic encephalopathies](#)

Assoc. Prof. **Brian Billups**, Australian National University (AU)

Title: [Amino acid transport for maintaining neurotransmission at glutamatergic synapses](#)

Prof. **Herman Wolosker**, Israel Institute of Technology (IL)

Title: [Slc38a5 is a physiological serine transporter at the blood-brain barrier and is critical for neurodevelopment](#)

Prof. **Kaspar Locher**, ETH Zürich (CH)

Title: [What distinguishes a multidrug from a lipid transporter?](#)

Thursday

08:45-08:50 Welcome

08:50-10:30 Session 7. **Lipid gymnastics - Highlights on lipids translocation mechanisms**

Chair: *Christine Ziegler, Univ. of Regensburg (DE)*

Asst. Prof. **Ahmad Reza Mehdipour**, Ghent University (BE)

Title: [Molecular dynamics of substrate recognition and transport in lipid transporters](#)

Prof. **Camilo Perez**, Univ. of Basel (CH)

Title: [Mechanism of a proton-dependent lipid transporter involved in teichoic acid synthesis](#)

Prof. **Cristina Paulino**, Univ. of Groningen (NL)

Title: [A true chimera: Transport mechanism and regulation of the KdpFABC complex](#)

Group leader **Chloé Martens**, Univ. Libre de Bruxelles (BE)

Title: [An embedded lipid in the efflux pump LmrP suggests a mechanism for multidrug recognition](#)

10:30-10:50 Coffee break

10:50-12:00 **Young Scientist Session**

1. Solveig Schmidt: The dopamine transporter antiports potassium to increase the uptake of dopamine (poster #61)
2. Jennifer Kahlhofer: Molecular mechanism of nutrient transporter degradation in human cells (Poster #35)
3. Imre Gonda: Conserved structural elements essential for siderophore import by the mycobacterial ABC transporter IrtAB (Poster #26)
4. Nataliia Dmitrieva: Exploring the transport cycle of a bacterial homolog of vesicular glutamate transporters (Poster #23)
5. Ágota Tóth: Structural and dynamical investigation of the MRP1 in different lipid bilayers by means of molecular dynamics (Poster #67)

12:00-13:45 Lunch & Poster Session 2

13:45-14:30 Keynote Speaker: **Raimund Dutzler**, Univ. of Zurich "The TMEM16 family of calcium activated lipid scramblases and ion channels"

14:30-14:50 Coffee break

14:50-16:30 Session 8. **Human ASCT2 - from structure to anticancer therapy**

Chair: Dirk Slotboom, Univ. of Groningen (NL)

Assoc Prof. **Avner Schlessinger**, Mount Sinai School of Medicine (US)

Title: [Harnessing Computational Approaches for Rational Design of ASCT2 Ligands](#)

Prof. **Renaë Ryan**, Univ. of Sidney (AU)

Title: [Characterising unexpected interactions of a glutamine transporter inhibitor with members of the SLC1A transporter family](#)

Prof. **Christof Grewer**, Univ. of Binghamton (US)

Title: [Mechanism of transport and inhibition of cancer-relevant glutamine transporters from the solute carrier 1 and 6 families](#)

Assoc Prof. **Albert Guskov**, Univ. of Groningen (NL)

Title: [A structural view onto disease-linked mutations in the human neutral amino acid exchanger ASCT1](#)

Friday

09:00-10:40

Session 9. **SLC4- and SLC9 family acid-base transport proteins - from structure to human disease**

Chair: Stine F. Pedersen, Univ. of Copenhagen (DK)

Prof. **Joe R. Casey**, Univ. of Alberta (CA)

Title: [Conformational changes in Band 3 \(SLC4A1/ AE1\) and possible significance for Erythrocyte Senescence Signaling](#)

Prof. **David Drew**, Stockholm Univ. (SE)

Title: [Elevating the molecular basis for sodium/proton exchange](#)

Prof. **Ebbe Bødtkjær**, Aarhus Univ. (DK)

Title: [Bicarbonate transporters and sensors in breast carcinogenesis](#)

Prof. **Stine F. Pedersen**, Univ. of Copenhagen (DK)

Title: [Structural and functional insights into NHE1 regulation](#)

10:40-11:00

Coffee break

11:00-12:40

Session 10. **Molecular mechanisms of Na⁺-coupled neurotransmitter transport**

Chair: Baruch Kanner, Hebrew Univ. Medical School (IL)

Prof. **Gary Rudnick**, Yale Univ. (US)

Title: [New developments in Neurotransmitter Transport Mechanisms](#)

Senior Investigator **Lucy R. Forrest**, Natl. Inst. on Neurological Disorders and Stroke - NIH (US)

Title: [Insights into neurotransmitter transport from modeling and simulation](#)

Group leader **Nicolas Reyes**, Université de Bordeaux/CNRS (FR)

Title: [Ion coupling mechanism in excitatory amino acid transporters](#)

Prof. **Christoph Fahlke**, Forschungszentrum Jülich (DE)

Title: [Mechanisms of K⁺-coupled glutamate transport](#)

12:40-13:45	Lunch
13:45-14:30	Keynote Speaker: Randy Blakely , Florida Atlantic Univ. " <i>Presynaptic Choline Transporters: From Gene to Mouse to Disease</i> "
14:30-14:50	Coffee break
14:50-16:55	<p>Session 11. <i>Emerging roles for transporters in dopamine dysfunction in CNS disorders</i> Chair: Nikhil Urs, Univ. of Florida (US), co-chair: Ulrik Gether, Univ. of Copenhagen (DK) Asst. Prof. Freja Herborg, Univ. of Copenhagen (DK) Title: Dopamine transport dysfunction in neuropsychiatric disease and parkinsonism Prof. Habibeh Khoshbouei, Univ. of Florida (US) Title: iPSc-derived human-like dopamine neurons reveal a potential therapeutic target for Parkinson's disease Asst. Prof. Thomas Steinkellner, Medical Univ. of Vienna (AT) Title: Role for VGLUT2 in dopamine neuron vulnerability and Parkinson's disease Asst. Prof. Nikhil Urs, Univ. of Florida (US) Title: Role of PFC catecholamine transporters in regulating striatal dopamine neurotransmission in health and disease Prof. Poul Nissen, Aarhus University (DK) Title: Structure and mechanism of human NKCC1</p>
18:00-?	Canal Tour & Farewell Dinner

Keynote abstracts

1. A new role of osmoregulated BCC secondary transporters in antibiotic-resistant pathogens

Prof. **Christine Ziegler**, University of Regensburg, Germany

Antibiotic resistance is a growing problem worldwide considered by the WHO as one of the greatest threats to world health. Pathogens resistant to several antibiotics such as *Acinetobacter baumannii* are feared as they particularly threaten patients with a weakened immune system. *A. baumannii* can remain infectious for a long time even in a dry environment surviving on clinical surfaces, on dry human skin or in body fluids, which contain relatively high concentrations of salts and other solutes. High salt, high osmolarity and dry environments would cause massive cell shrinkage and death as water is pulled out of the cells at low water activities, however, *A. baumannii* has developed an amazing stress defense machinery. It accumulates compatible solutes such as betaine or its precursor choline by stress-regulated Betaine-Choline-Carnitine Transporters (BCCTs), but in addition it synthesized *de novo* glutamate and the sugar alcohol mannitol, that binds water very strongly. Interestingly, in the antibiotic-resistant pathogen *Acinetobacter baumannii* the stress protectant trehalose is synthesized, too. Trehalose not only impacts virulence and growth at high temperatures and high salinities, it seems to be also the key in drug resistance.

Recently, we have solved the atomic structures of a suite of six BCCTs from the highly virulent *A. baumannii* strain AYE by cryo-EM and X-ray crystallography. We could identify unique additional domains in these LeuT-fold transporters and describe a new trehalose sensing mechanism for one of the osmoregulated betaine specific BCC symporters. Based on our structural and functional data we suggest a regulatory role of BCCTs in trehalose synthesis affecting the virulence of *A. baumannii* strains. As trehalose being an abundant disaccharide in bacterial pathogens, but not in mammals, can be considered an attractive target for the development of antimicrobial drugs, our molecular insights into the complex regulation machinery orchestrated by BCCTs might be a first step to fight *A. baumannii* infections.

2. The Allosteric Regulation of Synaptic Vesicle Glutamate Transport

Prof. **Robert H. Edwards**, UCSF School of Medicine, UCSF School of Medicine, USA

Proteins of Solute Carrier Family 17 (SLC17) use diverse mechanisms to transport anions. Originally identified as Na⁺-dependent phosphate transporters, they were subsequently found to transport organic anions. The lysosomal SLC17 protein sialin couples H⁺ to cotransport of sialic acid, and the resulting electroneutrality enables efflux from lysosomes driven by the outwardly directed pH gradient. Several structures of bacterial H⁺/D-galactonate transporter DgoT identify the residues crucial for substrate recognition and indicate the mode of coupling to H⁺. In contrast, the vesicular glutamate transporters (VGLUTs) are driven by membrane potential and undergo allosteric regulation by lumenal H⁺ as well as Cl⁻, which appears to coordinate VGLUT activity with the changing ionic conditions of cycling synaptic vesicles. We have also determined multiple structures of VGLUT2, with as well as without substrate. In addition to the central role of two arginines in recognition of the glutamate carboxyl groups and a phenylamine in recognition of the amine group through a cation- π interaction, the structures identify an asymmetry in the pathway to the substrate recognition site from both sides of the membrane and palmitoylation that regulates retrieval by endocytosis. The structures also reveal an extensive cytosolic charge network that acts as the cytosolic gate. We find that the strength of this gate dictates the nature of allosteric regulation by H⁺ on the other, lumenal side of the membrane, suggesting a general mechanism for the allosteric regulation of membrane transport.

3. The TMEM16 family of calcium-activated lipid scramblases and ion channels

Prof. **Raimund Dutzler**, Dept. of Biochemistry, University of Zurich, Zurich, Switzerland.

The TMEM16 proteins constitute a family of membrane proteins, which comprises lipid scramblases and Cl⁻ channels that are involved in diverse physiological processes. Whereas TMEM16 channels contribute to Cl⁻ secretion in airway epithelia and electrical signaling in smooth muscles and certain neurons, TMEM16 scramblases participate in blood clotting and the fusion of trophoblasts, osteoclasts and myoblasts. Members of both functional branches share a common architecture and are activated by intracellular Ca²⁺ by a similar mechanism. TMEM16 proteins form homodimers of subunits that are composed of ten membrane-spanning helices. The subunits are functionally independent and contain a regulatory Ca²⁺-binding site embedded within the transmembrane domain and a close-by site of catalysis located at the periphery of the protein, which either facilitates ion or lipid permeation.

In the fungal lipid scramblase nhTMEM16, Ca²⁺-binding opens a hydrophilic membrane-spanning furrow, which is hidden in the Ca²⁺-free protein. This furrow provides a pathway for lipid headgroups to move between both leaflets of the bilayer. In contrast, in the anion channel TMEM16A, Ca²⁺-binding triggers the opening of a protein-enclosed ion conduction pore located in the same region, which remains shielded from the membrane in its activated state. In this case, Ca²⁺ serves a dual role in promoting a conformational change to release a gate that impedes conduction in the closed state and by shaping the electrostatics to enhance anion permeation. Finally, in the lipid scramblase TMEM16F, we find both functions as lipid scramblase and ion channel contained within the same protein. Despite its close relationship to the anion channel

4. Presynaptic Choline Transporters: From Gene to Mouse to Disease

Prof. **Randy D. Blakely**, Executive Director, FAU Stiles-Nicholson Brain Institute

Acetylcholine, the first identified neurotransmitter, and perhaps the best defined in terms of synthesis, inactivation and signaling, still poses questions whose resolution mean the difference between a healthy, happy life and a life of emotional, cognitive, autonomic, and even fatal disorders. These questions encouraged us some two decades ago to clone and characterize human and mouse choline transporters. Unlike other neurotransmitters that are removed from the synapse after release by high-affinity transport mechanisms, acetylcholine is rapidly inactivated after release by the enzyme acetylcholinesterase, which produces acetate and choline, the latter the biosynthetic precursor to acetylcholine. Indeed, it is the uptake of choline that is rate limiting in the synthesis of acetylcholine, particularly under states where acetylcholine release is high and/or sustained. We have characterized the regulatory mechanisms that position sufficient choline transporters at the presynaptic membrane to achieve optimum recapture of choline, efforts that reveal a remarkable coupling between the acetylcholine release process and choline transport. Moreover, through studies of choline transporter deficient mice, we have demonstrated the essential role the transporter plays in cholinergic signaling and extended these efforts to identify humans with transporter dysfunction. My lecture will trace the arc of these studies, ending with most recent work that demonstrates cognitive deficits arising in humans as well as genetically engineered mice expressing a choline transporter coding variant found in ~10% of the U.S. population and a sizeable number of individuals worldwide.

Session 1. New kids on the monoamine transporter block - Implications for treatment of psychiatric and substance use disorders

Chairs: Lynette Daws, University of Texas Health Science Center at San Antonio (US)

Co-Chair: Sonja Sucic, Medical University of Vienna (AT)

INTRO: Serotonin and dopamine transporters (SERT and DAT) have long been implicated in the etiology and treatment of psychiatric and substance use disorders. However, a major concern is that therapeutics targeting these transporters provide suboptimal, or no therapeutic benefit for many. In this symposium we will provide new insights into novel targets to treat these debilitating disorders, including organic cation transporter 3, the plasma membrane monoamine transporter and novel regulators of DAT.

1. Unfaithful transporters: Insights into mechanisms of action of therapeutic and abused drugs

Prof. **Lynette C. Daws**, Departments of Cellular & Integrative Physiology and Pharmacology, University of Texas Health Science Center at San Antonio, USA

Data from our lab, and others, have revealed an important role for “uptake-2” cation transporters, low-affinity, but high-capacity transporters for monoamines, in regulating monoamine homeostasis. These transporters are polyspecific, taking up dopamine (DA), norepinephrine (NE) and serotonin (5-HT), with varying affinities, depending on class. Two “uptake-2” transporters most prominent in this regard are organic cation transporter 3 (OCT3) and plasma membrane monoamine transporter (PMAT). These are located in limbic brain regions, with evidence for their existence on neurons and glia. Here we provide a brief overview of our past findings revealing an important role for these transporters in actions of therapeutic (e.g. antidepressant) and abused (e.g. psychostimulant) drugs. We then focus on our recent, unpublished findings using constitutive, conditional and viral knock out (KO) approaches showing a crucial role for OCT3 in the actions of amphetamine and ethanol, and in fear memory. We show that amphetamine-evoked DA release (constitutive, inducible or viral), amphetamine self-administration (constitutive) and conditioned place preference (CPP) for amphetamine (constitutive, inducible) are markedly reduced in mice with OCT3 KO. We show that ethanol interacts with OCT3 to inhibit uptake of DA, and potentiates the ability of cocaine to inhibit DA uptake and produce CPP in wild-type mice, effects that are lost in constitutive OCT3 KO mice. Finally we show that KO of OCT3 from serotonergic neurons in basolateral amygdala (BLA) results in markedly slowed clearance of 5-HT from BLA. Moreover, mice with loss of OCT3 on 5-HT neurons globally, or virally knocked down locally in BLA, show increased fear memory and anxiety-like behaviors. Overall, data presented here provide compelling evidence for continued exploration of OCT3, and putatively PMAT, as targets for improving treatments for psychiatric and substance use disorders.

2. Impact of plasma membrane constituents on transporters responsible for monoamine clearance

Prof. **Harald H. Sitte**, Medical University of Vienna, Austria

Several transporters in the central nervous system take care of clearing the extrasynaptic space from monoamine neurotransmitters such as serotonin, dopamine and norepinephrine. The clinically relevant target of antidepressant drugs and a number of important psychostimulants are typically the monoamine transporters DAT, NET and SERT. These compounds inhibit the reuptake of monoamines by competitively blocking the transporters' action or acting as substrates and induce a reversal of the transport direction, so-called non-exocytotic neurotransmitter release or transporter-mediated efflux. In addition, organic cation transporters also contribute to this clearing mechanism as transporters with lower affinity but high capacity. Especially, OCT3 was suggested as a putative target for drug discovery since it may contribute to an antidepressive effect in the CNS.

Recent advancement in the understanding of the structural and molecular mechanisms of psychostimulant-induced efflux via these transporters will be discussed, mainly amphetamine- and cathinone. Finally, the important regulatory role of membrane lipids in these processes, mainly phosphoinositides such as PIP₂ and cholesterol, will also be highlighted.

3. Pharmacologically unmasking the functional contribution of PMAT to monoamine uptake

Asst. Prof. **T. Lee Gilman**, Kent State University, Kent, OH, USA

Plasma membrane monoamine transporter (PMAT, *Slc29a4*) transports monoamine neurotransmitters, including dopamine and serotonin, faster than more studied monoamine transporters, e.g., dopamine transporter (DAT), serotonin transporter (SERT). A considerable challenge in understanding PMAT's monoamine clearance contributions is that no current drugs selectively inhibit PMAT. To advance knowledge about PMAT's monoamine uptake role, and to circumvent this present challenge, we investigated how drugs that selectively block DAT/SERT influence behavioral readouts in PMAT wildtype, heterozygote, and knockout mice of both sexes. Drugs typically used as antidepressants (escitalopram, bupropion) were administered acutely for readouts in tail suspension test and locomotion. Drugs with psychostimulant properties (cocaine, D-amphetamine) were administered repeatedly to assess initial locomotor responses plus psychostimulant-induced locomotor sensitization. Though we hypothesized PMAT-deficient mice would exhibit augmented responses to antidepressant and psychostimulant drugs due to constitutively attenuated monoamine uptake, we instead observed sex-selective responses to antidepressant drugs in opposing directions, and subtle sex-specific reductions in psychostimulant-induced locomotor sensitization. These results suggest PMAT functions differently across sexes, and support hypotheses that PMAT's monoamine clearance contribution emerges when frontline transporters (e.g., DAT, SERT) are saturated and/or blocked. Thus, known human polymorphisms that reduce PMAT function could be worth investigating as contributors to varied antidepressant and psychostimulant responses.

4. Dopamine transporters out of control: Anomalous dopamine efflux and a potential remedy via manipulation of kappa opioid signaling

Post doc **Felix P. Mayer**, Florida Atlantic University, USA

Adele Stewart¹, Michelle Velez¹, Paul J. Gresch¹, Maximillian J. Rabil¹, Roxanne A. Vaughan², Lynette C. Daws³, Sammanda Rammamoorthy⁴ and Randy D. Blakely^{1,5}

¹ Department of Biomedical Science, Florida Atlantic University, Jupiter, FL, USA

² Department of Biomedical Sciences, University of North Dakota School of Medicine and Health Sciences, Grand Forks, North Dakota, United States

³ Department of Cellular & Integrative Physiology, University of Texas Health Science Center at San Antonio, San Antonio, TX, United States

⁴ Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, Virginia, United States.

⁵ Stiles-Nicholsen Brain Institute, Florida Atlantic University, Jupiter, FL, USA

Extracellular dopamine (DA) is tightly regulated by the presynaptic dopamine (DA) transporter (DAT). The rare DAT Val559 variant has been identified in individuals with attention-deficit hyperactivity disorder (ADHD), bipolar disorder and autism. Behavioral alterations of male Val559 mice include waiting impulsivity, enhanced motivation for reward, working memory deficits and altered locomotor responses to psychostimulants. The DAT Val559 variant mediates a persistent outward leak of DA (anomalous DA efflux, ADE) that supports continuous stimulation of presynaptic D₂-type autoreceptors (D₂ARs), leading to elevated surface levels of DAT Val559 in the male dorsal striatum. As presynaptic kappa opioid receptors (KORs) have been shown to regulate DAT surface expression and activity, we are exploring the potential of KOR antagonism to ameliorate biochemical, physiological and behavioral phenotypes associated with DAT Val559 expression, using biochemical approaches with acute striatal brain slices from WT and male DAT Val559 mice. In ongoing studies, we find that KOR agonists elevate DAT Thr53 phosphorylation (a site that has been linked to DAT-dependent amphetamine-induced DA efflux and DAT function) and increase DAT surface expression in the dorsal striatum of DAT WT mice. Conversely, the KOR antagonist nor-binaltorphimine (norBNI) reduces both Thr53 phosphorylation and surface trafficking of DAT Val559, but has no effect on WT DAT in acute striatal slices. *In vivo*, we observe that systemic administration of norBNI normalizes the vesicular DA release deficits observed in male Val559 knock-in mice, as revealed by the DAT inhibitor cocaine. Finally, systemic administration of norBNI appears to reverse working memory impairments of male Val559 mice and to normalize their aberrant motor behavior in the open field. Together, these studies support the ability of KOR antagonism to offset multiple deficits associated with DAT-mediated ADE and suggest that pharmacological modulation of KOR-activity may provide a novel treatment for disorders linked to altered DA signaling.

5. Structure activity relationship of DAT pharmacological chaperones

Assoc. Prof. **Ali Salahpour**, Department of Pharmacology and Toxicology, University of Toronto, Canada

Coding variants in the dopamine transporter (DAT) have been implicated in many human diseases. Among these is the infantile parkinsonism-dystonia known as Dopamine Transporter Deficiency Syndrome (DTDS). Afflicted individuals have minimal to no functional dopamine transporter protein primarily due to retention of misfolded disease-causing variants. Though no treatment is currently available, pharmacological chaperones targeting the dopamine transporter have been shown to rescue select DTDS disease-causing variants.

Previous work has identified two DAT pharmacological chaperones with moderate potency and efficacy: bupropion and ibogaine. In this study, we carried out structure-activity relationships (SARs) for bupropion and ibogaine with the goal of identifying the chemical features required for pharmacological chaperone activity. Our results show that the isoquinuclidine substituent of ibogaine and its analogues is an important feature for ibogaine pharmacological chaperone efficacy. For bupropion, the secondary amine group is essential for pharmacological chaperone activity. Lastly, we describe additional ibogaine and bupropion analogues with varying chemical modifications and variable pharmacological chaperone efficacies at the dopamine transporter. Our results contribute to the design and refinement of future dopamine transporter pharmacological chaperones with improved efficacies and potencies.

Session 2. Structural and functional dynamics of transporters

Chair: Ute Hellmich, Friedrich Schiller University Jena, Germany

INTRO: To properly carry out their function, membrane transporters have to move. Complementary methods, both in the wet lab and in silico, are suited to investigate various aspects of membrane protein function dynamics, on different time scales and in different environments – the ultimate dream being to understand an individual transporter in the cell at atomic detail. In addition, it is becoming less and less clear that all transporters follow a “smooth” transport cycle, rather, it seems likely that they occasionally populate off-cycle states that are nonetheless important to understand the playing field a protein occupies. This session will highlight some of the recent methodological advances to study these aspects in ABC and SLC transporters as well as P- and V-type ATPases.

1. Conformational dynamics of ABC transporters: From single molecules to in vivo studies

Assoc. Prof. **Oded Lewinson**, Technion, Israel

ABC transporters are members of an ancient superfamily of transport proteins that play diverse and vital roles in all kingdoms of life. They couple the energy derived from ATP hydrolysis to the transport of a wide range of biomolecules. Although they share a common basic architecture, they have evolved to perform many different functions. In this talk, I will present recent results that explain how this common architecture is fine-tuned by conformational dynamics and allosteric connectivity to give rise to distinct transport mechanisms and functional adaptations.

2. Dynamics of ABC transporters in cellular membranes: a milestone achievable with EPR

Prof. **Enrica Bordignon**¹, University of Geneva, Switzerland (CH)

Laura Galazzo¹, Gianmarco Meier², Dovile Janulienė³, Kristian Parey³, Dario De Vecchis⁴, Bianca Striednig², Hubert Hilbi², Lars V. Schäfer⁴, Ilya Kuprov⁵, Arne Moeller³, Markus A. Seeger²

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The structural properties of ABC transporters are currently mostly investigated after detergent extraction from native cellular membranes and reconstitution into artificial liposomes or nanodiscs, thereby removing them from their physiological environment. However, to truly understand the biophysical properties of membrane proteins, they should be ideally investigated within living cells. Here, we utilize Electron Paramagnetic Resonance methods using a spin-labeled nanobody as conformational reporter to interrogate the conformational cycle of the wild type ABC transporter MsbA in detergent micelles, liposomes, nanodiscs, inside-out vesicles and *E. coli* cells. Surprisingly, the wide inward-open conformation of MsbA, commonly considered a non-physiological state, was found to be prominently populated in metabolically active *E. coli* cells. The EPR study is complemented by cryo-EM analysis of MsbA in nanodiscs in the absence and presence of spin-labeled nanobodies and by molecular dynamics simulations, which revealed that extensive lateral portal opening is essential to provide access of its large natural substrate core lipid A to the binding cavity. Our work paves the way to investigate the conformational landscape of membrane proteins directly in cells (1,2).

(1) Galazzo, Meier et al., PNAS 2020 117(5):2441-2448 (2) Galazzo, Meier et al., 2022, under revision

3. Off-cycle transport states in function and regulation

Prof. **Dimitrios Stamou**, University of Copenhagen, Denmark

We have recently developed a method to resolve ionic currents with a sensitivity that is one million times higher than patch clamp.¹ This technical innovation allowed us to resolve for the first time the ionic currents mediated by primary and secondary active transporters at the single molecule level. The main finding emerging from these studies is that transporters venture outside the canonical transport cycle to states we refer to as “off-cycle states” and which can be inactive, sub-conducting or leaky. Here, I will discuss unpublished data highlighting the role of off-cycle states in the function and regulation of P-type ATPases, and the V-type ATPase.

(1) Veshaguri, S.; Christensen, S. M.; Kemmer, G. C.; Ghale, G.; Møller, M. P.; Lohr, C.; Christensen, A. L.; Justesen, B. H.; Jorgensen, I. L.; Schiller, J.; Hatzakis, N. S.; Grabe, M.; Pomorski, T. G.; Stamou, D. *Science* **2016**, *351* (6280), 1469.

4. Using MD simulations to investigate transporter function at all atom resolution.

Assoc. Prof. **Thomas Stockner**, Medical University of Vienna, Austria

Membrane transporters translocate substrates across membranes, while moving through a series of conformational states. The conceptual basis to allow for transport was initially proposed by Jardetzky as the alternating access hypothesis. Several transporters have now been resolved in at least two conformations, but evidence is accumulating that the transport cycle contains multiple (intermediate) state, both for primary active and secondary active transporters. Essential for substrate transport against an existing concentration gradient is energy input and an energetic downhill process that allows the transporter to move along the path of intermediate conformations of the transport cycle. I will show data on the dynamics and energetics of the primary active multidrug resistance transport ABCB1 and the secondary active serotonin transporter SERT. In particular, on the power stroke of ABCB1 that converts the chemical energy stored in ATP into mechanical forces and the first step of the transport cycle of SERT, which leads to substrate occlusion.

Session 3. Heteromeric Amino acid Transporters

Chair: Manuel Palacín, Univ. de Barcelona, Spain

INTRO: Heteromeric Amino acid Transporters (HATs) are composed by a heavy or ancillary subunit (SLC3 family) and a light or transporter subunit (SLC7 family). The physiological relevance of HATs is highlighted by their role in cancer, several inherited diseases and metabolism.

This session will cover physiology, pathophysiology, structural biology and mechanisms of transport and pharmacology of HAT transporters.

1. Molecular basis for redox control by the cystine/glutamate antiporter xCT.

Prof. **Simon Newstead**, Biochemistry, University of Oxford, United Kingdom

Cysteine plays an essential role in cellular redox homeostasis as a key constituent of the tripeptide glutathione (GSH). A rate limiting step in cellular GSH synthesis is the availability of cysteine. However, circulating cysteine exists in the blood as the oxidised di-peptide cystine, requiring specialised transport systems for its import into the cell. System x_c^- is a dedicated cystine transporter, importing cystine in exchange for intracellular glutamate. To counteract elevated levels of reactive oxygen species in cancerous cells system x_c^- is frequently upregulated, making it an attractive target for anticancer therapies. However, the molecular basis for ligand recognition remains elusive, hampering efforts to specifically target this transport system. Here we present the cryo-EM structure of system x_c^- in both the apo and glutamate bound states. Structural comparisons reveal an allosteric mechanism for ligand discrimination, supported by molecular dynamics and cell-based assays, establishing a mechanism for cystine transport in human cells.

2. Structural determinants of substrate interaction in Heteromeric amino acid transporters

Prof. **Manuel Palacín**, Institute for Research in Biomedicine-Barcelona, University of Barcelona and CIBERER

Heteromeric amino acid transporters (HATs) are one of the ten types of amino acid transporters present in the human body. Growing interest in the pathophysiological role of this group of transporters in rare and complex diseases and cancer has brought about the recent resolution of various structures of human HATs and bacterial homologues at atomic level. This knowledge sheds light on the mechanisms of transport used by these molecules. I will present the identified molecular bases underlying substrate binding asymmetry in HATs and substrate preference in HATs that transport only neutral amino acids.

3. Structural and cellular signaling bases of anti-cancer therapeutics targeting amino acid transporter LAT1/CD98hc

Prof. **Yoshikatsu Kanai**, Department of Bio-system Pharmacology, Graduate School of Medicine, Osaka University, Osaka, Japan

Since molecular identification, the heteromeric complex of LAT1 (SLC7A5) and 4F2hc (CD98hc) has been shown to be highly expressed in tumor cells and cells stimulated by pathological conditions. Thus, the role of LAT1-4F2hc in the pathogenesis of diseases has been investigated from various aspects. We have shown that LAT1 is upregulated with high specificity for cancer cells, and its expression level is related to the prognosis of cancer patients. We have studied LAT1 as a target for cancer diagnosis and treatment and generated LAT1-specific substrates and inhibitors based on the structure-activity relationship analysis of LAT1 ligands. We have explored the applications of LAT1-specific substrates for PET diagnosis with high cancer specificity, boron neutron capture therapy, and nuclear medicine treatment with astatine-labeled compounds. The LAT1-specific high-affinity inhibitors we developed are currently in clinical trials as anticancer drugs. Because LAT1 is responsible for the cellular supply of essential amino acids, including leucine that stimulates mTORC1-mediated signaling, LAT1 is supposed to contribute to the metabolic regulation of cells. We demonstrated it by employing phosphoproteomics and comprehensively analyzing changes in cellular signaling caused by LAT1 inhibitors. Finally, to further optimize the LAT1 substrates and inhibitors for clinical use, it is essential to reveal the structure of the compounds' binding sites on LAT1 and how the compounds affect the post-binding LAT1 conformation. The cryo-electron microscopy analysis has revealed both apo structure and substrates/inhibitors-bound structures. We have conducted a site-directed mutagenesis study and revealed residues whose alteration influences the substrate selectivity of LAT1, which would be essential for recognizing the side chain of the substrates.

4. The role of *Slc7a5* during a critical developmental window of cortical refinement and metabolic adaptation in the brain

Lisa Knaus, IST Austria; PhD student in the Novarino group

Large neutral amino acids are key players of fundamental cellular processes including metabolism and ATP production. This implies that a loss of these substrates during critical developmental windows could have profound effects on organogenesis including brain formation. In line with this hypothesis, we recently identified mutations in the gene *SLC7A5*,

encoding a large neutral amino acid transporter (LAT1), as a rare cause of autism spectrum disorders and microcephaly. We found that in neural cells the expression of *Slc7a5* is temporally integrated with the postnatal period of cortical network refinement, indicating that this process is highly dependent on sufficient amino acid supply. Currently, however, little is known about the metabolic reprogramming that neuronal cells have to undergo during differentiation and maturation processes especially during the period where no glial metabolic support is available. Using metabolomics, proteomics and single cell RNA sequencing analysis we found that throughout development neurons undergo significant metabolic remodeling and that *Slc7a5* expression essentially contributes to a unique postnatal metabolic state. A deregulation of this perinatal metabolic transition leads to a stage- and cell-type-specific change in neuronal activity patterns and decreased neuronal survival.

Session 4. The neglected SLC6 transporters: Structure and function of GABA, glycine and creatine transporters

Chair: Prof. Petrine Wellendorph, University of Copenhagen, Denmark

Co-Chair: Dr. Stefanie Kicking, University of Copenhagen, Denmark

INTRO: While GABA and glycine are the two main inhibitory neurotransmitters in the brain, creatine plays a fundamental role in ATP homeostasis of tissues with high energy demands such as the brain. Thus, a tight control of glycine, GABA and creatine levels is essential. This is governed by their cognate transporters (GATs, GlyT and CreaT, respectively) positioned at presynaptic neurons/surrounding glial cells (GATs and GlyT) and the blood-brain barrier (CreaT). Given the range of pathologies associated with an imbalance in GABA and glycine neurotransmission as well as creatine deficiency, it is somewhat surprising that no more research is going into these fields. Consequently, this session aims to stimulate more interest by providing an overview of state-of-the-art GABA, glycine and creatine transporter research in order to shed light on structural and functional aspects.

1. Structural insights into the mechanism of glycine transport and inhibition

Asst. Prof. **Azadeh Shahsavari**, Aarhus University, Denmark

Peter Stohler³, Gleb Bourenkov², Iwan Zimmermann^{4,5}, Martin Siegrist³, Wolfgang Guba³, Emmanuel Pinard³, Clara N. Pedersen¹, Steffen Sinning⁶, Markus A. Seeger⁴, Thomas R. Schneider², Roger J. P. Dawson^{3,5}, Poul Nissen¹

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⁶Department of Forensic Medicine, Aarhus University, Aarhus, Denmark.

Glycine transporter GlyT1 is the main regulator of neuronal excitation and inhibition mediated by neurotransmitter glycine in the brain. Prolonging glycinergic signalling through selective inhibition of GlyT1 has been pursued extensively over the past two decades as a key strategy for the treatment of a broad range of neurological/psychiatric disorders including schizophrenia. GlyT1 inhibitors achieve antipsychotic and pro-cognitive effects against many

symptoms of schizophrenia, however a successful drug candidate has to come. To elucidate structure-based mechanisms for inhibition and transport in GlyT1, we have investigated its complexes with a benzoylpiperazine chemotype inhibitor and substrate glycine. Using an inhibition state-specific sybody and a serial synchrotron crystallography (SSX) approach, we have determined the structure of GlyT1 at 3.4 Å resolution to reveal the selective inhibitor-bound state, adopting an inward-open conformation. More recently, we have determined the cryo-electron microscopy (cryo-EM) structure of GlyT1 at 3.0 Å resolution showing the glycine-bound inward-facing occluded conformation. The data unveil a dual nature of non-competitive inhibitors of functional transport exhibiting also competitive binding to the substrate binding site of glycine. The results provide detailed insight into the mechanism of glycine transport and reuptake inhibition and help re-evaluate efforts for the development of efficacious GlyT1 inhibitors.

2. Identification of GAT1 inhibitors by means of MS Binding Assays in combination with affinity selection mass spectrometry

Prof. **Klaus T. Wanner**, Department of Pharmacy – Center for Drug Research, Ludwig-Maximilians-Universität München, Butenandtstr. 5-13, 81377 Munich, Germany

MS Binding Assays represent a label-free alternative to radioligand binding assays, providing basically the same capabilities as the latter. Thereby, *MS Binding Assays* have the advantage that any ligand addressing the respective target with suitable affinity and potency may be selected as reporter ligand provided its quantification by mass spectrometry (MS) allows a high enough sensitivity. As radioligand binding assays, *MS Binding Assays* can be carried out in form of saturation experiments or as kinetic experiments. Competition experiments finally allow to indirectly measure the affinity of test compounds by monitoring the binding of the *MS Marker*.

MS Binding Assays are, in addition, also well suited for library screening by utilizing competitive binding experiments revealing active sublibraries by the reduction of binding of a reporter ligand. In a subsequent step, hits of active sublibraries may be identified by testing individual library constituents in competitive binding experiments.

A considerable improvement of the efficiency of the hit identification step can be achieved by supplementing MS Binding Assays with the concept of affinity selection mass spectrometry (ASMS). In that case, only a single competitive MS binding experiment is performed with the individual sublibrary. As before, a sublibrary is considered as active, when the binding of reporter ligand has been reduced to a predefined extent. However, as in the sample used for quantification of the reporter ligand, also the hit compounds are contained, this sample is additionally employed for hit identification by MS – as in ASMS assays.

This concept has now been applied to the identification of inhibitors of the GABA transporter subtype 1 (GAT1) by screening various compound libraries.

3. Structural determinants of binding of the creatine transporter inform the functional annotation of the SLC6 transporters family

Dr. **Claire Colas**, University of Vienna, Austria and current affiliation Solgate GmbH, Austria

The solute carrier 6 (SLC6) family of transporters comprises 20 members with great pharmacological impact, as they transport neurotransmitters, amino acids, betaine, taurine and creatine across the cells. Most of the time, transporters are studied individually or by subgroup. Notably, members of the monoamine subgroup - containing the norepinephrine transporter NET (SLC6A2), the dopamine transporter DAT (SLC6A3) and the serotonin transporter SERT (SLC6A4) - have been extensively studied. These findings provide precious

insight into the three-dimensional architectures of these transporters, as well as their mechanism of transport and modulation. However, the structural determinants defining the substrate specificities across the entire SLC6 family are still poorly understood. We have previously built homology models of the creatine transporter CreaT (SLC6A8), belonging to the GABA transporter subgroup (GATs), using the structures of two templates, i.e. the human serotonin transporter SERT and the prokaryotic leucine transporter LeuT. Our models permitted us to identify key features responsible for the unique substrate specificity profile of CreaT and provide a framework for a deeper understanding of the distinct substrate specificities of the SLC6 family members.

Here, we gather the commonalities in the form of a generalized structural and functional annotation of the transporters of distinct SLC6 subgroups and create a holistic knowledge of the entire family. We describe a conserved molecular organization of SLC6 orthosteric sites and provide further insight into the structural determinants responsible for substrates selectivity within the family and other transporters sharing a similar fold, such as the SLC7 family.

This work paves the way towards a systematic and general understanding of the SLC structural determinants underlying their interactions with ligands, that will advance drug discovery for this therapeutically important protein family.

4. Molecular Characterization of extrasynaptic GABA transporters and their pharmacological potential in ischemic stroke

Dr. **Andrew Clarkson**, University of Otago, New Zealand

Functional recovery after ischemic stroke has been found to involve a chronic elevation in tonic inhibition mediated by extrasynaptic GABA_A receptors.¹ The stroke-induced increase in tonic inhibition is due to impaired function and expression of the GABA transporter, GAT3 (SLC6A11), whereas GAT1 is unaffected.¹ The role of the GABA/betaine transporter, BGT1 (SLC6A12), remains unknown. We hypothesized that substrates of GAT3 and BGT1 would increase GAT3 and BGT1 cell surface expression, thereby decreasing extracellular GABA levels. Thus, the aim of this study was to investigate the effects of GAT3 and BGT1 substrates on functional recovery after stroke in mice. By means of focal ischemic stroke induced by photo-thrombosis to the sensorimotor cortex of mice, we tested the GAT3 substrate, L-isoserine (IC₅₀ 38 μM), and the BGT1 substrate ATPCA (IC₅₀ 29 μM).² Compounds were administered from day 5 after stroke, directly in the ischemic core, via micro-osmotic pumps at doses corresponding to their IC₅₀ and 10xIC₅₀, respectively. Sensorimotor function was assessed one-week prior to stroke and then at weeks 1, 2, 4 and 6 post-stroke on both the grid-walking and cylinder tasks. Stroke volumes were assessed at week 6. We observed significant forelimb deficits (P<0.001) contralateral to the hemisphere with stroke for at least 6-weeks post-insult on both the grid-walking and cylinder task. Chronic delayed treatment with L-isoserine and ATPCA improved sensorimotor function from day 7 (high dose) and day 14 (low dose) in the grid-walking task and from day 14 (low and high doses) in the cylinder task. Treatment with L-isoserine and compound 9 did not affect stroke volumes. This study is the first to report improved functional recovery after stroke following a delayed long-term treatment with substrates for the GABA transporters, GAT3 and BGT1, in a mouse model of focal ischemic stroke.

Session 5. Cancer metabolism: shaping metabolic flux through membrane transport”

Chair: Dr. Louise Fets, MRC London Institute of Medical Sciences, United Kingdom

INTRO: Metabolic adaptation of cancer cells is essential in order to maintain high proliferation rates within an often-hostile micro-environment, and it is becoming clear that altered transporter expression patterns play an important role in fuelling these metabolic changes. Using models ranging from cell culture, to Drosophila, to mice, and techniques including genetics and stable-isotope labelling metabolomics, this session will explore the influence of transporters on tumour metabolism, how they mediate the effects of external influences such as diet, as well as the impact that their expression patterns may have on tumour drug sensitivity. In addition, we look at how our understanding of transporter influence on tumour metabolism can be applied to better understand their roles in other pathological contexts such as heart failure.

1. Controlling the flow - exploiting pyruvate and lactate transport to treat cardiac hypertrophy

Post doc **Ahmad Cluntun**, Rutter Lab, Department of Biochemistry, University of Utah, Salt Lake City, USA

The metabolic rewiring of cardiomyocytes is a widely accepted hallmark of heart failure (HF). These metabolic changes include a decrease in mitochondrial pyruvate oxidation and an increased export of lactate. We identify the mitochondrial pyruvate carrier (MPC) and the cellular lactate exporter monocarboxylate transporter 4 (MCT4) as pivotal nodes in this metabolic axis. We observed that cardiac assist device-induced myocardial recovery in chronic HF patients was coincident with increased myocardial expression of the MPC. Moreover, the genetic ablation of the MPC in cultured cardiomyocytes and in adult murine hearts was sufficient to induce hypertrophy and HF. Conversely, MPC overexpression attenuated drug-induced hypertrophy in a cell-autonomous manner. We also introduced a novel, highly potent MCT4 inhibitor that mitigated hypertrophy in cultured cardiomyocytes and in mice. Together, we find that alteration of the pyruvate-lactate axis is a fundamental and early feature of cardiac hypertrophy and failure.

2. Source matters; handling of exogenous versus endogenous proline suggests transporters as a selective therapeutic target

Prof. **Daniel Tennant**, University of Birmingham, United Kingdom

Proline is a non-essential amino acid with essential roles in both protein structure and redox homeostasis. The cellular enzymes that synthesise proline – pyrroline 5-carboxylate reductase 1-3 (PYCR1-3) – use NAD(P)H as part of their mechanism. Endogenous proline production therefore contributes to the redox state of the cell. However, the peripheral plasma contains between 100-200 μ M proline, which is freely available to cells by diffusion through members of the SLC36 and SLC38 families of plasma membrane transporters. I will discuss how cells differentially use proline derived from endogenous and exogenous sources, and whether a specific member of the SLC38 family may represent a putative therapeutic target in malignant brain tumours.

3. The role of transporters on host-tumour metabolic interactions

Group leader **Susumu Hirabayashi**, MRC London Institute of Medical Sciences, United Kingdom

Cancer is increasingly viewed as a systemic disease associated with a range of host metabolic changes including obesity, diabetes, and cachexia/muscle wasting syndrome, each of which alters the host metabolic and nutritional environment. Cancer cells actively acquire nutrients from the extracellular space to support their growth, but how cancer cells sense and respond to changes in systemic nutrient availability remains incompletely understood. We leverage the fruit fly *Drosophila melanogaster* as a model system to explore host-tumour metabolic and nutritional interactions. I present how our *Drosophila* studies have started to reveal mechanisms by which tumours respond to systemic metabolic changes through modulating the expression of transmembrane transporters.

4. Investigating the therapeutic implications of SLC transporters and metabolism in cancer

Post doc **Emily Barnes**, Fets Lab, MRC London Institute of Medical Sciences, United Kingdom

Oncogene-driven changes in solute carrier (SLC) transporter expression facilitates altered flux of metabolites across cell membranes, and is often seen as part of reprogrammed cellular metabolism in cancer. In addition to their endogenous metabolic substrates, SLCs may also engage with exogenous targets including pharmacological compounds. While it is well established that SLC transporters play a major role in drug pharmacokinetics, the impact of their heterogenous expression in tumours on drug sensitivity and resistance is not fully characterised. We investigate whether SLC transporters may influence drug response directly, by mediating transport, or indirectly, through modulation of the metabolic state of the cell. I will present how we combine drug sensitivity assays with metabolomics approaches, including stable isotope labelling and mass spectrometry, to elucidate the relationship between SLC transporter expression, drug response and metabolic state in cancer cells.

Session 6. Slc38 transporters in neuronal signaling and brain diseases

Chair: Prof. Farrukh A. Chaudhry, Univ. of Oslo, Norway

INTRO: Glutamine sustains fast neuronal signaling in the adult brain as well as normal brain development. Although glutamine is the primary precursor for glutamate and GABA, the glutamine transporters involved remain elusive. This session will reveal the transporters and mechanisms involved and their impact on synaptic transmission, brain development and brain pathology. In addition, high-resolution structures of four ABC transporters using single particle cryo-EM will be shown.

1. Ammonium ion (NH₄⁺) perturbs SLC38 glutamine transporter function which underpins neurotoxicity in hyperammonemic encephalopathies

Prof. **Farrukh A. Chaudhry**, University of Oslo and Oslo University Hospital, Norway
E. H. Hamdani^{1,3}, M. Popek⁴, M. Frontczak-Baniewicz, M. N. Moen¹, T. P. Utheim², J. Albrecht⁴, M. Zielinska⁴

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In liver failure, ammonia escapes detoxification in the liver and accumulates in the cerebrospinal fluid (CSF) where it is considered to be the main pathogenic toxin in hepatic encephalopathy (HE), a neuropsychiatric disorder belonging to the group of diseases classified as hyperammonemic encephalopathies. However, the molecular mechanisms involved have been disputed. We have previously shown that four members of the solute carrier 38 (Slc38) family of amino acid transporters contribute to astroglial-to-neuronal shuttling of glutamine for the synthesis of glutamate and GABA. As HE is associated with dysfunctional glutamatergic and GABAergic neurotransmission, we investigated whether ammonia perturbs the Slc38 transporters and whether this contributes to HE pathophysiology. We now show by two-electrode voltage-clamp electrophysiology on *Xenopus laevis* oocytes expressing slc38 transporters that ammonium ion competes at the glutamine binding sites of the system N transporters Slc38a3 and Slc38a5 and thereby inhibits bidirectional astroglial glutamine transport. It also permeates through the same transporters in competition with H⁺, Na⁺ and K⁺ and may perturb astroglial intracellular pH, membrane potential and K⁺-buffering. To further test a relation between Slc38a3 and HE, we knocked-down Slc38a3 by transcranial injection of *vivo morpholino* (VM) oligomers in mice. This results in cerebral cortical edema, lower extracellular glutamine content and reduced total brain glutamate, indicative of disrupted neurotransmitter synthesis, all mimicking events contributing to HE development. Finally, in the azoxymethane (AOM) mouse model of acute liver failure (ALF) we demonstrate downregulation of Slc38a3 protein and impeded astroglial glutamine release followed by cytotoxic edema. Altogether, our data mechanistically demonstrate differential regulation of four Slc38 glutamine transporters by NH₄⁺ and suggest a primary role of these transporters in the pathophysiology of HE.

2. Amino Acid Transport for Maintaining Neurotransmission at Glutamatergic Synapses

Assoc. Prof. **Brian Billups**, The Australian National University, Australia
Michael L. Castenares¹, Angela L. Nicoli¹, Sarah R. Hulme¹, Matthew J. Kenna¹, Shaam A. Al-Abed¹, Abhijit Das^{2,3}, Angelika Broer⁴, Caroline D. Rae^{2,3}, Stefan Broer⁴

¹ Eccles Institute of Neuroscience, The Australian National University; ² Neuroscience Research Australia; ³ School of Medical Sciences, The University of New South Wales; ⁴ Research School of Biology, The Australian National University

Glutamate is a ubiquitous neurotransmitter that mediates the vast majority of excitatory neurotransmission in the central nervous system. Following its release from presynaptic terminals it must be rapidly recycled or replenished to allow continuous, uninterrupted neuronal communication. It is thought that neuronal glutamate recycling involves the release of glutamine from neighbouring glia, which is sequestered by presynaptic terminals for the re-synthesis of glutamate. Despite the wide acceptance of this hypotheses, the nature of the presynaptic glutamine transporter has never been determined.

We hypothesise that the presynaptic glutamine transporter is a system A transporter (*Slc38a1* or *Slc38a2*), which are electrogenic neutral amino acid transporters known to be present in

the central nervous system. By whole-cell patch-clamp recording directly from presynaptic terminals in acutely isolated mouse brain slices, we have investigated the nature of presynaptic glutamine transport.

Recordings from the calyx of Held presynaptic terminal in the auditory brainstem demonstrate glutamine transport that is partially sensitive to the substrate MeAIB, but also partially sensitive to leucine. This indicates the presence of *Slc38* transport with the addition of another glutamine transport mechanism. In contrast, recordings from mossy fibre terminals in the hippocampus show glutamine transport that is entirely sensitive to leucine and hence is not mediated by an *Slc38* transporter. A possible candidate for this presynaptic glutamine transport in the hippocampus is the *Slc6* transporter NTT4 (*Slc6a17*). We therefore created *Slc6a17* knock-out mice (NTT4-KO), which we show lack this presynaptic glutamine transport. NTT4-KO mice show reduced synaptic communication at high frequencies of neurotransmission, reduced reliance of astrocytic glutamine for glutamate production, and impaired memory consolidation. These data thus demonstrate that NTT4 (*Slc6a17*) is a functional presynaptic glutamine transporter that is vital for normal nervous system function.

3. Slc38a5 (SNAT5/SN2) is a Physiological Serine Transporter at the Blood-Brain Barrier and is Critical for Neurodevelopment

Prof. **Herman Wolosker**, Department of Biochemistry, Technion-Israel Institute of Technology, Rappaport Faculty of Medicine, Haifa 31096, Israel. E-mail: hwoloske@technion.ac.il

D-Serine is a gatekeeper of N-methyl-D-aspartate receptors (NMDARs), which play an essential role in synaptic plasticity, neurodegeneration, and psychiatric disorders. We have shown that D-serine signaling is regulated by several *Slc38* transporters, including system A (*Slc38a1* and *Slc38a2*), which removes D-serine from the synapse and affects synaptic plasticity. D-serine synthesis and NMDAR activity are also controlled by a metabolic interplay termed the "serine shuttle," in which L-serine is exported from astrocytes to fuel the neuronal production of D-serine. We now show that D-serine production in the brain in the early postnatal period also requires the import of L-serine across the blood-brain barrier (BBB). We identified *Slc38a5* as a serine transporter enriched at the BBB that mediates the import of serine during early postnatal development. *Slc38a5* knockout mice (KO) exhibit lower brain serine content, behavioral and motor abnormalities, reduced brain volume, and impaired neurogenesis and synaptogenesis. Although *Slc38a5* is widely known as a glutamine transporter, glutamine levels were unchanged in *Slc38a5*-KO, suggesting that this transporter is not required for the maintenance of glutamine in the brain. Our observations suggest that members of the *Slc38* transporter family, including *Slc38a5*, are important regulators of serine metabolism and are critical for optimal NMDAR function and neurodevelopment.

4. What distinguishes a multidrug from a lipid transporter?

Prof. **Kaspar Locher**, Institute of Molecular Biology and Biophysics, ETH Zürich, Switzerland

The human proteins ABCB1 (P-glycoprotein) and ABCG2 (BCRP) are multidrug ABC transporters that are expressed in various tissues and tissue barriers and exhibit an extraordinarily broad substrate specificity. In contrast, the related ABCB4 and ABCG1 proteins are specific transporters of phospholipids (ABCB4) or cholesterol (ABCG1). Using single particle cryo-EM, we have determined high-resolution structures of all four of these ABC transporters, which provided insight into the structural basis of the observed substrate specificity. The presentation will focus on structural differences in ABCB1 and ABCB4 and attempts to rationalize and modify the substrate specificity.

Session 7. Lipid gymnastics - Highlights on lipids translocation mechanisms

Chair: Prof. Christine Ziegler, University of Regensburg, Germany

INTRO: Lately, we have entered an exciting era in which various mechanisms of membrane transport processes emerge by a sophisticated combination of structural biology, spectroscopy, and computational methods. In this session we will cover different substrate classes ranging from K^+ ions to lipid molecules. We will discuss determinants of substrate recognition and specificity and learn about the interaction of transporters with lipids and subunits, respectively, to facilitate and regulate transport. We will underline and compare mechanisms of different transporter families including P-type ATPases, ABC transporters, and secondary transporters. Moreover, we want to share new advances on membrane proteins integrative structural biology methods in particular Molecular dynamics simulations, Cryo-EM, X-ray crystallography, EPR and structural Mass Spectrometry.

1. Molecular dynamics of substrate recognition and transport in lipid transporters

Asst. Prof. Ahmad Reza Mehdipour, Center for Molecular Modeling, Ghent University, Ghent, Belgium

Membrane proteins known as lipid transport proteins actively transport lipid molecules between the two leaflets of biological membranes. These proteins can move lipids between membranes via cavities that protect the lipids from the aqueous environment during transport. There are many membrane protein families that involved in this process, including primary active transporters (ABC transporters and P-type ATPases), and secondary active transporter from MATE and MFS families. In the recent years, we used different computational tools, such as molecular docking and molecular dynamics simulations, to look at the processes at which these transporters recognize the substrates (1) and then the transport process occurs (2,3). Furthermore, using molecular dynamics simulations, we identified different mechanisms that these transporters are using to flip the lipid or lipid-linked molecules depending on the nature of the substrates (2,3).

1) Perez et al. Structure of outward-facing PglK and molecular dynamics of lipid-linked oligosaccharide recognition and translocation. *Structure*, 2019.

2) Lambert et al. Evidence for a trap-and-flip mechanism in a proton-dependent lipid transporter. *Nat Commun*, 2022.

3) Zakrzewska et al. Inward-facing conformation of a multidrug resistance MATE family transporter. *PNAS*, 2019.

2. Mechanism of a proton-dependent lipid transporter involved in teichoic acid synthesis

Prof. Camilo Perez, University of Basel, Switzerland

Transport of lipids across membranes is fundamental for diverse biological pathways in cells. Multiple ion-coupled transporters take part in lipid translocation, but their mechanisms remain largely unknown. Major facilitator superfamily (MFS) lipid transporters play central roles in cell wall synthesis, brain development and function, lipids recycling, and cell signaling. LtaA is a flippase that mediates the translocation of the lipid-anchor of lipoteichoic acid, an essential

cell wall biopolymer in the Gram-positive pathogen *Staphylococcus aureus*. We used X-ray crystallography, cysteine disulfide trapping, molecular dynamics simulations, mutagenesis analysis, and transport assays *in vitro* and *in vivo*, to elucidate the mechanism of LtaA and reveal its importance for bacterial fitness. We demonstrate that while the entire amphipathic central cavity of LtaA contributes to lipid binding, its hydrophilic pocket dictates substrate specificity. Cycling of LtaA through outward- and inward-facing conformations is essential for transport, while LtaA lateral openings are asymmetric in their function. We propose that LtaA catalyzes lipid translocation by a 'trap-and-flip' mechanism that might be shared among MFS lipid transporters.

3. A true chimera: Transport mechanism and regulation of the KdpFABC complex

Prof. **Cristina Paulino**, Electron Microscopy and Membrane Enzymology Group, University of Groningen, The Netherlands

My research group aims at elucidating the mechanism of action of membrane transporters on a molecular level. To this end we use an interdisciplinary approach with cryo-EM as a central technique. Several of our projects focus on membrane proteins that fall out-of-the-box, challenging conceptual boundaries present when classifying them into merely primary-active transporters, secondary-active transporters, or channels. They demonstrate how in the course of evolution, conserved protein architectures not only evolved from one another, but can merge together to adapt to different environmental and cellular requirements. A prime example for this is our research line on the bacterial emergency K⁺-uptake system KdpFABC. The complex is composed of four subunits, whereby the KdpA resembles a K⁺-channel and KdpB is a P-type ATPase (primary-active transporter). While it was assumed that K⁺ is transported solely by the channel-like subunit^{1,2}, we were able to demonstrate a different and so far unprecedented transport mechanism, where K⁺ is translocated through both subunits via two half-channels³. Our data show how KdpFABC functions as a true chimera, synergizing the best features of otherwise separately evolved transport mechanisms: ATP driven pumping of a P-type ATPase with the high affinity and selectivity of an ion channel. Combining cryo-EM with biochemical and MD simulation data, allowed us to elucidate in more depth how both subunits are coupled⁴ and how they are regulated by an off-cycle state, strengthening the idea that KdpB is an early descendant of a common ancestor of cation pumps.

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2. Huang, C.-S., Pedersen, B. P. & Stokes, D. L. Crystal structure of the potassium-importing KdpFABC membrane complex. *Nature* 90, 705 - 685, doi:10.1038/nature22970 PMID - 28636601 (2017).
3. Stock, C. et al. Cryo-EM structures of KdpFABC suggest a K⁺ transport mechanism via two inter-subunit half-channels. *Nat Comms* 9, 4971, doi:10.1038/s41467-018-07319-2 PMID - 30478378 (2018).
4. Silberberg, J. M. et al. Deciphering ion transport and ATPase coupling in the intersubunit tunnel of KdpFABC. *Nat Commun* 12, 5098, doi:10.1038/s41467-021-25242-x (2021).

4. An embedded lipid in the efflux pump LmrP suggests a mechanism for multidrug recognition

Group Leader **Chloé Martens**, Centre for Structural Biology and Bioinformatics, Université Libre de Bruxelles, 1050 Brussels, Belgium

Expression of multidrug (MDR) transporters plays an important role in sustaining bacterial antibiotic resistance yet the molecular basis underlying multidrug recognition is poorly understood. The physiological role of bacterial MDR efflux pumps is speculative but the

recognition of hydrophobic compounds such as lipids from the membrane supports a number of essential metabolic functions. We investigate the nature of substrate binding within *Lactococcus lactis* LmrP, a prototypical multidrug transporter from the major facilitator superfamily. We determined the crystal structure of LmrP in a ligand-bound outward-open state and observed an embedded lipid in the binding cavity of LmrP, an observation supported by native mass spectrometry analysis. Molecular dynamics simulations suggest that the anionic lipid stabilizes the observed ligand-bound structure. Mutants engineered to disrupt binding of the embedded lipid display reduced transport of some, but not all, antibiotic substrates. Our results suggest that a lipid within the binding cavity provides a malleable hydrophobic component that allows adaptation to the presence of different substrates, helping to explain the broad specificity of this protein and possibly other transporters.

Session 8. Human ASCT2 - from structure to anticancer therapy

Chair: Dirk Slotboom, University of Groningen, The Netherlands

INTRO: *The SLC1 family of solute carrier proteins comprises two subfamilies in humans – excitatory amino acid transporters (EAAT1-5) and neutral amino-acid transporters (ASCT1&2). While EAATs accumulate the transported amino acid harnessing the membrane gradients of Na⁺, K⁺ and H⁺, ASCT1&2 are strict exchangers that “harmonize” the amino acid pools. Both ASCTs are linked to diseases, and ASCT2 has long been considered as a potential target for anti cancer drugs. In this session, new structural and mechanistic insights on ASCTs, and development and characterization of inhibitors will be presented.*

1. Harnessing Computational Approaches for Rational Design of ASCT2 Ligands

Assoc. Prof. **Avner Schlessinger**, Department of Pharmacological Sciences, Icahn School of Medicine at Mount Sinai, New York, United States.

Solute Carrier (SLC) transporters can play a major role in mediating nutrient delivery in reprogrammed cancer metabolism networks. For example, the amino acid transporters LAT-1 (SLC7A5) and ASCT2 (SLC1A5) are upregulated in a variety of cancer types, where they supply the growing tumor cells with essential amino acids that are used as nutrients to build biomass and signaling molecules to enhance proliferation. Thus, Nutrient deprivation via the inhibition of these transporters provides a potential strategy for cancer therapy. Here, we apply a range of computational modeling methods, including homology modeling, ligand docking, and MD simulations to develop strategies to modulate nutrient SLC transporters. We use modeling, virtual screening, and functional testing to identify conformation-specific inhibitors for ASCT2. Initial hits are then refined through iteration of molecular docking, estimation of free energy of binding, and medicinal chemistry, as well as biochemical and structural approaches. Finally, metainference MD simulations guide the identification of cryptic subpockets and further compound optimization. Our results provide useful chemical tools to characterize reprogrammed metabolic networks, as well as a framework for developing efficacious lead compounds against ASCT2 and other SLC transporters. Our studies also highlight the utility of combining state-of-the-art computational and experimental approaches in characterizing challenging human membrane protein targets.

2. Characterising unexpected interactions of a glutamine transporter inhibitor with members of the SLC1A transporter family

Prof. **Renae M Ryan**, University of Sydney, Australia

Natasha Freidman*, Chelsea Briot*, [Renae M Ryan](#)

*School of Medical Sciences, Faculty of Medicine and Health, University of Sydney, NSW, Australia

*Authors contributed equally to this work.

The Solute Carrier 1A (SLC1A) family comprises a group of membrane proteins that act as dual-function amino acid transporters and chloride channels. It includes the alanine serine cysteine transporters (ASCTs) and the human glutamate transporters known as excitatory amino acid transporters (EAATs). ASCT2 transports a range of neutral amino acids including glutamine and is regarded as a promising target for cancer therapy as it is upregulated in a range of solid tumours. L- γ -glutamyl-p-nitroanilide (GPNA) is a compound widely used in studies probing the role of ASCT2 in cancer biology. Here, we demonstrate that GPNA activates the chloride conductance of ASCT2 to the same extent as a transported substrate, whilst not undergoing the full transport cycle. This is a previously unreported phenomenon for inhibitors of the SLC1A family and corroborates a body of literature suggesting that the structural requirements for transport are distinct from those for chloride channel formation. We also show that in addition to its currently known targets, GPNA inhibits several of the glutamate transporters (EAATs). Together, these findings raise questions surrounding the true mechanisms of its anticancer effects and reinforces the need for the development of selective ASCT2 inhibitors.

3. Mechanism of transport and inhibition of cancer-relevant glutamine transporters from the solute carrier 1 and 6 families.

Prof. **Christof Grewer**, Department of Chemistry, Binghamton University, Binghamton, NY, USA

Alanine serine cysteine transporters ASCT1/2, and amino acid transporter ATB⁰⁺ are neutral (and basic) amino acid transporters belonging to the solute carrier (SLC) 1 and 6 families. ASCT2 (SLC1A5) and ATB⁰⁺ (SLC6A14) were shown to be highly upregulated in cancer cells, and, for ASCT2, inhibition results in the reduction of intracellular glutamine levels and cell growth. While SLC6A14 is a symporter, transporting neutral amino acids, such as glutamine, and basic amino acids into cells driven by the transmembrane electrochemical concentration gradient of Na⁺, ASCTs function as exchangers, taking up neutral amino acid in homo- or hetero-exchange with intracellular amino acid, in a Na⁺-dependent manner. However, the detailed molecular understanding of the structure/function and substrate/inhibitor recognition of these transporters is still lacking. Here, we discuss some of our recent work involving functional and kinetic analysis of transport mechanism, as well as characterization of competitive inhibitors, and an allosteric binding site on ASCT2 for non-competitive inhibitors. Examples for the results obtained are: 1) Turnover rates for substrate transport were determined for both SLC6A14 and ASCT2, using pre-steady-state kinetic methods with sub-millisecond time resolution, revealing rapid turnover rates of >80 s⁻¹. 2) For ASCT2, the glutamate transporter non-competitive inhibitor, UCPH-101 was found to be a partial inhibitor. Full inhibition could be restored after site-directed mutagenesis to the predicted binding site, indicating that the allosteric binding site at the subunit interface is conserved between the ASCT2 and excitatory amino acid transporter 1 (EAAT1) members of the SLC1 family. Overall, our results highlight recent advancements in our understanding of transport mechanism and substrate recognition of two important glutamine transporters.

4. A structural view onto disease-linked mutations in the human neutral amino acid exchanger ASCT1

Assoc. Prof. **Albert Guskov**, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, The Netherlands

SLC1 family of solute carrier proteins comprises two subfamilies – excitatory amino acid transporters (EAATs) and neutral amino-acid exchangers, named ASCTs after their substrate specificity. ASCT2 has been largely in focus due to its direct link to the carcinogenesis, but it seems to be a redundant drug target. In the framework of pathophysiology, ASCT1 is a more attractive target for investigations, as it plays a prominent role in the transport of both L- and D-isomers of serine, essential for the normal functioning of the central nervous system in mammals.

A number of mutations in ASCT1 (namely E256K, G381R, R457W) have been linked to severe neurodevelopmental disorders, generally termed as spastic tetraplegia, thin corpus callosum, and progressive microcephaly (SPATCCM) disease. SPATCCM is an autosomal recessive neurodevelopmental disorder, leading to severely impaired global development in early infancy. Most patients are unable to achieve independent walking or speech.

In this contribution I will report cryo-EM structure of ASCT1, which we have used to map the aforementioned mutations. Their impact on substrate transport was characterized both with the functional assays (via radioactive substrate uptakes) and by the Molecular Dynamics simulations. Our studies revealed that the given mutations lead to the significantly diminished transport capability of ASCT1 caused by instability of a transporter and due to the ‘locked’ conformations which severely impede the transport cycle.

Session 9. SLC4- and SLC9 family acid-base transport proteins: from structure to human disease

Chair: Stine Falsig Pedersen, University of Copenhagen (DK)

INTRO: Acid-base transport is pivotal to normal cell function in all our tissues, and dysregulation of acid-base transporters plays a central role in many diseases, including cancer, cardiovascular- and neurological diseases. However, until recently, no structural knowledge about the mammalian acid-base transporters has been available, and detailed mechanistic insight into their regulation is only beginning to emerge.

The aim of this session is to provide a timely and highly interdisciplinary update on the structure and regulation of two major families of acid-base transporters: the SLC9A family of Na⁺/H⁺ exchangers, and the SLC4 family of Na⁺, HCO₃⁻ cotransporters. The specific topics range from high resolution structural analyses (Drew, Pedersen) over detailed mechanistic insight into their cellular regulation (Casey, Boedtkjer, Pedersen), to animal studies (Boedtkjer) and human patient mutations (Casey).

1. Conformational changes in Band 3 (SLC4A1/ AE1) and possible significance for Erythrocyte Senescence Signaling

Prof. **Joseph Casey**, Department of Biochemistry, University of Alberta, Canada

Band 3 (SLC4A1, Anion Exchanger 1, AE1), the predominant protein of erythrocyte membranes, facilitates $\text{Cl}^-/\text{HCO}_3^-$ exchange and anchors the plasma membrane to the cytoskeleton. The Band 3 crystal structure revealed the amino acid 812-830 region as intracellular, conflicting with protein chemical data that suggested extracellular disposition. Further, circulating senescent cell auto-antibody that cannot enter erythrocytes, binds two regions of Band 3: residues 538-554 and 812-830. To reconcile this discrepancy, we assessed localization of residues 812-830 with Band 3 expressed in HEK293 cells and human erythrocytes, using chemical labeling probes and an antibody against residues 812-830. Antibody and chemical probes revealed reorientation of 812-830 region between extracellular and intracellular. This dramatic conformational change is an intrinsic property of the Band 3 molecule, occurring when expressed in HEK293 cells and without the damage that occurs during erythrocyte circulation. Conditions used to crystallize Band 3 for structural determination did not alter conformational dynamics. Collectively, these data reveal large Band 3 conformational dynamics localized to a region previously identified as an erythrocyte senescence epitope. Surface exposure of the senescence epitope (812-830), limited by conformational dynamics, may act as the “molecular clock” in erythrocyte senescence.

2. Elevating the molecular basis for sodium/proton exchange

Prof. **David Drew**, Department of Biochemistry and Biophysics. Stockholm University, Sweden

The regulation of intracellular pH is a fundamental process in most living organisms. Almost all cells have transport proteins, known as NHEs, that couple the movement of protons against sodium ions to fine-tune the cells internal pH, sodium levels and cell volume. The dysfunction of NHEs has been linked to many diseases such as cancer, hypertension, heart failure, diabetes, and epilepsy. Despite their fundamental importance to cell homeostasis and human physiology, structural information for the mammalian NHEs was lacking. Here, I will present the cryogenic electron microscopy structures of mammalian NHE isoform 9 (SLC9A9) and NHA2 (SLC9B2), which are associated with autism spectrum (ASD) and attention deficit hyperactivity (ADHD) disorders and hypertension, respectively. I will outline the variation in their ion-binding sites, their elevator-like structural transitions, and the role of phosphoinositide lipids to promote homodimerization that, together, have important physiological ramifications. EMBO J (2020) e105908; NSMB (2022) 108–120.

3. Bicarbonate transporters and sensors in breast carcinogenesis

Prof. **Ebbe Boedtkjer**, Department of Biomedicine, Aarhus University, Denmark

Cellular metabolism and the associated acid load increase during breast carcinogenesis. The temporal patterns of acid-base regulation that protect cancer cells against intracellular acidification are starting to unravel, but the underlying molecular mechanisms responding to acid-base disturbances in the tumor microenvironment remain largely unknown. In this talk, I will discuss the role of HCO_3^- transporters and sensing mechanisms in breast tissue during malignant transformation based on recent evidence from mouse models of breast carcinogenesis and biopsies of human breast cancer tissue.

The electroneutral $\text{Na}^+, \text{HCO}_3^-$ -cotransporter NBCn1 is up to 7-fold upregulated during breast carcinogenesis, and NBCn1 elevates intracellular pH and the capacity for net acid extrusion in breast cancer tissue. Disrupted NBCn1 expression delays breast cancer development. In human breast cancer patients, NBCn1 expression and measures of $\text{Na}^+, \text{HCO}_3^-$ -cotransport activity independently predict high proliferative rates in primary breast carcinomas and occurrence of regional lymph node metastasis.

Expression of the putative extracellular HCO_3^- sensor Receptor Protein Tyrosine Phosphatase (RPTP) γ declines from normal to malignant breast tissue and is particularly low in higher malignancy grade breast cancer tissue. Loss of RPTP γ promotes pre-malignant changes in macroscopically normal breast tissue, accelerates breast cancer development, and shifts breast carcinogenesis towards more malignant histopathologies. Preceding these changes in histopathology, disruption of RPTP γ increases the expression of NBCn1, augments the capacity for net acid extrusion, and elevates intracellular pH in breast tissue.

High NBCn1 and low RPTP γ expression each reduce survival of women with luminal A and triple-negative breast cancer.

In conclusion, HCO_3^- plays multiple roles in breast tissue during neoplastic development. NBCn1 actively imports HCO_3^- into cancer cells to defend against intracellular acidification. We further propose that RPTP γ senses HCO_3^- in the extracellular environment and in turn adjusts the capacity for net acid extrusion. Together, these mechanisms fundamentally influence breast cancer risk and prognosis.

4. Structural and functional insights into NHE1 regulation

Prof. **Stine Falsig Pedersen**, Department of Biology, University of Copenhagen, Denmark

In mammals, the ubiquitous Na^+/H^+ exchanger SLC9A1 (NHE1) regulates numerous physiological processes and its dysregulation contributes to many diseases. NHE1 activity is tuned by a complex network of events including phosphorylation-/dephosphorylation, protein- and lipid interactions, yet very little is known about the molecular mechanisms involved.

Here, I present our recent findings on mechanisms underlying NHE1 regulation by the Ser/Thr protein phosphatase calcineurin (CN), the Ca^{2+} -binding protein calmodulin (CaM), and interactions with the plasma membrane.

NHE1 is phosphorylated at multiple sites. We recently identified a novel such site, Thr779, phosphorylation of which increases NHE1 activity. Studying its regulation, we found that NHE1 interacts with CN via two classical CN binding motifs, PxlIT and LxVP, in the disordered NHE1 C-tail, and that Thr779 is rapidly and specifically dephosphorylated by CN. The mechanism relies on a combination of recognition motifs, dynamic charge-charge interactions and a specific substrate interaction pocket.

NHE1 interacts with CaM in a non-conventional mode. We determined the NMR solution structure of CaM linking two NHE1 C-tails. This interaction is highly dynamic, with multiple accessible states of different stoichiometries, which were tunable by variations in NHE1:CaM ratio and $[\text{Ca}^{2+}]$ and by phosphorylation of Ser648 in the first CaM-binding α -helix. In cells, Ca^{2+} -CaM-induced NHE1 activity was reduced by mimicking S648 phosphorylation and by mutation of the first CaM-binding α -helix.

Finally, NHE1 carries a lipid-interacting domain (LID) in its membrane-proximal C-tail region. We found that the NHE1-LID forms a helical hairpin co-structure with the membrane, anchoring the regulatory domain in close proximity to the transport domain. Disintegration of this structure reduced steady-state pHi and the rate of pHi recovery after acid loading.

In conclusion, a network of interactions fine-tune NHE1 activity, allowing it to respond sensitively to changes in the state of cells.

Session 10. Molecular mechanisms of Na⁺-coupled neurotransmitter transport

Chair: Prof. Baruch Kanner, Hebrew Univ. Medical School, Isreal

INTRO: Sodium-coupled neurotransmitter transporters play a crucial role in the process of neurotransmission. These transporters remove the neurotransmitters from the synaptic cleft and as a result the signaling by the neurotransmitter is terminated. Inhibition of transport activity prolongs neurotransmitter signaling, and therefore is a key strategy in the development of therapies for a broad range of disorders of the central nervous system including depression, schizophrenia and epilepsy. In this symposium, new neurotransmitter transporter structures combined with functional and computational approaches will be presented. This is expected to deepen our insights into the transport mechanism and hopefully will also help to design more specific neurotransmitter transporter inhibitors and modulators.

1. New developments in Neurotransmitter Transport Mechanisms

Prof. **Gary Rudnick**, Yale University, USA

Ion-coupled transport requires coordination of conformational changes that allow substrates to pass through the membrane with stoichiometric coupling of net substrate and ion fluxes. The coordination involves some binding interactions that allow specific conformational changes to occur and others that inhibit those changes. Coupled transport also requires coordinated movements that prevent slippage and leakage from dissipating the ionic driving forces. Recent structural and biochemical studies investigating the transporters LeuT, GlyT1 and SERT illustrate how these processes work together to couple ion and substrate movements in the NSS family of transporters. Other studies investigating ligands that influence conformational changes in SERT have identified ligands with unique conformational properties and therapeutic potential.

2. Insights into neurotransmitter transport from modelling and simulation

Senior investigator **Lucy R. Forrest**, Natl. Inst. on Neurological Disorders and Stroke - NIH (US)

Monoamine transport through the plasma membrane occurs via neurotransmitter:sodium symporters. Surprising differences exist in the ion dependencies of the different members of this family. In particular, human serotonin transporter, SERT only co-transport a single sodium ion, rather than two; can utilize potassium ions or protons in the return step of the cycle; and its bound chloride ion appears to remain bound throughout the entire cycle, rather than being transported. In recent years, X-ray crystallographic and cryo-EM data have been reported for hSERT in several different conformations, although the interaction sites for potassium and protons, and the molecular determinants of cation selectivity and stoichiometry remain unclear. We describe modeling and molecular simulation studies based on these structural data and their use in interrogating the chloride and cation binding sites in hSERT.

3. Ion coupling mechanism in excitatory amino acid transporters

Group Leader **Nicolas Reyes**, Université de Bordeaux/CNRS (FR)

Human excitatory amino acid transporters (EAATs) maintain steep gradients of glutamate in the brain essential to neurotransmission, and to prevent neuronal death. EAATs use ionic gradients as the energy source, and co-transport the transmitter into the cytoplasm with Na^+ and H^+ , while counter-transport K^+ to re-initiate the transport cycle. However, the molecular mechanisms underlying ion-coupled transport remain incompletely understood. In this talk, I will present structural and thermodynamic analyses of EAAT1 in different ion-bound conformations, including elusive counter-transport ion bound states. Binding energies of co-transported Na^+ and H^+ are coupled to neurotransmitter binding and occlusion within the protein core. Two Na^+ and one H^+ are able to bind apo transporters and contribute to form the transmitter binding site, while binding of a third Na^+ and protonation of a conserved glutamate residue are required for transmitter occlusion. Ca^{2+} competes with Na^+ for a conserved binding site, and it is thermodynamically-coupled to neurotransmitter binding, suggesting a regulatory role of Ca^{2+} in glutamate transport at the tripartite synapse. The counter-transported ion binding site overlaps with that of glutamate, revealing the K^+ mechanism to exclude the transmitter during the transport cycle, and to prevent its neurotoxic release on the extracellular side.

4. Mechanisms of K^+ -coupled glutamate transport

Prof. **Christoph Fahlke**, Forschungszentrum Jülich (DE)
Jan-Philipp Machtens, Forschungszentrum Jülich (DE)

Excitatory amino acid transporters (EAATs) are dual function proteins that remove glutamate from the synaptic cleft as secondary active glutamate transporters and also function as anion-selective channels. We use molecular simulations together with electrophysiology and fluorescence spectroscopy to describe mechanisms of coupled transport and anion conduction in mammalian EAATs and the prokaryotic homolog Glt_{Ph} .

In the apo state, hairpin loop 2 (HP2) - that regulates access to the glutamate/aspartate-binding site - is in dynamic equilibrium between open and closed conformations. Occupation of Na1 and Na3 sites stabilizes HP2 gate opening via *conformational selection* and ensures together with electrostatic coupling of aspartate to Na^+ binding a fixed Na^+ :substrate stoichiometry. Substrate selection is based on an *induced-fit mechanism*. Substrate association and occupation of Na2 induces HP2 closure, with closure rates depending on the bound substrate. This permits preferential L-aspartate transport, which binds with lower affinity than other substrates, but closes HP2 most effectively.

The final step of the transport cycle is a K^+ -dependent re-translocation in mammalian EAATs; by contrast, prokaryotic transporters are K^+ independent. MD simulations identified a K^+ -binding site, which is conserved between prokaryotic and mammalian EAATs. Differences in K^+ coupling are based on distinct allosteric K^+ -HP2 interactions: whereas HP2 can close also in the apo state and thus permit K^+ -free translocation of Glt_{Ph} , K^+ binding is required for HP2 closure and transmembrane translocation in mammalian EAATs.

Although the molecular details of anion conduction in glutamate transporters are well understood, the exact relationship between substrate translocation and channel opening are still unclear. Based on extensive MD simulations, we developed Markov state models of elevator translocation to identify intermediate translocation states and their kinetics. These results allow us linking substrate translocation with ion permeability and help to explain how EAATs can operate as either secondary activate transporters or ion channels

Session 11. Emerging roles for transporters in dopamine dysfunction in CNS disorders

Chair: Nikhil Urs, University of Florida; USA

Co-Chair: Ulrik Gether, University of Copenhagen, Denmark

INTRO: Dopamine (DA) through slow-synaptic transmission is an important regulator of CNS function, and dopamine dysfunction has been implicated in the etiology of many CNS disorders such as drug addiction, Parkinson's disease and ADHD. Although slow-synaptic neurotransmission is achieved through action of dopamine on postsynaptic receptors, extracellular clearance and vesicular packaging are equally important processes in the regulation of neurotransmission. Studies in rodent models have shown the importance of transporters, but recent studies highlight the importance of new emerging molecular and circuit mechanisms in regulating dopamine neurotransmission. However, the role of these molecular and circuit mechanisms in dopamine dysfunction are not fully understood. The speakers in this session will address dopamine dysfunction from various perspectives using population genetics, region-specific catecholamine regulation, vesicular glutamate transporters, and receptors/ion channels in dopamine neurons. This session highlights new avenues of transporter-related research in dopamine neurotransmission and its impact on CNS disorders.

1. Dopamine transport dysfunction in neuropsychiatric disease and parkinsonism

Asst. Prof. **Freja Herborg**, University of Copenhagen

Faculty Of Health Sciences, Department of Neuroscience, Molecular Neuropharmacology Group.

The dopamine transporter (DAT) is critical for dopamine (DA) homeostasis in the brain as it exert tight control over synaptic dopamine dynamics by mediating high affinity reuptake. We recently described the first patient with atypical dopamine transporter syndrome, characterized by adult early-onset parkinsonism and comorbid ADHD. Although psychiatric and neurodegenerative diseases are known to constitute mutual risk factors, our understanding of the mechanisms underlying this comorbidity remains limited. We have now generated the first construct valid mouse model of atypical DTDS to uncover pathobiological processes that link DAT dysfunction to both parkinsonism and psychiatric disease. In addition, we have leverage large-scale exome sequencing data from patients with mental diseases to further interrogate the genetic basis for DA transport dysfunction in psychiatric disease. Thus, this talk will present new investigations of the genetic and mechanistic link between disrupted dopamine transport and neuropsychiatric diseases and parkinsonism.

2. iPSc-derived human-like dopamine neurons reveal a potential therapeutic target for Parkinson's disease

Prof. **Habibeh Khoshbouei**, Department of Neuroscience, University of Florida

Pathophysiological changes in dopamine neurons precede their demise and contribute to the early phases of Parkinson's disease (PD). Intracellular pathological inclusions of the protein α -synuclein within dopaminergic neurons are a cardinal feature of PD, but the mechanisms by which α -synuclein contributes to dopaminergic neuron vulnerability remain unknown. The inaccessibility to diseased tissue has been a limitation in studying progression of

pathophysiology prior to degeneration of dopamine neurons. To address these issues, we differentiated induced pluripotent stem cells (iPSCs) from a PD patient carrying the α -synuclein triplication mutation (AST) and an unaffected first-degree relative (NAS) into dopaminergic neurons. In human-like dopamine neurons α -synuclein overexpression reduced the functional availability of D2 receptors, resulting in a stark dysregulation in firing activity, dopamine release, and neuronal morphology. We back-translated these findings into primary mouse neurons overexpressing α -synuclein and found a similar phenotype, supporting the causal role for α -synuclein. Importantly, application of D2 receptor agonist, quinpirole, restored the altered firing activity of AST-derived dopaminergic neurons to normal levels. These results provide novel insights into the pre-degenerative pathophysiological neuro-phenotype induced by α -synuclein overexpression and introduce a potential mechanism for the long-established clinical efficacy of D2 receptor agonists in the treatment of PD.

3. Role for VGLUT2 in dopamine neuron vulnerability and Parkinson's disease

Asst. Prof. **Thomas Steinkellner**, Institute of Pharmacology, Center for Physiology and Pharmacology, Medical University of Vienna

Though many neuronal populations are affected in Parkinson's disease (PD), its cardinal motor symptoms are a consequence of dopamine (DA) neuron loss in the substantia nigra (SNc). The precise mechanisms underlying DA neuron vulnerability remain unclear, but include oxidative stress, and aggregation of alpha-synuclein. More recently, a glutamate driven process has been implicated in disease progression, and there is now proof that DA neurons express the vesicular glutamate transporter VGLUT2 and co-release glutamate. Further, there is evidence for a presynaptic role of VGLUT2, whereby VGLUT2 can increase the driving force for loading DA into synaptic vesicles, which may enable tuning of DA release in response to activity changes. We recently discovered that the majority of SNc DA neurons transiently express VGLUT2 in development, but most shut down expression in the adult. Interestingly, VGLUT2 can re-emerge in response to insult. Reemergent VGLUT2 may provide a beneficial compensatory adaptation and contribute to the native resistance of ventral tegmental area DA neurons that express more VGLUT2. Consistent with this, we find that DA neurons are more sensitive to toxin-induced cell death in mice that lack VGLUT2 in DA neurons; and DA neurons expressing VGLUT2 are more resilient to neuronal injury in animals and in human PD. On the other hand, we find that ectopic expression of VGLUT2 causes profound toxicity to SNc DA but not other neuronal populations.

We propose that VGLUT2 confers protection to DA neurons with low VGLUT2 levels in adult DA neurons and little co-release restricted to synaptic release sites. In response to increased metabolic demand, neural activity, aging or injury, VGLUT2 transcription temporarily increases to sustain DA transmission or to sequester toxic substrates to vesicles. However, severe or sustained injury leads to prolonged or high-level expression of VGLUT2 in DA neurons and vulnerable populations cannot cope with the consequences.

4. Role of PFC catecholamine transporters in regulating striatal dopamine neurotransmission in health and disease

Asst. Prof. **Nikhil Urs**, Department of Pharmacology and Therapeutics, University of Florida, Gainesville, FL USA

Striatal dopamine signaling is implicated in locomotion, motivation, and reinforcement learning but its upstream circuit regulation is not well understood. A potential mechanism of striatal dopamine regulation is highlighted by studies with psychostimulants, their paradoxical calming

effects and the prefrontal cortex (PFC). Studies using dopamine (DA) transporter (DAT) knockout (KO) mice suggest a role for serotonin (5-HT) whereas, newer non-stimulant drugs, such as atomoxetine and guanfacine, suggest a role for norepinephrine (NE) in the PFC, in this paradoxical calming effect. Thus, we sought to clarify the mechanism of this paradoxical calming action. Our ex vivo efflux experiments reveal that NE transporter (NET) blocker desipramine elevates norepinephrine (NE) and dopamine (DA), whereas fluoxetine (SERT blocker) elevates 5-HT in the PFC of DAT-KO mice. Systemic administration of desipramine or fluoxetine inhibits hyperactivity in DAT-KO mice, whereas local PFC infusion of only desipramine produced this same effect. In contrast, pharmacological NE depletion and DA elevation using nescastat also inhibits hyperactivity in DAT-KO mice. Together, these data suggest elevation of PFC DA and not NE or 5-HT, as a convergent mechanism for the paradoxical effects of psychostimulants on hyperlocomotion. In addition to hyperlocomotion, chronic NET blockade (NET KO mice) also enhances cognitive behaviors like reversal learning and goal-directed behavior. Furthermore, our data using a retrograde AAV-based approach suggest that PFC catecholamines could regulate striatal dopamine through topographically organized distinct sub populations of D1R+ and D2R+ pyramidal neuron circuits. Overall our studies will elucidate and identify novel molecular and circuit mechanisms for cortical catecholamines and their regulation of striatal dopamine dynamics.

5. Structure and mechanism of human NKCC1

Prof. **Poul Nissen**, DANDRITE – Nordic EMBL Partnership for Molecular Medicine. Aarhus. University, Dept. Molecular Biology and Genetics

The sodium-potassium-chloride transporter NKCC1 belongs to the SLC12 family, also denoted Cation-Chloride Cotransporters (CCC). It performs the electroneutral uptake of 2Cl^- ions and 1K^+ ion along with 1Na^+ ion. NKCC1 is important for regulating cell volume, hearing, blood pressure maintenance, and regulation of chloride currents in GABAergic and glycinergic signaling in CNS. We have performed functional studies of human NKCC1 and determined a 2.6 Å resolution cryo-EM structure of a substrate-loaded, inward-facing conformation (1) – an occluded state that has also been observed for the SLC6 type transporters MhsT and LeuT (2,3). Cl^- binding at the Cl-1 site together with the nearby K^+ ion provide a crucial bridge between the LeuT-fold scaffold and bundle domains. The Cl-2 site seems to undertake a structural role similar to a conserved glutamate of SLC6 transporters. It has a solvated access from the cytoplasm and may serve for chloride-sensitive regulation of transport in other CCC's. Supported by mutagenesis and computational simulations, we describe a putative Na^+ release pathway dependent on release of Cl^- from the reversible Cl-2 site.

Abstracts of Poster Sessions

All posters with even numbers will be presented on Wednesday, June 8 at 12:30 – 14:15. All posters with odd numbers will be presented on Thursday, June 9 at 12:00 – 13:45

1. Novel anti-AML drugs as inhibitors of ABC transporters

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Despite the advances in understanding the biology of acute myeloid leukemia (AML), poor treatment outcome and the low survival rate of patients remains an issue. However, drug resistance is a significant problem in the treatment of AML. One of the primary mechanisms of resistance in anthracycline-treated AML is the drug efflux through ATP-binding cassette (ABC) transporters. Recently, besides FLT3-ITD inhibitors, other new drugs have been developed and introduced as potential anti-AML therapeutics. Our study aimed to evaluate three of these novel and investigational drugs, glasdegib, edicotinib, bemcentinib, and their interactions with ABC transporters as mechanisms of multidrug resistance (MDR) and important causative factor of pharmacokinetic drug-drug interactions. Hoechst accumulation assay using MDCK-ABCG2 cells identified glasdegib and edicotinib as ABCG2 inhibitors, which was further confirmed with mitoxantrone accumulation assay in HL60-ABCG2 (and respective non-resistant control) cell lines. Subsequently, XTT proliferation assays, performed on the A431-ABCG2 cell line further demonstrated that glasdegib can reverse mitoxantrone resistance mediated by ABCG2. Advantageously, the mRNA level of ABCB1, ABCG2, and ABCC1 were not influenced after 24 h exposure of LS174T cells to glasdegib, whereas bemcentinib was revealed as a potential ABCG2 inducer. To conclude, glasdegib seems to be a promising drug in terms of the possible overcoming of the transporter mediated MDR as its side effect in the treatment of AML.

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2. Analysis of the Dopamine and Serotonin Transporter Substrate Selectivity

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An efficient and regulated monoaminergic neurotransmission is essential for the overall neurotransmitter homeostasis in the nervous system. Monoamine transporters (MATs) comprise dopamine (DA), serotonin (5-HT) and norepinephrine (NE) transporters (SERT, DAT and NET, respectively). Upon exocytotic release of monoamines in the synaptic cleft, MATs contribute to their fast reuptake, being able to terminate both synaptic and volume transmissions. MATs can be targeted by substances that act as blockers, which inhibit their function, or by substances that act as substrates, which are instead internalized by the transporter and can elicit non-exocytotic monoamine release through complex mechanisms. Drugs that interact with SERT as substrate to induce release have recently gained attention for the treatment of different neuropsychiatric disorders such as post-traumatic stress disorder,

social anxiety and Dravet syndrome, but the basis of DAT-SERT substrate selectivity still remains enigmatic.

Here, we study the determinants of DAT and SERT substrate selectivity by using radiotracer assays and transporter electrophysiology. Both transporters are capable to transport both substrates. We show that the transport of the alternative substrate is inefficient mainly because of low affinity. In addition, DA and 5-HT are both capable of inducing reverse transport at both transporters. Our data contributes to previous findings suggesting that DA and 5-HT, under physiological conditions, interact mainly with their respective transporter.

Overall, our results contribute to a broader understanding of the physiological conditions in which this alternative inefficient transport by DAT or SERT might occur, as well as in which conditions non-exocytotic release arises. Moreover, they provide additional information for the development of selective SERT releasers, which can potentially impact the treatment of neuropsychiatric disorders.

3. The atomic features that underlie the occlusion mechanism of the human dopamine transporter

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The Neurotransmitter:Sodium Symporter (NSS) family includes the human dopamine transporter (hDAT), which is expressed in neuronal cells. By recycling the released neurotransmitter into the presynaptic cell, hDAT is critical for dopamine homeostasis and dopaminergic signaling. Furthermore, hDAT malfunction has been associated with a variety of psychiatric disorders, including attention deficit hyperactivity disorder (ADHD), Parkinson's and Alzheimer's diseases. Currently, drugs that target hDAT for treatment of patients have limited efficacy and considerable side effects. Building a complete understanding of dopamine transport at the atomic level is a critical step in overcoming these limitations. In the present study, we show preliminary results from μ s-long molecular dynamics simulations. We conducted five independent simulations of hDAT in the *apo* state as well as with the bound substrate dopamine, applying the AMBER99SB-ildn force field. In all simulations, hDAT shows structural stability, and the co-transported ions (Na1, Na2, and Cl) stay firmly bound. The outer vestibule and the outer gate salt bridge between residues R85 and D476 stay open in the *apo* simulations. Dopamine, on the other hand, triggers transporter occlusion, revealing the conformational changes recognized as the initial step in the transport cycle. Additionally, through structural analyses, we identified the major structural changes associated with the occlusion of hDAT that are induced by dopamine binding.

4. Functional analysis of the glutamate/aspartate-proton symporter GltP

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L-glutamate is the primary mediator of excitatory signals in the mammalian central nervous system. Glutamate transporters (excitatory amino acid transporters (EAATs)) regulate the concentration of glutamate in the synaptic cleft. Transport involves the symport of three Na⁺ and one H⁺ with each glutamate (or aspartate) molecule taken up, and an exchange of one K⁺. *Escherichia coli*'s glutamate/aspartate-proton symporter GltP is a member of the same family as EAATs. Different from the other glutamate transporters, GltP uses only protons as symporter ions. Here, we aim to characterize the proton dependence. Reversal potential measurement showed that one glutamate is taken up together with three protons. The same stoichiometry has been reported for sodium ion coupled prokaryotic homologues such as Glt_{TK} and Glt_{PH}, which might indicate that protons in GltP bind at the same sites where sodium ions bind in the homologues. We hypothesized that competition between sodium ions and protons for the same binding sites might occur. However, we did not find any dependence on sodium ions in a wide range of conditions tested. Using a new method for generating a long-lasting electrical potential gradient in proteoliposomes, we show that the electrical and pH gradients across the membrane differently affect the transport activity rate in GltP reconstituted in liposomes. Protons also have an essential part in stabilizing and activating the transport.

5. Transport Mechanism of LATs - the use of nanobodies as a tool for structural and functional studies

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L-Amino acid Transporters (LATs) play key roles in human physiology and are involved in several human pathologies such as primary inherited aminoacidurias (Torrents et al. 1999; L Feliubadaló et al. 1999), autism spectrum disease (Tărlungeanu et al. 2016) and age-related hearing loss (Guarch et al. 2018) among others. However, their complete and detailed transport mechanism is still unknown as the structures solved until now are most of them in the same conformational state, open-to-in, with an exception of LAT1 bound to inhibitors that showed for the first time an open-to-out conformation (Yan et al., 2021). To decipher LATs transport mechanism we study the bacterial LAT alanine-serine-cysteine exchanger BasC as a LAT model. BasC has been already characterized (Bartoccioni et al. 2019) and 3D structurally solved in complex with a specific nanobody (Nb74) (Errasti-Murugarren et al. 2019). Single-molecule Fluorescence Resonance Energy Transfer (smFRET) studies in solution permitted us to track the TM1a tilt upon substrate amino acids (L-Ala, L-Ser) addition. These dynamical studies permitted us to conclude that the cytosolic gate closes triggered by substrates while the Lysinuric Protein Intolerance (LPI) disease variant (K154A) is not able to close it properly. Moreover, the 29 different Nb against BasC were screened and permitted us to classify them by their different inhibition profiles and interaction side. Selected Nb and substrates were also assayed by smFRET to reveal BasC transport cycle conformational changes. Some Nb (e.g., Nb51, Nb53, Nb58, Nb71 and Nb78) have been found to force BasC to achieve distinct conformations regarding the TM1a tilt within the cytosolic gate in comparison with the inward-facing Apo state and, furthermore, some of them blocked the

substrate effect on the TM1a tilt assessed by smFRET while others still permitted its closing. Our study shows that the combination of smFRET with structural techniques (X-ray and cryo-EM) is an appropriate approach to study the molecular mechanism of a LAT through a bacterial model, trying to find out the detailed mechanistic defect of K154A mutation causing LPI.

6. Investigating the role of membrane transporters in metabolic adaptation to low pH in the tumour microenvironment

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Cancer is a highly heterogeneous disease with significant variability in gene expression occurring between tumour types, and even between patients with the same tumour type. One important factor contributing to this heterogeneity is the tumour microenvironment (TME). As a result of poorly developed vascular systems, the TME can often be a hostile environment with areas of low nutrient availability, hypoxia and acidosis. In addition, it hosts a mixture of cell types including cancer associated fibroblasts, monocytes, dendritic cells, tumour associated macrophages, T cells and other cells of the immune system. The nutrient needs and metabolic waste products of these cells create a unique metabolic environment, providing a pressure for the metabolic adaptation of cancer cells, in order to survive and grow.

A number of interactions between cancer cells the tumour microenvironment are facilitated by solute carrier (SLC) transporters. These include nutrient acquisition, efflux of waste products and inter-cell-type metabolic cross-talk that can mediate immune modulation. We hypothesised that the expression patterns of SLCs may change in response to low pH in the tumour microenvironment. We looked at publicly available RNA sequencing data from clinical samples in The Cancer Genome Atlas (TCGA) database and assessed SLC expression patterns across 12 cancer types. Using gene sets from literature to identify tumours with acidic transcriptional signatures, we identified tumour type-specific changes in SLC expression signatures. As a complementary, unbiased approach, we performed RNA sequencing on a panel of lung and colon adenocarcinoma cell lines grown at either pH 7.4 or pH 6.8. Here, we combine our *in silico* TCGA analysis with the gene expression changes and metabolomics analysis of tumour cells *in vitro*, to study the key metabolic changes required for adaptation to a low pH environment.

7. Sugar binding and translocation in SGLT1: SSM-based electrophysiology reveals sugar occluded intermediates

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Recently we described an electrophysiological assay revealing pre steady-state charge translocations in the human Na⁺/sugar cotransporter SGLT1 upon sugar binding. We postulated this correlates with local conformational transitions that transfer charged protein residues across the membrane. In general, this may be attributed to mechanisms such as induced fit, substrate occlusion, alternating access or the closure of the extracellular gate.

Here we applied SSM-based electrophysiology for a detailed functional characterization of SGLT1 revealing kinetic properties of sugar binding and translocation, including rate and

equilibrium constants, apparent KM values and relative I_{max} values for different sugar species. Based on the data we conclude a kinetic model describing the sugar translocation pathway in SGLT1, revealing new insights into the transport mechanism: (1) Sugar is able to bind to the outward facing, empty carrier. Hence, substrate binding may happen in random order, under high sugar and low sodium conditions. (2) Not sodium binding, but sugar binding leads to an electrogenic conformational transition within SGLT1 under 0 mV conditions, likely representing sugar occlusion with $k_{+}=60$ s⁻¹ and $k_{-}=200$ s⁻¹. (3) Following sugar occlusion, the rate limiting step in sugar translocation is the transition into the inward open conformation. It occurs at the slowest rate for D-glucose, but at higher rates for sugars with lower apparent affinities. (4) At 0 mV and saturating sugar concentrations, all reactions within the sugar translocation pathway are fast compared to the empty carrier translocation. After sugar release, sodium release limits the slow transition of the empty carrier from the inward to the outward open state.

A 10-state kinetic model has been established to describe the sugar translocation pathway involving three substrate induced electrogenic transitions. Further work is required to merge this model with existing models based on the knowledge about voltage driven transitions observed with conventional electrophysiology.

8. Fear processing in mice with PMAT deficiency

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The plasma membrane monoamine transporter (PMAT, Slc29a4) is a polyspecific cation transporter that, in the brain, predominantly takes up monoamine neurotransmitters like dopamine and serotonin. PMAT function is hypothesized to emerge when other monoamine transporter function is impaired, such as under conditions of heightened monoamine neurotransmitter release in response to stressors. In humans, common genetic polymorphisms can reduce PMAT function, but these have exclusively been studied in the context of metformin treatment response under diabetic conditions. Nothing is currently known about how reduced PMAT function affects fear processing, nor any other aspects of emotional learning or memory. Our lab sought to begin examining the effects of PMAT deficiency on fear processing by using mice with constitutive reductions in PMAT function (i.e., heterozygotes), and comparing these to wildtype mice. Specifically, we evaluated both cued and contextual fear processing. Based on evidence that chronic impairments in serotonin uptake decrease fear expression, we hypothesized heterozygote mice would display lower levels of both cued and contextual fear relative to wildtypes. Surprisingly, we observed a trend ($p=0.08$) for male heterozygotes to exhibit attenuated cued fear expression. However, their acquisition, extinction retention, and cued fear renewal were indistinguishable from wildtypes, as was their contextual fear processing. Females exhibited no effects of PMAT deficiency upon contextual or cued fear processing. Four weeks after fear processing behavior tests concluded, mice were subjected to swim stresses, and blood was collected for stress hormone analyses. These data are still being analyzed, but we hypothesize that heterotypic stressor exposure will unmask the behavioral sequelae of reduced PMAT function. Our current findings are consistent with previous reports of sex-selective effects of PMAT deficiency on behavioral responses to stressor exposure. Continued investigation into how reduced PMAT function affects emotion processing and stress responsiveness could have translational implications for people with functional PMAT polymorphisms.

9. The WAT1 protein in the transport and homeostasis of the plant hormone auxin

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This project aims to decipher the regulation of auxin-transporting proteins in plants where WAT1 would be its focus. Auxins are a class of plant hormones that play a crucial role in plant growth and almost every aspect of plant development from embryogenesis to fruit formation. The recombinant WAT1 protein will be purified using metal ion affinity chromatography and size exclusion chromatography (SEC). However, the structural basis of auxin transport will be a major concern, in particular, Cryo-electron microscopy and X-ray crystallographic methods will be used to structurally characterize its transport by WAT1 at different steps. Mutants of residues suspected of being involved in auxin transport using site-directed mutagenesis will be generated and biochemically characterized, all correlated with kinetic assays using known substrates and compared to the wild type will allow us to elucidate and decipher the molecular mechanism of auxin transport. In particular, electrophysiology experiments using the SURFE2R N1 instrument (Nanon Technologies GmbH) which applies on a solid-supported membrane (SSM)-based electrophysiology will provide an additional functional characterization of auxin transport. Furthermore, biophysical characterization will also be expanded to include protein/protein and protein/ligand interactions together with affinity constants through ITC (Isothermal titration calorimetry) and MST (Microscale Thermophoresis).

10. Exploring human MRP4 protein binding sites and elucidating inhibition mechanisms by extensive molecular docking

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Organ transplantation have saved countless lives in the last decades; however, graft rejection is still a major issue with multifactorial causes and unpredictable outcome. In particular, the chronic administration of xenobiotics (e.g., immunosuppressants, antiviral drugs) to transplanted patients can result in cytotoxicity caused by intracellular accumulation, a plausible cause of graft rejection. The human ABCC4/MRP4 effluxes endogenous substrates and xenobiotics resulting in multidrug resistance, however the inhibition of this efflux may lead to xenobiotics accumulation inside kidney cells. Therefore, rationalizing the molecular interaction between MRP4 and these xenobiotics is of utmost importance to predict cytotoxicity, to avoid graft rejection.

The structure of human MRP4 was obtained from Alphafold, embedded in a POPC and cholesterol lipid bilayer. The model was further refined by microsecond long molecular dynamics (MD) simulations. Key protein structures were selected along MD simulations. A thorough molecular docking approach was performed on all experimentally known ligands (i.e., endogenous molecules, drugs, inhibitors, and polyphenols), on the whole protein, for all selected structures. We identified binding sites for substrates and inhibitors, in correlation with

experimental knowledge of key aminoacids. These binding sites highlight plausible mechanisms for substrate recognition, competitive and non-competitive inhibitions.

11. Another substrate of GABA Transporter 1 (GAT1), the novel therapeutic Betaine!

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GABA, γ -aminobutyric acid is the primary inhibitory neurotransmitter in the central nervous system (CNS), and its concentration is regulated by GABA transporters (GATs). To date, four GATs have been identified (GAT1-3, BGT-1 (*SLC6a1,13,11,12* respectively)). GAT1 is responsible for re-uptake of ~75-80% of GABA released in the CNS. This Na⁺/Cl⁻ dependent cotransporter has the highest affinity for GABA and is mainly localized on the neuronal membrane. It has been associated with the disorders caused by GABA imbalance in the CNS such as epilepsy, stroke, and depression. Recent findings show that regulated betaine supplementation improves the brain condition of patients with neurodegenerative diseases like Alzheimer's, Parkinson's, and dementia, and neuropathological like epilepsy. However, the mechanism behind this amelioration by betaine is not yet clear. The betaine/GABA transporter (BGT-1) could be involved, but its low expression levels in the brain raises questions about its effectiveness suggesting a possible role of GAT1.

By using *Xenopus laevis* oocytes heterologously expressing rGAT1 and two-electrode voltage clamp, we show that betaine induces inward transport current via rGAT1. The currents are dose and voltage-dependent with the apparent $K_{0.5} \approx 11.87 \text{ mM}$ and $I_{\text{max}} \approx 76.14 \text{ nA}$ at -60 mV . The current is absent in non-injected and non-GAT1 expressing oocytes. Moreover, SKF89976a, a potent GAT1 blocker, inhibits the betaine-induced currents, confirming transport via rGAT1. We also performed release experiments on HEK293 cells expressing YrGAT1. Betaine induced an efflux in ³[H]GABA pre-loaded cells with an affinity similar to that obtained by electrophysiological experiments ($K_{0.5} \approx 14.62 \text{ mM}$). We also performed experiments in the presence of GABA and betaine at different concentrations suggesting a dual role of betaine on the GAT1 transporter. In conclusion, GAT1 can transport betaine when it is present at mM concentration, compatible with its role of an osmolyte, but betaine seems also to regulate GABA transport, when added at μM concentrations in the presence of GABA, suggesting a possible allosteric modulation of GAT1.

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12. Dynamics of sugar transporter in malaria parasite: an integrated approach

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Sugar porters are responsible of the translocation of glucose and other monosaccharides across cell membranes via an alternating-access mechanism, where conformational changes in conserved helical bundles modulate the accessibility of the substrate to the two sides of the membrane. The hexose transporter from the malaria parasite *Plasmodium falciparum* (PfHT1) is a promiscuous sugar porter which shares the structural features of specialized sugar transporters but is able to transport both glucose and fructose. Glucose-bound PfHT1 is the first of sugar transporters to have been captured in an occluded conformation, which represents an intermediate state in the translocation cycle [1]. Sugar binding and gating have been shown to be allosterically coupled in PfHT1, suggesting a role of gating dynamics in substrate specificity and in promoting the sampling of the occluded conformation. Here we combine cryo-EM, NMR and fluorescence experiments with molecular dynamics simulations for the characterization of substrate-modulated conformational dynamics of PfHT1, in order to establish the mechanistic basis for sugar-catalyzed transport and substrate specificity.

[1] Qureshi, A.A., Suades, A., Matsuoka, R. et al. The molecular basis for sugar import in malaria parasites. *Nature* 578, 321–325 (2020)

13. Localization and function of the Na⁺/H⁺ exchanger NHE6 in primary neurons from different parts of the brain

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The Na⁺/H⁺ exchanger NHE6 (SLC9A6) is one the most frequently mutated loci associated with X-linked neuronal and intellectual disabilities, including Christianson Syndrome (CS). NHE6 localizes to early and recycling endosomes (EE, RE), where it regulates luminal pH, which in turn is important for endosomal trafficking and thus internalization and recycling of signaling receptors. Loss of NHE6 function causes excessive acidification of EE, consistent with a role of NHE6 as an endosomal “leak” H⁺ pathway [1]. However, mechanistic understanding of the physiological functions of NHE6 in the brain is lacking.

To address this question, cerebellar, cortical, and hippocampal primary neurons were isolated from NMRI mice and used to investigate NHE6 localization using immunofluorescence analysis and confocal microscopy. We found no difference in the localization of wild type (WT) NHE6 to EE and RE between these three types of primary neurons, as assessed by Manders analysis of colocalization with the Transferrin Receptor. In contrast, NHE6 localization to EE

and RE was increased from xx% in the soma to yy% in neuronal outgrowths of cerebellar and hippocampal neurons.

Next, we asked if signaling by the endosomally internalized plasma membrane receptor TrkB was dependent on NHE6. Treatment with the TrkB ligand Brain-derived Neurotrophic Factor (BDNF) elicited rapid activation of TrkB, Akt and ERK kinases, and the CREB transcription factor in cerebellar and hippocampal neurons. Transient siRNA-mediated knockdown (KD) of NHE6 in the neuroblastoma cell line SH-SY5Y reduced Akt activation and decreased long-term activation of CREB by xx%. To further explore this, we created a CRISPR/Cas9 NHE6-KO SH-SY5Y cell line in which mechanisms involved in this NHE6 dependence are currently being evaluated.

In conclusion, our results suggest that NHE6 is highly localized to neuronal outgrowths in cerebellar and hippocampal neurons, where it could be important for CREB-driven transcriptional events through regulation of TrkB recycling.

1. Ilie, A., et al., Assorted dysfunctions of endosomal alkali cation/proton exchanger SLC9A6 variants linked to Christianson syndrome. *The Journal of biological chemistry*, 2020. **295**(20): p. 7075-7095.

14. Microfluidic platform for screening of compounds on monoamine transporters

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Key-words: dopamine, neurotransmitters, novel psychoactive substances, microfluidics, efflux

Neurons propagate signals by releasing neurotransmitters in the synaptic cleft, the action is terminated thanks to the reuptake of these molecule carried out by membrane transporters. The neurotransmitter sodium symporters family (NSS) is of great interest for its link to mental and behavioral disorders such as depression, anxiety epilepsy and obesity. Among this group of transporters there are the dopamine, DAT, serotonin, SERT and norepinephrine transporter, NET. The aim of this project is to develop a novel high throughput microfluidic system for the characterization of compounds on the three monoamine transporters mentioned above.

The microfluidic system was constructed using the Elveflow range of microfluidic instruments and it allows to perfuse transfected HEK293 cells seeded in a microfluidic chip with different solutions. The system allows to perfuse 2 chips at one time for a total of 12 channels.

For this project tool compounds were used to validate the platform. We tested a blocker and a releasing agent for each of the 3 monoamine transporters, carrying out superfusion assays with the automated microfluidic set up. The assay allows to study of monoamine transporter-mediated substrate efflux with the advantage of avoiding substrate reuptake: the substrate is washed away via continuous flow.

D-Amphetamine was tested as a releasing agent at hNET and hDAT, while pCA for hSERT. The blockers used were GBR12909 for hNET and hDAT and paroxetine for hSERT. All the compounds tested show the expected effect on the monoamine transporters thus validating the system.

15. Study on the functional activities of MDR-associated ABC transporters in the *ex vivo* lung tumor explants

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Lung cancer is the leading cause of cancer mortality in both genders. Multidrug resistance (MDR) results in the failure of anticancer treatments in oncological patients. This phenomenon is often mediated by pharmacokinetic mechanisms, including efflux of anticancer drugs from the tumor cells through ATP-binding cassette (ABC) transporters. Although few expression studies in lung cancer tissues were reported in the past, functional activities of MDR-related ABC transporters have not yet been elucidated in detail. In the present study, we introduced a protocol for the generation of patient-derived non-small cell lung cancer (NSCLC) explants and evaluated expressions and activities of ABCB1, ABCG2, and ABCC1 transporters in them. In the initial step, sixteen primary cultures have been established from NSCLC biopsies excised by the pathologist immediately after lobectomy. In the western blotting experiments, transporters' expression levels have been detected showing substantial interindividual variability. Additionally, the functional activities of examined transporters were assessed in the explants by employing the accumulation flow-cytometric studies with doxorubicin and mitoxantrone. Specific model inhibitors (LY335979, Ko143, and MK-571) were used as positive inhibitory controls. Importantly, we found an association between the expression levels of ABC transporters and outcomes of accumulation studies. In explants with low transporters' expression levels, model inhibitors caused insignificant changes in the accumulation of model cytostatic substrates and vice versa. In conclusion, we demonstrate that ABCB1, ABCG2 and ABCC1 might play a significant role in MDR to conventional chemotherapeutics in NSCLC patients.

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16. Illuminating the monoamine transporters: High affinity fluorescent probes for selective visualization of DAT and NET

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Monoamine transmission is controlled by dopamine (DA), serotonin (5-HT) and norepinephrine (NE) transporters (DAT, SERT and NET respectively), wherein these transmembrane proteins mediate the re-uptake of monoamine neurotransmitters from the extracellular space into the cell. Proper function is necessary to achieve homeostasis as

dysregulation of monoamine transporters (MATs) is linked to several neuropsychiatric disorders, including substance use disorder. Fluorescently-tagged small molecules have proven useful as pharmacological tools to visualize protein expression, localization and distribution in distinct cell systems. The previously reported fluorescent cocaine-based analogue JHC1-064, has been extensively used as a tool to study MATs due to its high affinity for all three transporters. Nevertheless, lack of selectivity of this fluorescent probe across MATs contributes to certain staining limitations. This has been overcome in SERT with an S-citalopram-based probe, VK2-83, but remains a challenge for DAT and NET. The design of these fluorescent ligands encompasses three structural features: 1) a high affinity parent ligand, 2) a linker of appropriate length, and 3) a fluorescent dye. In this study, utilizing the NET selective inhibitor nisoxetine and the atypical DAT inhibitor JJC8-087 as parent compounds, rhodamine-labeled fluorescent probes, with high affinity and preferential binding for either NET or DAT have been designed and synthesized, with the goal of obtaining selective fluorescent tools to study expression and distribution of these MATs in distinct cell systems, including brain tissue.

17. Systematic characterization of human dopamine transporter missense mutations from a Danish cohort of psychiatric patients

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The dopamine transporter (DAT) shapes extracellular dopamine (DA) levels through high-affinity Na⁺-dependent reuptake of DA. Several reports suggest that missense mutations in the DAT gene (SLC6A3) are associated with neuropsychiatric diseases including ADHD, autism and bipolar disorder. DAT mutations have also been implicated in both infantile and early-onset parkinsonism. Here, we systematically characterize disease-associated DAT mutations in vitro to identify and classify mutational phenotypes. From an exome-sequenced Danish cohort of 19,005 individuals (iPSYCH2012), we chose 53 DAT mutants predominantly identified in patients with ADHD, ASD, schizophrenia or bipolar disorder. The mutants were expressed in HEK293 cells and evaluated using classical [³H]dopamine uptake assays and a new “sniffer cell” assay exploiting T-Rex 293 cells expressing genetically encoded DA sensors. A large fraction of mutants was functionally impaired with lowered V_{max} and/or increased K_m values. For 14 mutations we were unable to detect any activity. Additionally, we identified two variants with enhanced uptake capacity. To further dissect the molecular phenotype, the mutants are being tested for altered surface expression/inhibitor binding using a novel, fluorescently tagged cocaine analogue, DG3-80. Moreover, by use of the sniffer cells, all mutants were screened for constitutive DA efflux, a phenotype earlier reported for disease-associated DAT mutants. Remarkably, constitutive efflux was only observed for the autism associated T356M variant that previously was shown to possess this phenotype. Summarized, our results provide an important framework for deciphering mechanisms underlying how perturbed DAT function may contribute to neuropsychiatric disease.

18. The allosteric glycine transporter 2 inhibitor ORG25543 has different molecular determinants of potency and reversibility

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Neuropathic pain is caused, in part, by disrupted glycinergic neurotransmission. It is hypothesised that inhibitors of the glycine transporter 2 (GlyT2) will transiently increase glycine concentrations to restore nociceptive control. ORG25543 (IC₅₀= 6 nM) is a selective, pseudo-irreversible and non-competitive inhibitor of GlyT2 that is analgesics in animal models of pain¹. We have used site directed mutagenesis to characterise the binding site and mechanism of action of ORG25543.

It has been proposed that ORG25543 binds to an extracellular vestibule site of GlyT2². This site, known as the vestibule allosteric site, is formed by transmembrane domains (TMs) 1b, 6a, 10 and 11. We have found that ORG25543 does not bind to this site, but to an allosteric lipid inhibitor binding site comprised of TMs 5, 7 and 8 and extracellular loop (EL) 4³. Contrary to previous studies, we have found that mutations in the vestibule allosteric site do not significantly alter the potency of ORG25543². We have identified several key residues in the lipid allosteric binding site that modulate the potency ORG25543, particularly in TM5. The transition from an outward-open conformation to an inward-open conformation is a crucial process in the transport cycle and is associated with partial unwinding of TM5⁴. We propose that ORG25543 inhibits GlyT2 by preventing the unwinding of TM5 and slows the transition to the inward facing state. Reversibility of the compound is impacted, to different extents, by mutations in the allosteric lipid binding site, the vestibule allosteric site and the substrate site. The mutations that have the greatest impact on potency do not have the greatest impact on reversibility. We suggest that ORG25543 binds to the allosteric lipid binding site, with reversibility being determined by the conformational flexibility of the transporter.

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19. First insights into the structure and dynamics of the creatine transporter using MD simulations

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The creatine transporter (CRT1) is a member of the solute carrier 6 (SLC6) family of transporters. It plays an essential role in buffering energy levels in the brain through uptake of creatine, and its malfunction results in creatine transporter deficiency syndrome (CTD) that is associated with intellectual disabilities and autism. Although advances have been made in identifying mutants of CRT1 which lead to CTD, thus far no structure of CRT1 has emerged, providing a barrier to structure-function analysis of the transporter. Here we present a homology model of CRT1 using the human serotonin transporter as a template and show through molecular dynamics simulations that this is stable once embedded in a membrane. Moreover, we probe the protonation states of key protonatable residues, including Asp-458 and Cys-144. We show that Asp-458 protonation is required to maintain the secondary structure of transmembrane helix 9 and prevent local deformations in the membrane environment. We also show that deprotonation of Cys-144, thought to be important for binding of creatine, changes the electrostatic character of the substrate binding cavity, resulting in additional ion density in the binding site. We verified our prediction by functional analysis showing that a C144S mutant has no effect on transporter function, ruling out the hypothesis of a deprotonated Cys-144. Our results provide an important step towards deciphering the structure of CRT1.

20. Deciphering the structural defects from SNP variants of ABCB4 transporters in hepatocytes by means of molecular dynamics simulations

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Bile secretion is an essential function of the liver, necessary for digestion of fat as well as the elimination of xenobiotics and endogenous metabolites. This function mostly relies on transporters located at the canalicular membrane of hepatocytes, such as the ATP-binding cassette (ABC) transporter ABCB4. Variations in the ABCB4 gene may be related to modulation of expression, intracellular traffic, function or stability of the transporter. These mutations may in turn be associated to severe liver diseases such as progressive familial intrahepatic cholestasis types 2/3 (PFIC3).

The present study aims to provide insights into structural defects of selected SNP mutants of ABCB4 by means of molecular dynamics (MD) simulations supported by cellular and molecular biology experiments.

Taking advantages of the recent cryo-EM resolutions of ABCB4 transporters, μ s-scaled MD simulations were performed considering (i) different bound states (*i.e.*, ATP- and/or substrate-bound ABCB4) and (ii) different mutations (I490T, I541F and L5556) for which modes were embedded in lipid bilayers. Local destabilization events arising from SNP mutation were monitored by means of alchemical calculations. Particular attention was paid to the local

structural impact from mutations in order to provide hints about function impairments supported by clinical and experimental observations.

21. NMR investigation of Neurotransmitter-Sodium Symporters: a case study of Leucine Transporter

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Neurotransmitter/Na⁺ symporters (NSSs) terminate neuronal signaling by recapturing neurotransmitters released into the synapse in a co-transport (symport) mechanism driven by the Na⁺ electrochemical gradient. Because of the critical roles played by NSS members in the spatiotemporal control of neurotransmitter levels, perturbation of transporter activity by mutations or by pharmacophores have been associated with Parkinson's disease, depression, schizophrenia, Tourette's syndrome, attention deficit hyperactivity disorder (ADHD), and orthostatic intolerance ^[1].

Nuclear magnetic resonance (NMR) is virtually absent in the toolkit of biochemical techniques exploited to study NSSs transporters. The large amount of purified protein required, and the poor-quality spectra produced by large protein-detergent system always represented a major halt in the study of membrane transporters. We sought to change this paradigm. Employing LeuT, a bacterial transporter from *A.Aeolicus*, whose structure is closely related to human NSSs ^[2] and Saturation Transfer Difference techniques (STD-NMR) ^[3] we investigated substrate binding in detergent micelles and proteoliposomes. We show how ligand binding and affinity can be probed by NMR and how it is dynamically affected by ion concentrations and transporter conformations.

As sodium ion co-transport is linked with transporter activity, the ability to closely monitor Na⁺ in/out concentrations could reveal how LeuT and other NSSs members couple electrochemical potential with substrate shuttling. Therefore, harnessing the potential of NMR to observe ²³Na nuclei and employing shift reagents in proteoliposomes, we were able to observe different resonances for Na⁺ inside and outside the lipidic compartment and we are studying how they are affected by LeuT transporter activity.

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22. LRRK2 G2019S plays a role on excitatory/inhibitory imbalance in Parkinson's disease

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Parkinson's disease (PD) is associated with general modifications in the circuitry of the basal ganglia; hence, a central role has been attributed to the impairment of synaptic neurotransmission. Recently, *Leucine-rich repeat kinase 2* (LRRK2) has been discovered to play a role in both monogenic and sporadic forms of PD, in which the occurrence of substitution G2019S has been observed at a high frequency.

By two-electrode voltage-clamp technique on *Xenopus laevis* oocytes, we investigated the possible modulation of LRRK2 on glutamatergic and GABAergic transmission. Since excitotoxicity is a feature of PD physiopathology, alterations of glutamate transporter (EAAT2) could play the main role in glutamate-reuptake impairment. Oocytes co-injected with EAAT2 and LRRK2 G2019S show a significant reduction of the glutamate transport current compared to that observed in presence of LRRK2 wild-type. Immunocytochemistry studies show that LRRK2 alters the transporter localization on the plasma membrane, exhibiting that the amount of exposed EAAT2 is reduced in the presence of the mutated kinase. Remarkably, inhibition of LRRK2 G2019S with MLI-2 blocker recovers the EAAT2 membrane localization and its transport current similarly to wild-type condition.

We also performed micro-transplantation of membranes from striatum tissues of the LRRK2-associated PD mouse model in *X. laevis* oocytes, to investigate the possible role of LRRK2 on GABAergic transmission. Interestingly, the data show a significant reduction of GABA evoked current in the LRRK2 G2019S tissue compared to the wild-type striatum. The reason behind this reduction is still unclear as the GABA_A receptors are functionally unaltered. Nevertheless, GABA_A receptors show slower desensitization in mutated striatum, suggesting the influence of LRRK2 on the distribution of GABA_A receptor isoforms on the membrane, enhancing tonic current.

Our results indicate a critical role of LRRK2 in the modulation of excitatory/inhibitory imbalance, prompting further studies that include the possible modulation of LRRK2 on the GABA reuptake process.

23. Exploring the transport cycle of a bacterial homolog of vesicular glutamate transporters

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The solute carrier 17 (SLC17) family belongs to the major facilitator superfamily (MFS) and encompasses vesicular glutamate transporters (VGLUTs), inorganic phosphate transporters, the lysosomal H⁺/sialic acid cotransporter sialin, and the vesicular nucleotide transporter (VNUT). SLC17 members mediate symport or exchange of various substrates with H⁺ at distinct stoichiometries. A bacterial D-galactonate transporter (DgoT) from *E. coli* [1] shows high sequence similarity with mammalian VGLUTs and sialin. Only four charged residues (D46, R47, R126 and E133) are present in the membrane spanning region and were suggested to be involved in transport activity.

We used a combination of experimental and computational approaches to describe DgoT transport mechanism. In solid-supported membrane (SSM)-based electrophysiology, rapid galactonate application triggers a transient current component generated by substrate binding and subsequent conformational changes, followed by a slower component due to electrogenic transport. Transport current at different pH values demonstrates inhibition of H⁺ release and binding with apparent pK values of 7.0 and 9.2, respectively. Measurements at different electrochemical gradients revealed that the transport current reverses at conditions that indicate coupled symport of galactonate and H⁺ at 1:2 stoichiometry. The D46N or E133Q mutations lead to loss of transport activity, while selective binding of galactonate remains intact. In our all-atom molecular dynamics simulations, protonation of key residues D46 and E133 leads to rearrangements of transmembrane (TM) helices TM1 and TM7 that gate access to the substrate-binding site from the extracellular side. Substrate binding then induces the closure of the periplasmic gate and induces transition to inward-open conformation, where substrate is released, similar to the alternating access mechanism used by other MFS transporters [2, 3]. Based on our experimental and computational results, we propose a detailed transport model for galactonate/H⁺ cotransport in DgoT.

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24. Expression of OAT Transporters in Placenta during Gestation and at Preterm Deliveries; Effect of Pro-inflammatory Environment

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OATP (SLCO) membrane transporters located in the placental tissue play an important role in exchange of various exogenous and endogenous substrates including cytokines between maternal blood and the developing fetus. During the placental development and maturation the tissue needs to react to (pato)physiological changes occurring during pregnancy, many of them being accompanied by inflammation, which can lead to several complications and

undesired preterm birth. The aim of our present work was to investigate the expression of OATP transporters in preterm delivered human placentas in comparison to first trimester and healthy term placentas. Another aim was to address possible effect of pro-inflammatory environment on the expression of selected SLC transporters using human first trimester trophoblast cell line HTR-8/SVneo, choriocarcinoma cell line BeWo and human term placental villous explants.

Using real-time qRT-PCR, we observed significantly higher expression of *OATP2A1* (*SLCO2A1*), *OATP3A1* (*SLCO3A1*), and *OATP2B1* (*SLCO2B1*), in term placental tissue compared to the first trimester placentas and, interestingly, an increase in the expression of two of the transporters, *OATP3A1* and *OATP2B1*, in the preterm delivered placentas compared to the healthy term placental tissue. Exposure of HTR-8/SVneo and BeWo cells to INF γ we lead to increased expression of *OATP2A1* transporter, while significant decrease was observed in the *OATP2B1* (*SLCO2B1*) in BeWo cells upon INF γ -mediated stimulation. Interestingly, in line with the results observed in the preterm placentas, placental villous explants demonstrated significant increase in the expression of *OATP3A1* (*SLCO3A1*) following exposition to TNF α and LPS as pro-inflammatory stimulants. Our results thereby indicate that placental proinflammatory environment can affect expression of some OATP membrane transporters and can thereby affect placental transport function and possibly fetal development as well as labor onset.

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25. A novel sodium-coupled neutral amino acid transporter 2 (SNAT2) inhibitor synergises with the glucose transport inhibitor Bay-876 in cancer cells

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SNAT2 (SLC38A2) is a sodium-dependent neutral amino acid transporter, which mediates the concentrative accumulation of amino acids to support protein synthesis, metabolic processes, cellular osmolarity and the activation of mTORC1. SNAT2 also provides cancer cells with glutamine necessary to drive glutaminolysis, thereby presenting itself as a potential oncotherapeutic target. Consequently, a high-throughput screening assay was developed to identify novel inhibitors of SNAT2. The assay exploited the inducible nature of SNAT2, whereby HCC1806 cells were starved of amino acids to yield high functional expression levels of the transporter. Using an optimized fluorescent membrane potential (FMP) assay, a curated chemical library of 33 934 compounds was screened to identify compound 57E (3-methyl-4-methylphenylsulfonylamino-*N*-(2-trifluoromethylbenzyl)-2-thiophenecarboxamide) as a potent inhibitor of SNAT2. From two different assays, an IC₅₀ of 0.8 – 3 μ M was determined and compound 57E was found not to inhibit SNAT1, a paralog of SNAT2. MDA-MB-231 and HPAFII cells tolerated the SNAT2 inhibitor up to 100 μ M in a cell proliferation assay but it was found to potently synergise with the glucose transport inhibitor Bay-876, suggesting that both glycolysis and glutaminolysis pathways can be synergistically co-targeted in cancer cells.

26. Conserved structural elements essential for siderophore import by the mycobacterial ABC transporter IrtAB

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The mycobacterial ABC transporter IrtAB is responsible for the uptake of iron-charged siderophores called mycobactins. A previously determined inward-facing structure of IrtAB revealed an ABC exporter fold, yet the transporter imports mycobactins across the inner membrane of mycobacteria. By conducting cryo-EM analyses in nanodiscs or detergent under ATP-hydrolyzing conditions, we show here that next to the inward-facing state, IrtAB also adopts a novel outward-occluded conformation featuring a large, enclosed cavity. Transition to the outward-occluded conformation results in a lateral opening of a crevice that may form an access route of mycobactins from the outer membrane leaflet to the internal cavity. We observed lipid and detergent binding at this crevice, and the introduction of bulky amino acids in this region resulted in elevated basal ATPase activity. Conformational changes at the crevice are structurally linked to a highly conserved triple histidine motif donated from two transmembrane helices, which coordinates a transition metal ion. Substitution of these histidines with alanines resulted in a decoupled transporter, which still hydrolyzes ATP, but lost its capacity to import radioactively labelled mycobactin into the cell. Our data support a transport mechanism in which IrtAB imports mycobactins from the outer membrane leaflet by alternating between its outward-occluded and inward-facing conformations in response to ATP binding and hydrolysis.

27. In Parkinson's disease patients Tyrosine Hydroxylase expression is increased in CD14+ monocytes and linked to TNF

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Peripheral immune cells (human and mouse) express tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine synthesis. Little is known about TH levels in peripheral immune cells. This knowledge gap is due, in part, to the lack of a sensitive assay to measure TH in immune cells containing low levels of this protein. We developed the TH-Bio-ELISA that can quantify picogram levels of TH protein. TH-Bio-Elisa is highly sensitive and quantifies picogram levels of TH in multiple TH expressing cells (primary human macrophages, murine dopamine neurons, PC12 cells and primary human monocytes). We then measured TH in monocytes of Parkinson's disease (PD) patients, and age-matched healthy subjects from both sexes (n=11/group). We hypothesized that the loss of TH⁺ neurons in the brains of PD patients is accompanied by lower TH in the peripheral monocytes of PD patients. Contrary to our hypothesis, we found that monocytes from PD patients express significantly more TH protein than healthy controls (P<0.01, two-tailed t-test). Next, we focused on understanding the mechanism of increased monocytic TH in PD. Tumor necrosis factor (TNF), a pro-inflammatory cytokine, is increased in the brains and peripheral circulation of PD patients and

animal models of PD. Therefore, we investigated a possible connection between higher levels of TH protein and increased TNF in PD. Monocytes isolated from healthy donors were treated with TNF or with TNF in the presence of an inhibitor. Tissue plasminogen activator (TPA) served as a positive control. TNF stimulation increased both the number of TH⁺ monocytes and the quantity of TH per monocyte (N=3-6 independent biological replicates per group, P<0.01), without increasing the total number of monocytes. Our data suggest TNF modifies monocytic TH production. TH-Bio-ELISA provides a sensitive approach to investigate the immune regulatory pathways between brain and peripheral immune system in PD.

28. Molecular mechanism of substrate occlusion in the human serotonin transporter

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The human serotonin transporter (hSERT) determines neurotransmission in the central nervous system by the fast re-uptake of serotonin (5HT) from the synaptic cleft. As a member of the solute carrier (SLC) 6 family, SERT uses the electrochemical gradient of sodium across the plasma membrane as driving force for substrate re-uptake. SERT is targeted by various clinical relevant drugs, because improper clearance of 5HT is associated with different neuropsychiatric diseases. Since high-resolution crystal structures of SLC6 members including LeuT and hSERT became available, investigations of the structure-function relationship has been immensely accelerated. The alternating access mechanism proposes that for substrate translocation several conformational states are visited leading to the transport cycle. After initial sodium binding that stabilises the outward-open conformation, serotonin binds to the central substrate binding site (S1). This induces substrate occlusion by forcing the bundle domain to move towards the scaffold domain. While comprehensive studies of LeuT have shown that occlusion is mainly achieved by the rearrangements of extracellular parts of transmembrane helices (TMH) which also contributes to closing the extracellular gate, the molecular mechanism of action remains enigmatic. To study this process in SERT, site-directed mutagenesis was used to introduce point-mutations in position 172, which contributes to the hydrophobic lid of the outer gate and covers the substrate in the S1. I172 is highly conserved among the SLC6 family and plays an important role in substrate binding. HEK293 cells stably expressing different mutations of SERT are used to perform uptake experiments with tritiated 5HT. Molecular dynamic simulations combined with experimental data show that i) I172 on TMH3 initiates substrate occlusion by interacting with F335 and F341 on TMH6 causing a de-wetting of 5HT and the S1 ii) mutations of I172 demonstrate different uptake rates that can be directly linked to structural and dynamic differences in the S1.

29. Aquaporin 7 (AQP7): what's old and new?

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In contrast to orthodox aquaporins as water channels, AQP7 is a glycerol channel abundantly expressed in human adipose tissue facilitating the glycerol efflux upon lipolytic signal. Structural information of AQP7 has been revealed by X-ray crystallography, suggesting that AQP7 forms tetramers as others AQPs and each monomer is a glycerol channel, however the role of central pore formed by four monomers is still unclear yet. Additionally, the inhibitor of AQPs is rarely studied although AQPs are physiologically important and relevant with cancers development. Here we report an AQP7 cryo-EM structure at 2.55 Å resolution in the formation of dimer of tetramers. And two tetramers adhere each other in a twisted way by extracellular loop C. Well-defined densities are identified in the central pore and restricted by the leucine filters. GC/MS analysis suggests glycerol-3-phosphate (Gro3P) presents in the protein sample and is compatible with densities in the central cavity. Thus, we propose rationally that AQP7, in addition to function as a glycerol channel, may serve as a junction protein. Furthermore, glycerol presents in all eight monomers in the cryo-EM structure, but to a varied degree, implying a dynamic glycerol channel. The channel is blocked structurally by AQP7 inhibitor, presented from the complex structure of AQP7 and inhibitor determined by cryo-EM. Interestingly, the inhibitor is coordinated by A91 and H92 in the glycerol channel, considerably conserved in the AQP family, thus providing the possibility for the application of the inhibitor into the other AQPs.

30. Unveiling the DAT interactome and proteomic characterization of dopamine release sites in health and disease

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The availability of dopamine in the extracellular space is strictly controlled by the dopamine transporter (DAT). Reuptake of released dopamine into the dopamine release sites, and thereby, precise termination of dopaminergic signaling is critical for the proper operation of multitudinous brain functions. Moreover, dopamine release sites show distinct functional and structural properties compared to that of other neurotransmitter systems, which are proposed to have important implications for the development of disorders of the dopamine system. In our series of targeted studies, we carried out identification of the DAT interactome in the striatum of mice via affinity-purification mass spectrometry. Acute amphetamine exposure of mice elicited rapid reorganization of the DAT complex leading to alteration in the net level of several signaling and scaffolding proteins. Members of the DAT complex were also assessed in mice possessing patient-derived mutations in DAT with a phenotype resembling attention-deficit hyperactivity disorder and early-onset parkinsonism. Significantly affected DAT interactome proteins are mostly linked to signaling, scaffolding, and organization of the presynaptic active zone. Moreover, a marked reduction was revealed in the level of TMEM24, a protein involved in the transport of the precursor of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) to the plasma membrane from the location of its synthesis. In addition, we established and validated a method for the selective analysis of purified dopamine release sites employing a highly specific fluorescent cocaine analog and fluorescence-activated synaptosome sorting. Our results provide proteomic clues for the unique physiology of

dopamine release sites, while our last series of experiments captured proteins affected by chronic amphetamine exposure-induced sensitization specifically in striatal dopamine release sites. In sum, we unveiled proteins of the DAT interactome affected by perturbed DAT function either induced by exposure to amphetamine or due to disease-associated mutations, and we also carried out the global proteomic profiling of dopamine release sites.

31. Toward the structural advances in Major Facilitator Superfamily mediated transport cycle

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Members of Major Facilitator Superfamily (MFS) constitute one of the most important classes of membrane proteins. They translocate a broad spectrum of xenobiotics and endogenous substances across cell membranes. Thus, they are of utmost importance from both pharmacological and physiological points of view. Pharmacologically speaking, MFS transporters participate in pharmacokinetics, hence affecting xenobiotic absorption, distribution, and elimination. The comprehension of their function is essential to understand how they modulate therapeutic/adverse effects of drugs.

Driven by secondary or tertiary active transport, MFS transporters translocate substrates down their concentration gradient or drive an uphill transport using the energy stored in electrochemical gradients. To carry out the substrate translocation, the transporter must display an alternation of its cavity opening toward either intracellular or extracellular compartments. Therefore, the transporter undergoes large structural changes exhibiting different conformational states. The determined structures as well as the machine-learning-based AlphaFold2 predictions of human glucose transporter 1 and 3 (*hGLUT1* and *hGLUT3*) and human Organic Anion Transporter 1 (*hOAT1*) provided several conformational states which may be used for deciphering general rules for the transport cycle of mammalian MFS proteins. By means of μ -second-scaled Molecular Dynamic (MD) simulations performed dynamics on *hGLUT1*, *hGLUT3* and *hOAT1* in inward-, outward-facing and occluded states. The simulations were considered as *apo* states but also as substrate- (β -D-glucose) or inhibitorbound (α -maltose) states. Alchemical Binding Free Energy Calculations were performed to assess the conformation-dependent binding free energy for substrate and inhibitor. Given that MFS proteins have been shown to display a strong dependency on lipid bilayer composition, we have embedded transporters either in a simplistic plasma membrane (POPC:POPE:Chol 2:1:1) or in realistic-like membrane rich in differential lipids displaying natural occurring asymmetry. The collected data were used to decipher conformational MFS structural descriptors.

32. The two-domain elevator-type mechanism of zinc-transporting ZIP proteins

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Zinc is essential for all organisms and yet detrimental at elevated levels. Hence homeostasis of this metal is tightly regulated. The Zrt/Irt-like proteins (ZIPs) represent the only zinc importers in metazoans. Mutations in human ZIPs cause serious disorders, but the mechanism by which ZIPs transfer zinc remains elusive. Hitherto, structural information is only available for the model member, BbZIP, and as a single, ion-bound, conformation, precluding mechanistic insights. Here, we elucidate an inward-open metal-free BbZIP structure, differing significantly in the relative positions of the two separate domains of ZIPs. With accompanying co-evolutional analyses, mutagenesis and uptake assays, the data point to an elevator-type transport mechanism, likely shared within the ZIP family, unifying earlier functional data. Moreover, the structure reveals a previously unknown ninth transmembrane segment that is essential for activity *in vivo*. Our findings outline the mechanistic principles governing ZIP-protein transport and enhance the molecular understanding of ZIP-related disorders.

33. Behavioral encoding across timescales by region-specific dopamine dynamics: Is it all about DAT?

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The dorsal (DS) and ventral striatum (VS) receive dense dopaminergic projections controlling motor functions and reward-related behavior. However, it remains poorly understood how dopamine release dynamics is coupled in these regions across temporal scales to modulation of behavioral outcomes. Here, we address this question by probing extracellular dopamine dynamics in freely moving mice concomitantly in DS and VS using the genetically encoded dopamine sensor dLight1.3b. Our data reveal highly different dopamine dynamics in the two regions with a rapidly fluctuating signal in the DS, carrying information across dopamine levels, and a much slower fluctuating signal in the VS, consisting mainly of slow-paced transients.

Using machine learning, we correlate these dynamics to behavioral syllables across timescales and observe striking coordination of the dopamine signals between the two regions during exploratory behavior. Disruption of dopamine dynamics with cocaine imposes drastic changes that leads to randomization of action selection sequencing and disturbance of DS-VS coordination. The data indicate that coordinated, distinct dopamine dynamics of DS and VS, on a sub-second to minutes timescale, orchestrate behavioral sequences where the DS signal modulates the stringing together of actions and the VS signal provides the motivation to initiate and sustain the selected action.

34. Understanding substrate promiscuity of peptide transporters

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In bacteria, several membrane transporters have evolved that are responsible for the uptake of short peptides into the cell. Among them are di- and tripeptide transporters of the major facilitator superfamily (MFS) that recognize a variety of substrates though little is known about their underlying selectivity.

Here we present the combination of structural, biochemical, in silico and systems biology approaches for an in-depth characterization of the Escherichia coli peptide transporter DtpB. We determined 14 structures of DtpB in complex with various peptides. Using thermal unfolding assays, we were able to determine the binding constants of a large peptide library and to use a subset of it to correlate peptide transport rates in order to understand which properties a peptide must possess for it to be recognised and transported by DtpB.

35. Molecular mechanism of nutrient transporter degradation in human cells

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Proliferating cells increase nutrient acquisition to fuel anabolic processes for biomass formation. Conversely, differentiated or quiescent cells adjust their nutrient uptake to maintain metabolic homeostasis for survival. A key strategy by which cells reconfigure nutrient uptake across the plasma membrane is the selective addition or removal nutrient transporters. How cells control nutrient uptake to increase biomass or to preserve homeostasis is not understood. The goal of this project is to identify the molecular mechanisms that control nutrient transporter endocytosis and degradation during proliferation and upon entry into quiescence and thereby determine the abundance of nutrient transporters at the plasma membrane.

Our results show that, in non-cancerous cell lines, exit from cell cycle and entry into quiescence correlated with the selective endocytic downregulation and lysosomal degradation of the glutamine transporter SLC1A5/ASCT2 and the neutral amino acid transporter

SLC7A5/LAT1. Upon re-entry into the cell cycle, SLC1A5 and SLC7A5 protein levels increased again. The protein levels of several other transporters were not regulated under these conditions. Based on these results we hypothesize that the selective regulation of nutrient transporter abundance is directly linked to cell growth and proliferation. To analyze the contribution of SLC1A5 and SLC7A5 to these processes, we generated CRISPR/Cas9-mediated knockout cell lines. While SLC1A5 and SLC7A5 were essential for efficient exit from quiescence and re-entry into the cell cycle, they were not required for entry into quiescence. Moreover, we could identify two α -arrestins, which seems to be involved in the selective regulation of either SLC1A5 or SLC7A5. Currently we are characterizing the underlying molecular mechanisms.

Our results will provide a better molecular understanding of how proliferating and quiescent cells control their nutrient transporter repertoire to adjust nutrient uptake accordingly.

36. Visualizing the binding mode of the antidepressant vilazodone on the serotonin transporter

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The serotonin transporter (SERT) belongs to the family of neurotransmitter sodium symporters (NSS) and is responsible for serotonin recycling and neuronal signal termination in the synaptic cleft. SERT is a therapeutic target for psychiatric disorders, such as major depression. The standard medical treatment involves administration of selective serotonin reuptake inhibitors (SSRIs) which outcompete serotonin binding to the orthosteric site (S1). However, non-competitive inhibition of SERT is likely to produce a more favorable therapeutic profile. SERT carries an additional allosteric site (S2), which is a promising avenue due to low sequence conservation, high specificity and thus, less adverse effects. Vilazodone is a novel SSRI with a distinct chemical scaffold to other SSRIs, raising questions about its binding mode and efficacy. In our recent work (Per Pledge et al. 2021), we report that vilazodone inhibits serotonin uptake and impedes dissociation of [3H]-imipramine from S1 at low nanomolar concentrations. Surprisingly, the Cryo-EM structure of the vilazodone-and-imipramine-bound SERT showed vilazodone binding in the extracellular cavity (S2) in a unique pose compared to previous S2 ligands. Our aim is to investigate the structure of the vilazodone-bound full-length SERT in the absence of orthosteric ligands. We developed a protocol for the expression and purification of SERT in detergents and lipid-nanodiscs. Their activity is then assessed by radioligand binding assays (SPA). Although the combination of DDM with CHS stabilized active SERT, it produced empty micelles – leading to unwanted noise during cryo-EM particle collection. With our optimized nanodisc samples and a SERT-specific FAB fragment, we envision of demystifying the unique binding pose of vilazodone using Cryo-EM compared to the double-occluded transporter. Our findings will inform on the molecular determinants of administering vilazodone in the presence or absence of another orthosteric drug and point towards new avenues of rational drug design for the treatment of psychiatric disorders.

37. Characterization of α -pyrrolidinovalerophenone derivatives' structure-related activity at monoamine transporters

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Synthetic cathinones have gained increasing popularity as recreational designer drugs over the past years. Pyrrolidinophenone cathinones in particular received attention due to abnormal behavior attributed to consumption of α -pyrrolidinovalerophenone (α -PVP) also known as 'flakka' or 'Zombie drug'. With recurring waves of new illicit drugs occurring on the street markets, it is vital to elucidate the structure-activity relationship at monoamine transporters and probable abuse liability.

In this study, we tested two yet uncharacterized derivatives of α -PVP, namely α -pyrrolidinoisohexaphenone (α -PiHP), containing a branched side-chain, and 3',4'-methylenedioxy- α -pyrrolidinohexiophenone (MDPHP). We compared these derivatives to α -PVP and other already characterized derivatives including α -pyrrolidinopropiophenone (α -PPP), methylenedioxypyrovalerone (MDPV) and 3',4'-methylenedioxy- α -pyrrolidinopropiophenone (MDPPP) with *in vitro* radiotracer uptake inhibition experiments in monoamine transporter expressing HEK293 cells.

α -PiHP showed more potent inhibition in the nano-molar range at the human dopamine transporter (hDAT) and the human norepinephrine transporter (hNET) compared to α -PPP, but not α -PVP. At the human serotonin transporter (hSERT) no relevant pharmacological activity was observed for α -PiHP, showing an even stronger selectivity for hDAT and hNET than α -PVP. MDPHP showed equipotent inhibition as MDPV at hSERT, hDAT and hNET, strongly inhibiting hDAT and hNET in the nano-molar range. MDPPP was ten times less potent at hSERT and hDAT and hundred times less potent at hNET than MDPV and MDPHP.

In conclusion, we showed that the interaction profile of the newly explored derivative MDPHP compares very well to MDPV. In contrast, α -PiHP showed similar uptake inhibition at hDAT and hNET but less potent inhibition at hSERT than α -PVP, which therefore may be associated with higher abuse liability.

38. Pharmacological characterization of conformationally restricted cyclic GABA analogs as potent and selective BGT1 inhibitors

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The betaine/GABA transporter 1 (BGT1, gene: SLC6A12) is a member of the prominent SLC6 transporter family with still elusive function, largely due to a lack of potent and selective tool compounds. We here present the synthesis and the pharmacological characterization of five novel conformationally restricted cyclic GABA analogs which were designed by modeling based on the previously reported BGT1 inhibitor (1S,2S,5R)-5-aminobicyclo [3.1.0]hexane-2-carboxylic acid (bicyclo-GABA). Using [³H]GABA radioligand uptake assays, the N-methylated analog (**2**) was identified as slightly less potent than bicyclo-GABA, but with no activity at recombinant $\alpha 1\beta 2\gamma 2$ GABA_A receptors. Additional pharmacological characterization of bicyclo-GABA and **2** in a fluorescence-based membrane potential (FMP) assay indicated that both compounds are not transported by BGT1 and thus function as inhibitors, which was validated by a Schild analysis for bicyclo-GABA. In order to decipher the molecular determinants driving activity of bicyclo-GABA and **2**, in silico-guided mutagenesis studies were performed. The non-conserved BGT1 residues Q299 and E52 were identified as critical for BGT1 activity and selectivity. The binding mode of bicyclo-GABA was further validated by the introduction of activity into the corresponding GAT3 mutant L314Q (38 times potency increase cf. wildtype). Furthermore, [³H]bicyclo-GABA was generated to assist with the pharmacological analysis which in contrast to the FMP assay indicated substrate behavior of the compound. Overall, this novel class of GABA analogs may serve as valuable tools to further study structure-function of BGT1 as well as the elusive pharmacological role of BGT1, both in the brain and in the periphery such as the liver and the kidneys.

39. Extrinsic loop domain modulation of the endosomal sodium/proton exchanger NHE9

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Na⁺/H⁺ exchangers (NHE) are found in all cells wherein their activities regulate intracellular pH, sodium levels and cell volume. The NHE isoform 9 (SLC9A9) is localized to late- and recycling- endosomes and its dysfunction has been linked to susceptibility of long COVID, and autism spectrum (ASD) and attention deficit hyperactivity (ADHD) disorders. NHE9 is highly expressed in glioblastoma cancer cells, therein modulating epidermal growth factor (EGF) turnover. Previously, the loop domain structure in NHE9 could not be modelled. Here, we present the cryo-EM structures of NHE9 the additional loop domain modelled. The loop domain has two domain-swapped β -hairpins situated ~ 15 Å above the dimerization interface. Six lysine and arginine residues are clustered in the β -hairpin domain and are well-situated for binding negatively-charged lipids. Consistent with previously measured native MS and lipid-binding analysis, we observe map density that fits two PIP₂ lipids. We conclude the additional loop domain has evolved as a binding site for binding negatively-charged lipids, that can likely regulate NHE9 activity by fine-tuning oligomerization dynamics.

40. Investigating the Role of Cholesterol in Regulating the Slc7 Family of Amino Acid Transporters

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SLC7 transporters are a family of membrane proteins from the amino acid polyamine cation (APC) superfamily, which control the relative concentration of amino acids inside and outside of the cell. Recent structural work (Yan, R. et al. 2019, Lee, Y. et al. 2019) has yielded structures of human SLC7 members with cholesterol densities. Given that several APC homologues (SLC7,36,38) are known to traffic between the differing environments of the plasma membrane, lysosome and the Golgi apparatus, we are investigating human SLC7 structures to analyse the role of lipids on the structural dynamics of these transporters. The analysis of cholesterol was undertaken as it had been shown to play an important role in stabilising the interaction between a single transmembrane helix protein with *GkApcT*, which although a bacterial transporter, is thought to associate with chemically similar hopanoid sterols (Jungnickel et al. 2018). Here we employ a robust serial-multiscale approach to study the molecular dynamics of selected human SLC7 members in cholesterol-enriched membranes. We use an occupancy-based metric supplemented with kinetically-derived binding residence times to identify lipid sites. Through this pipeline, we report several conserved cholesterol sites for SLC7s, most of which coincide with available experimental densities. For each of the transporters studied, cholesterol sites display a varying degree of affinity, as calculated with umbrella sampling/potential of mean force calculations, from medium to high affinity (ca. -30 kcal/mol). Interestingly, conformation-dependent cholesterol binding was recorded for one of the members (LAT1), giving insight to the relationship of cholesterol binding with local helical packing. Together these data show that cholesterol is interacting at specific sites on the SLC7 transporters and these interactions likely play a role in modulating structural dynamics and function.

41. A rebel with(out) a cause: on folding & intracellular trafficking of the human creatine transporter 1

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The human creatine transporter 1 (CRT1, SLC6A8) is a member of the sodium-dependent neurotransmitter transporter (NTT) protein family. Creatine transporter deficiency (CTD) is a rare disease manifested by intellectual disability, epilepsy, development delay and motor dysfunction. Folding and trafficking defects in SLC6 proteins frequently lead to pathological conditions, e.g. mutations in the human creatine, dopamine and GABA transporters trigger CTD, Parkinsonism and epilepsy, respectively. Interestingly, treatment with a renowned chemical chaperone 4-phenylbutyrate (4-PBA) rescued the surface expression and function of several folding-deficient CTD variants. Hence, it is vital to decipher the arcane molecular machinery behind the protein folding and intracellular trafficking of hCRT1. Using biochemical and pharmacological approaches, we observed that CRT1 is the only NTT intolerant to introducing a yellow fluorescent protein (YFP)-tag at its N-terminus, i.e. resulting in ER-retention. We generated serial truncations along the N-tail of CRT1, and found that the Δ N60-CRT1 mutation abolished creatine uptake. The ER-localization of N-tail YFP-tagged hCRT1 and Δ N60-CRT1 was observed in transiently transfected HEK293 cells. These findings break a hallmark rule, previously established for SLC6 transporter-relatives of hCRT1, that their amino tails are virtually dispensable to their folding and trafficking (cell surface expression) or

even their substrate uptake activity. Our data provide novel insights into the molecular and physiological features underlying the non-conforming folding and trafficking routes of CRT1. These ought to impart crucial details relevant to the role and regulation of CRT1 in disease (e.g. CTD and cancer).

42. Characterizing sodium binding to the human GABA transporter hGAT1

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The human GABA transporter (hGAT1) is a membrane protein that mediates the re-uptake of the neurotransmitter GABA from the synaptic cleft into neurons and glial cells. Dysregulation in the transport cycle has been associated with epilepsy and neuropsychiatric disorders, highlighting the crucial role of the transporter in maintaining brain neurotransmitter levels homeostasis. From a mechanistic point of view, the transport cycle is defined as secondary active, in which the unfavourable translocation of substrate is energized by the co-transport of sodium and chloride ions along their electrochemical gradient. To investigate the process of sodium binding, we carried out in parallel 100 independent full-atom molecular dynamics simulations of hGAT1 in a physiologically relevant membrane environment, each trajectory 250ns long. Analysis indicated i) a clear sodium entry path ii) defined the temporary and stationary binding sites and iii) K_{on} of sodium binding. Data showed that binding of sodium ions to hGAT1 induced local and global structural adjustments, thereby providing a framework for understanding the energetics associated with the process ion binding and transport.

43. Structures of ferroportin in complex with the small molecule inhibitor vamifeport

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A major regulatory mechanism of iron homeostasis in humans is mediated by ferroportin (FPN), the only cellular iron exporter, and the peptide hormone hepcidin, which inhibits Fe^{2+} transport and induces internalization and degradation of FPN. Dysregulation of this key regulatory axis leads to multiple pathological conditions, and consequently, pharmacological FPN inhibitors that block FPN-mediated iron transport are of high clinical interest. Here, we determined cryo-EM structures of human FPN in complex with synthetic nanobodies and vamifeport (VIT-2763), the first clinical-stage oral FPN inhibitor. Vamifeport competes with hepcidin for FPN binding and is currently in clinical development for β -thalassemia and sickle cell disease. The structures revealed two distinct conformations, an outward-facing and an occluded state of FPN. The binding site of vamifeport is in the center of the transporter and overlaps with the hepcidin binding site explaining the competitive binding of the two ligands. The introduction of single point mutations in the vamifeport binding pocket reduced the affinity of vamifeport to FPN in fluorescence polarization assays, validating our structural data. Together, our data provide first insights into the conformational rearrangement of FPN during the transport cycle and its pharmacological inhibition by vamifeport.

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44. Structural and functional properties of a plant NRAMP related aluminum transporter

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The transport of transition metal ions by members of the SLC11/NRAMP family constitutes a ubiquitous mechanism for the uptake of Fe^{2+} and Mn^{2+} . Despite the evolutionary conservation of the family, two of its branches have evolved a distinct substrate preference with one mediating Mg^{2+} transport into prokaryotes and another the transport of Al^{3+} into plant cells. Here we have addressed the structural and functional properties of a putative Al^{3+} transporter from the plant *Setaria italica*. We show that the protein transports diverse divalent metal ions and binds the trivalent ions Al^{3+} and Ga^{3+} , which are both presumable substrates. Its cryo-EM structure reveals an occluded conformation that is closer to an inward- than an outward-facing state with a binding site that is remodeled to accommodate the increased charge density of its transported substrate.

45. A new GlyT2 variant associated to hyperekplexia

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Hyperekplexia (OMIM 149400) is a sensorimotor syndrome of great perinatal clinical relevance. Neonates present energetic startles in response to trivial stimuli, generally tactile or acoustic, that may be lethal due to apnea episodes. The disease is caused by total or partial blockade of inhibitory glycinergic neurotransmission. The neuronal glycine transporter GlyT2 removes the neurotransmitter from the synaptic cleft, recycling it to the presynapse and supplying glycine for the filling of the synaptic vesicles. The function of GlyT2 maintains the strength of glycinergic neurotransmission in vertebrates, and its dysfunction causes a presynaptic form of hyperekplexia. Some mutations in the GlyT2 gene (SLC6A5) are the second most common cause of human hyperekplexia. Here we describe a new GlyT2 variant localized in an infantile patient diagnosed with hyperekplexia. The SLC6A5 gene in the patient contains a missense mutation in the open reading frame of the GlyT2 gene inherited in homozygosity. The sequences of the glycine receptor genes GLRA1 and GLRB did not show abnormalities. The substitution has been classified as “probably damaging” with the highest score in the bioinformatics software PolyPhen-2 (Ramensky et al., 2002). This is because the

substituted residue is totally conserved among the phylogenetic scale. In order to characterize the new mutant transporter, we expressed the recombinant GlyT2 variant in heterologous cells and analysed the pathogenic mechanism of the mutation. We prove the mutant is prematurely degraded from the ER and, therefore, it is inactive. The consequences of mutant-wild-type co-expression are being analysed.

46. Probing the determinants of sugar-catalysed state transitions in the mammalian fructose transporter GLUT5

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In mammals, glucose transporters (GLUT) control organism-wide blood glucose homeostasis. In human, this is accomplished by fourteen different GLUT isoforms, that transport glucose and other monosaccharides with varying substrate preferences and kinetics. Nevertheless, sugar coordinating residues in GLUT1, GLUT3 and GLUT5 structures have proven to be very similar. Indeed, there is little difference between the sugar-coordinating residues in the GLUT proteins and even the malarial *plasmodium falciparum* transporter PfHT1, which is uniquely able to transport a wide range of different sugars. PfHT1 was captured in an intermediate “occluded” state, revealing how the extracellular gating helix TM7b has moved to break over the sugar-binding site. Based on sequence difference and kinetics, it was concluded that the TM7b gating helix dynamics and interactions are evolving to enable substrate promiscuity in PfHT1, rather than the sugar-binding site itself. It was unclear, however, if the TM7b structural transitions observed in PfHT1 would be similar in the other GLUT proteins. Here, using enhanced sampling molecular dynamics simulations, we show that the fructose transporter GLUT5 spontaneously transitions through an occluded state that closely resembles PfHT1. We demonstrate how fructose lowers the energetic barriers between outward and inward-facing states, and how fructose binding is coupled to TM7b gating by a strictly-conserved asparagine residue and a GLUT5-specific tyrosine-histidine pairing. We conclude that substrate translocation in GLUT proteins is driven by conformational selection and that TM7b should be considered as an extension of the sugar-binding site.

47. Novel positive modulator of dopamine transport discovered in Australian desert plant

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Dopaminergic signaling is essential for movement, cognition, reward and motivation, mediated by action at multiple dopamine receptor subtypes. This activity is tightly regulated by the dopamine transporter (DAT). In a range of conditions such as Parkinson's disease, schizophrenia, addiction and ADHD, symptoms are alleviated with medications that modulates the dopaminergic system, and discovery of novel modulators is essential for developing new improved therapies.

In our continuous screening of novel plant-derived molecules for their effects on monoamine transport, we identified (2*E*,4*Z*,6*E*)-5-(acetoxymethyl)tetradeca-2,4,6-trienoic acid (5ATTA) as a positive modulator of DAT activity. The compound was isolated from the leaves of *Eremophila oppositifolia* subsp. *Angustifolia*, endemic to the Australian desert. In cell lines transfected to over express the human or *Drosophila* (fruit fly) variant of DAT, we found that 5ATTA selectively elevated uptake of a sub-saturating [³H]dopamine concentration to 165% and 440% of control, respectively. We did not observe any noticeable increase in transport rate when applied to its nearest homologues, the transporters for norepinephrine, serotonin and GABA. The effect of 5ATTA was blocked by the selective DAT inhibitor nomifensine and found to non-competitively inhibit the binding of the cocaine-analogue [³H](–)-2-β-Carbomethoxy-3-β-(4-fluorophenyl)tropane (CFT, WING35,428). Together these data could suggest that the observed potentiating effect of 5ATTA on DAT transport is the result of direct action on DAT, however further experiments are being undertaken to confirm this and unravel details on this novel mode of action.

To the best of our knowledge, this novel action for 5ATTA, distinguishes it from all existing therapies targeting the DA system. Thus, the present discovery could pave the way for new strategies to modulate dopaminergic signaling and ultimately alleviate or treat conditions specifically associated with altered DA levels.

48. The effect of natural and artificial mutations on ABCG2 function and expression

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ABCG2 (ATP-Binding Cassette Subfamily G Member 2) is a multidrug transporter protein responsible for the export of endo- and xenobiotics in various cells in the human body. It has a major protective role in tissue barriers such as the blood brain barrier, stem cells, the gut or the placenta. Among its substrates are a wide range of commonly used drugs and chemotherapeutics and thus contributes to multidrug resistance in cancer cells. ABCG2 has a broad range of substrates that can be different in size and other attributes, for this reason it is compulsory to examine the possible interaction with ABCG2 when a new drug is developed and authorized. ABCG2 takes part in renal urate secretion and, according to GWA studies, a common missense variant is an important genetic factor in the development of gout.

Several naturally occurring mutations have an effect on the expression, function or trafficking of the ABCG2 membrane protein and the aim of this project was to examine natural and artificial mutant variants of this protein.

We have selected two regions in the ABCG2 structure to examine. First, we decided to investigate the role a disordered loop in the protein that is affected by a naturally occurring mutation when one of four lysines is deleted (K360del). Several artificial mutant variants were created to examine the role of this disordered loop in the protein. Other mutations introduced here affect two leucines (L554 and L555) that separate the central and upper cavities of the ABCG2 dimer according to the Cryo-EM protein structure model. HEK and HeLa cells were transfected with the ABCG2-protein coding plasmids, then the expression and function of the ABCG2 variants were measured after antibody labeling and a flow cytometry-based transport assay in live cells.

The results should contribute to a better understanding of the relationship between the structure and function of ABCG2.

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49. Investigating substrate-dependent conformational changes of the dicarboxylate transporter, VcINDY.

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Citrate and dicarboxylates are major drivers governing energy homeostasis, and the main route for uptake of these anions in humans is through the SLC13 transporters that belong to the divalent anion Na⁺ symporter (DASS) family. The prototype DASS transporter is VcINDY from *Vibrio cholerae*, which can use the electrochemical Na⁺ gradient to power transport of succinate across the membrane. Here, using a combination of cryo-EM structures, ligand binding assays, and site-specific cysteine alkylation assays, we demonstrate that VcINDY couples Na⁺ and succinate through a novel cooperative mechanism. In the absence of bound Na⁺ ions, key regions contributing to the succinate binding site are substantially more flexible effectively abolishing the succinate binding site. However, when Na⁺ binds these key regions rigidify to form the succinate binding site. These data suggest that VcINDY, and likely all DASS transporters, use this conformational selection mechanism to couple substrate and ion binding, which is a critical step in transport cycle.

50. Enantioselective Drug-binding kinetics shape the psychostimulant effect of dopamine transporter inhibitors

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The pharmacology of different compounds is normally assessed under thermodynamic equilibrium, however, in the human body, the drug-target interaction is influenced by the constant flux of fluids and a variety of physiological processes. Hence, the in vivo properties of a drug differ from the in vitro thermodynamic equilibrium. α -Pyrrolidinovalerophenone (α PVP) is a psychostimulant and drug of abuse associated with severe intoxications in humans. α PVP exerts long-lasting psychostimulant effects, when compared to the classical dopamine transporter (DAT) inhibitor cocaine. We used radiotracer assays, computational

approaches, transporter electrophysiology and behavioural assays in mice to investigate the role of binding kinetics in the psychostimulant effect elicited by α PVP enantiomers. We found that α PVP enantiomers substantially differ from the DAT-inhibitor cocaine in their binding kinetics. The two enantiomers differ from each other in their association rates, but they show similar slow dissociation rates leading to pseudo-irreversible binding kinetics at DAT. The pseudo-irreversible binding kinetics of α PVP is responsible for the observed non-competitive pharmacology, correlates with persistent psychostimulant effects in mice and differ from the fast-acting DAT-inhibitor cocaine. Our work shows that drug binding kinetics at DAT can have a significant impact on the psychostimulant effects of drugs in vivo. Given this information, a more detailed assessment of drug-binding kinetics for DAT inhibitors might provide insights for the design of novel DAT inhibitors with improved clinical utility. Furthermore, it shows how psychopharmacological research on illicit drugs may help to reach a better understanding of physiological and toxicological processes.

51. The structure of a tripartite ATP-independent periplasmic (TRAP) transporter provides the molecular basis for function

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Tripartite ATP-independent periplasmic (TRAP) transporters are a major class of secondary transporters found only in bacteria and archaea. Unlike other secondary transporters, TRAP transporters cannot receive their substrates directly, but do so by using a secreted soluble substrate-binding protein. How a sodium-driven secondary active transporter is strictly coupled to a substrate-binding protein is poorly understood. We report the single-particle cryo-EM structure of the sialic acid TRAP transporter SiaQM from *Photobacterium profundum* at 2.97 Å resolution. SiaQM forms a heterodimeric complex, with four transmembrane segments from the Q-subunit and twelve from the M-subunit. SiaM is comprised of a “transport” domain and a “scaffold” domain. SiaQ forms intimate contacts with SiaM to extend the size of the scaffold domain, indicating TRAP transporters may operate as monomers, rather than the typically observed oligomers for elevator-type transporters. We have identified potential Na⁺ and sialic acid binding sites in SiaM and confirmed a strict dependence on its substrate-binding protein for uptake. Our crystal structure of the cognate sialic acid bound substrate-binding protein, together with co-evolution driven docking studies, provides a molecular basis for how sialic acid is delivered to the SiaQM transporter complex. We conclude that TRAP proteins are conceptually a blend between an ABC importer and a secondary transporter, which we describe as an ‘elevator-with-an-operator’. These data provide a molecular basis for TRAP function, able to inform design of new antimicrobials against pathogens that use TRAPs during infection.

52. Characterization of the Human Serotonin Transporter with Fluorescent Unnatural Amino Acid

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Neurotransmitter: Sodium Symporters are responsible for the Na⁺ driven reuptake of released neurotransmitters from the synaptic space. The uptake of serotonin is facilitated by the serotonin transporter (SERT), which is targeted by numerous antidepressants and psychostimulants. However, despite the pharmacological significance of SERT, little is known about the conformational shifts induced upon inhibitor and substrate binding, as the nature of the transporter renders conventional labelling strategies inefficient. Here, we incorporate site-specifically the non-canonical fluorescent amino acid L-ANAP into SERT by amber stop-codon suppression, using large-scale transient transfection of suspension HEK293 cells. We solubilize and purify the mutated transporter and record changes in the conformational dynamics by fluorescence spectroscopy. We show that SERT tolerates the incorporation of L-ANAP on several positions and that the integrity of the protein and fluorophore is preserved upon purification. Based on spectral changes in L-ANAP fluorescence for F556TAG, we directly probe the binding of different ligands, substrate and ions. We show that Na⁺ uniquely stabilizes a conformation different from those of NMDG⁺, K⁺ and Li⁺ and binds with an affinity of ~17 mM. These findings represent, to the best of our knowledge, the first characterization of a purified membrane protein encoding an unnatural amino acid. This will pave the way for future *in vivo* and *in vitro* studies, including patch-clamp fluorometry and transition metal ion FRET studies for which inserted L-ANAP will be combined with a correspondingly small FRET acceptor.

53. Structural basis for antifolate recognition by the proton coupled folate transporter PCFT.

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Folate is an essential vitamin (B9) that plays a critical role in cellular metabolism as the starting point in the synthesis of nucleic acids, amino acids and the universal methylating agent S-adenylmethionine. Folate deficiency is associated with numerous developmental, immune and neurological disorders. Mammals cannot synthesise folates *de novo*; therefore, several systems have evolved to uptake folates from the diet and distribute these within the body. The proton-coupled folate transporter (PCFT, SLC46A1) mediates folate uptake across the intestinal brush border membrane and the choroid plexus and is an important route for antifolate delivery in cancer chemotherapy. How PCFT recognizes folates or antifolates however is currently unclear. Here we present cryo-EM structures of PCFT in a substrate-free state and in complex with the new generation antifolate, pemetrexed. Our results provide a structural basis for understanding antifolate recognition and provide insights into the pH regulated mechanism of PCFT-mediated folate transport.

54. DAT-interacting protein, Synptogyrin-3, Modulates Dopamine Release and Selectively Reduces Cocaine Self-administration

Emily G. Peck and Sara R. Jones

Synptogyrin-3 (SYG3) is a synaptic vesicle protein highly expressed in dopamine-containing neurons that directly interacts with the dopamine transporter (DAT), suggesting a role in synaptic dopamine dynamics. The DAT is the primary reinforcing site of cocaine, and chronic cocaine exposure alters DAT function, expression, and dopamine release dynamics. We tested the hypothesis that chronic cocaine exposure disrupts SYG3 function, resulting in DAT alterations that drive excessive cocaine taking. Rats were trained to self-administer cocaine and tested on a progressive ratio (PR) schedule of reinforcement. Western blots showed a significant positive correlation between SYG3 and DAT protein levels and a significant negative correlation between SYG3 and PR breakpoint in the ventral tegmental area and nucleus accumbens. Thus, we virally overexpressed SYG3 in VTA dopamine neurons of cocaine-naïve rats to assess the effects on cocaine and sucrose self-administration, anxiety-like behavior, and learning assays. Additionally, nucleus accumbens dopamine terminal function was measured using *ex vivo* fast-scan cyclic voltammetry. SYG3 overexpression resulted in reduced cocaine responding on an extended access schedule and a lower PR breakpoint, suggesting decreased cocaine reinforcement and motivated cocaine responding. SYG3 overexpression also reduced anxiety-like without altering sucrose self-administration, sucrose preference, or pairwise discrimination. While SYG3 overexpression did not affect dopamine uptake rate in cocaine-naïve rats, it bimodally altered dopamine release—blunting release in response to high stimulation intensities but increasing release at low stimulation intensities. Together, these data provide evidence for SYG3's role as a regulator of dopamine kinetics and a potential target for pharmacotherapeutics to treat cocaine use disorder.

55. Towards Directly Visualising Conformational Changes in the Sodium Symporter Family

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Neurotransmitter: sodium symporters (NSSs) play a crucial role in the nervous system by regulating neurotransmitter signaling and homeostasis. NSSs terminate synaptic transmission and recycle sodium-dependent neurotransmitters. These transporters are also targets for the treatment of neuropsychiatric disorders. Previous structural studies have yielded structures of a series of putative intermediates in the proposed alternating access transport mechanism. However, all structural studies to date are carried out in the absence of physiologically relevant gradients and, to date, no time-resolved structural characterisation of the transport cycle has been performed. We are developing novel methodological approaches using X-ray and electron crystallography to directly tackle these questions.

Key words: NSSs, time-resolved crystallography, transport

56. Functional modulation of a pH-sensitive ion channel by a transporter family

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The acid-sensing ion channel 1a (ASIC1a) is a trimeric ligand-gated ion channel. Its activation by protons results in influx of Na⁺ and, to a lesser extent, Ca²⁺ before the channel rapidly desensitizes. ASIC1a-mediated currents contribute to normal brain function, including long term potentiation, which is important for learning and memory. However, elevated activity of ASIC1a has been directly implicated in the pathogenesis of multiple neuropathological conditions, such as ischemic brain injury, pain and Alzheimer's disease. Recent evidence suggests that during such neuropathological conditions, association with protein interaction partners can affect ASIC1a activity, thereby contributing to neuronal damage and cell death. Consequently, there is an increasing interest in understanding the regulation of ASIC1a by protein-interaction partners, which remain largely enigmatic. Here, we have used a combination of mass spectrometry, biochemistry, fluorescence spectroscopy and electrophysiology to identify a family of transporters that directly interacts with ASIC1a and regulates its function. Depending on the specific transporter isoform present in the channel-transporter complex, the activation of ASIC1a is either prolonged and potentiated or decreased. We anticipate this work to offer promising starting points for therapeutic targeting of ASIC1a-containing macromolecular complexes in various diseases.

57. An essential role of a non-essential amino acid: brain development depends on the entry of serine through the blood-brain barrier

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L-Serine is a non-essential amino acid synthesized in the brain from glucose via the "phosphorylated" pathway. A defect in any of the three enzymes that synthesize L-serine results in L-serine deficiency and causes congenital microcephaly, seizures, and severe psychomotor retardation. Although dietary L-serine intake is not thought to be required for brain function and development, there are several potential serine transporters in the blood-brain barrier (BBB). One of them, Slc38a5 (SN2 or SNAT5), is a Na⁺-coupled transporter that belongs to system N and is generally thought to physiologically transport L-glutamine. We now found that Slc38a5 uses L-serine more efficiently than L-glutamine both *in vitro* and *in vivo*. To investigate the contribution of exogenous L-serine to brain development, we generated Slc38a5-KO mice. We found that the brain Slc38a5 transporter is highly and exclusively expressed in the endothelial cells of the BBB during the first two weeks of mice postnatal development and subsequently decreases in adulthood. HPLC analysis of brain amino acids revealed reduced levels of L- and D-serine in PND5 and 11 mice, but not of L-glutamine. These changes were attenuated in older mice. We found that Slc38a5-KO mice exhibit lower brain weight, and brain MRI analysis shows microcephaly. L-Serine deficiency caused the accumulation of abnormal sphingolipids, impaired neurogenesis in the dentate gyrus, and decreased the size of postsynaptic densities. Young Slc38a5-KO mice showed decreased import of L-serine across the BBB and significant behavioral impairments, such as abnormal ultrasonic vocalization and motor dysfunction. In addition, administration of L-serine to dams during pregnancy and lactation prevented the behavioral abnormalities. Although L-serine is

synthesized endogenously throughout life, our data suggest that the import of L-serine across the BBB by Slc38a5 is essential for brain development.

58. Evaluating drug transport heterogeneity using multimodal molecular tissue imaging

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Cancer heterogeneity represents one of the greatest challenges to address in the era of precision medicine. Intra-tumour heterogeneity is defined as the presence of multiple sub-clonal populations with varied molecular makeup within the tumour. This transcriptional diversity in the bulk population can modulate drug efficacy and fuel the development of drug resistance when applying treatment-specific selective pressure.

Transmembrane transporter expression patterns are one factor that might play a significant role in drug efficacy. SLC-mediated drug uptake has been previously described for a number of compounds (e.g. gemcitabine [ENT1], YM155 [SLC35F2], 3-bromopyruvate [MCT1], MOG [MCT2]). Drug efflux via certain ABC transporters can also affect drug intracellular concentration. For example, MDR1 has been linked to resistance to paclitaxel and platinum as well as the Poly (ADP Ribose) Polymerase (PARP) inhibitor Olaparib in ovarian cancer (OVCA). Alongside vascularity, the heterogeneous expression of transporter proteins could determine drug distribution spatially within the tumour, giving rise to a disparate treatment response. It is therefore imperative to understand which transporters play a role in drug uptake in order to predict their impact on both efficacy and off-target or toxic effects.

We propose multimodal molecular tissue imaging (MMTI) of patient-derived OVCA explants as a novel approach to study the relationship between transporter expression, drug distribution, and efficacy. Atmospheric-Pressure Matrix-Assisted Laser-Desorption Ionisation (AP-MALDI) Mass Spectrometry Imaging (MSI) has been implemented for the detection of PARP inhibitor drugs in surgical explants that have been dosed *ex vivo*. MSI-measured drug uptake can be correlated to markers of drug efficacy and transporter expression through immunohistochemical analysis. Moreover, the use of spatially resolved transcriptomics might allow for the identification of novel drug-transporters, potentially providing biomarkers of toxicity and sensitivity to treatment in the context of personalised medicine.

59. Sonidegib acts as a pharmacokinetic resistance modulator in patient-derived NSCLC explants *ex vivo*

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Sonidegib (trade name Odomzo) is a novel medication approved for the therapy of basal cell carcinoma. Next to its primary indication, it has been investigated as a potential treatment for non-small cell lung cancer (NSCLC). In our previous study, we found that this Hedgehog pathway inhibitor potently inhibits ABCB1 and ABCG2 transporters and sensitizes cancer cells to conventional chemotherapeutics *in vitro*. The present study aimed to verify the possible clinical value of these preceding *in vitro* observations in four patient-derived non-small cell lung cancer (NSCLC) explants. In the initial step, primary cultures have been established from NSCLC biopsies excised by the pathologist immediately after lobectomy. In the western blotting studies, ABCB1 and ABCG2 expressions have been detected in these explants showing substantial interindividual variability. Subsequently, accumulation and drug combination assays were performed. Importantly, we found an association between ABCB1/ABCG2 levels and outcomes of accumulation/combo experiments. In sample with low transporters' expression levels, sonidegib caused insignificant changes in the accumulation of model cytostatic substrates and their combination effects were mainly defined as antagonism or additivity. In contrast, accumulation of daunorubicin and mitoxantrone was significantly increased following sonidegib treatment in remaining three samples with higher expression of ABCB1/ABCG2. Moreover, synergism was the predominant drug combination effect observed in these primary NSCLC explants. In conclusion, our data introduced sonidegib as an effective ABCB1- and ABCG2-targeting MDR modulator *ex vivo*. We believe that our findings might be useful as a foundation for future *in vivo* research evaluating sonidegib as a part of the combination therapy of Hedgehog pathway/ABCB1/ABCG2 co-expressing tumors.

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60. Discovery and Pharmacological Characterization of a High-Affinity Allosteric Inhibitor of the Serotonin Transporter

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Neurotransmitter: Sodium Symporters (NSS) are a family of transporters responsible for Na⁺ dependent reuptake of neurotransmitters in the synaptic cleft. An important member of the NSS family is the serotonin transporter (SERT), as this is one of the most drugged transport proteins in the human body. Tricyclic antidepressants (TCA) and selective serotonin reuptake inhibitors (SSRI) are two classes of SERT inhibitors prescribed for common mood disorders such as depression, anxiety, obsessive-compulsive disorders and post-traumatic stress disorders. Both classes work by targeting the orthosteric site (S1) in SERT. Unfortunately, the use of these inhibitors is not without side effects.

Almost four decades ago the allosteric site (S2) in SERT was proposed, but it was not until 2020 that a high-affinity ligand, Lu AF60097, was discovered for this site. Unfortunately, Lu AF60097 will never become a drug due to a high distribution volume. In this study we therefore search for a druggable allosteric SERT inhibitor with a distinct pharmacological profile. By inhibiting dissociation of a radioligand bound in the S1 site, we screen eight analogue compounds provided by Lundbeck A/S. We here find one compound, Lu AF88273, to show high allosteric potency in SERT. By performing uptake experiments we suggest Lu AF88273

to inhibit SERT in a non-competitive way. Binding experiments prove Lu AF88273 to displace [³H]S-citalopram and [³H]imipramine in a dose-response manner. Whether this is by binding to the S1, S2 or a combination of both sites remains inconclusive. Upon molecular dynamics simulations we observe residue Glu493 and Phe556 to be important for Lu AF88273 S2 binding when imipramine is bound in the S1 site. By performing a small structure activity relationship study, we suggest the unsubstituted indole in Lu AF88273 to be important for binding, this correlating well with the simulation.

61. The dopamine transporter antiports potassium to increase the uptake of dopamine

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The dopamine transporter (DAT) facilitates dopamine reuptake from the extracellular space to terminate neurotransmission. Pharmacological compounds such as amphetamine and cocaine can modify its uptake. DAT belongs to the neurotransmitter:sodium symporter(NSS)/the solute carrier 6 transporter family, which includes transporters for serotonin, norepinephrine, and GABA that utilize the Na⁺ gradient to drive the uptake of substrate via binding to Na⁺- and ligand binding sites. Decades ago, it was shown that the serotonin transporter also antiports K⁺, but investigations of K⁺-coupled transport in other NSSs have been sparse. Here, we show that Na⁺-dependent ligand binding to the *Drosophila*(d)- and human DAT is inhibited by K⁺. We also show that the conformational dynamics of dDAT in K⁺ are divergent from both the apo- and Na⁺-bound states by hydrogen-deuterium exchange coupled to mass spectrometry. Furthermore, we reconstitute active dDAT into liposomes and find that intra-proteoliposomal K⁺ increases the dopamine uptake capacity and maximum uptake velocity. We also reconstitute dDAT into liposomes containing fluorescent Na⁺- or K⁺ indicators and thereby visualize Na⁺ and K⁺ fluxes in single proteoliposomes. We find that the rates of K⁺ efflux are increased under dopamine uptake conditions and the rates of Na⁺ influx are increased by the combination of dopamine and intra-proteoliposomal K⁺. We suggest that K⁺ can bind to and be antiported by DAT, and that this increases the overall rate of the dopamine transport cycle. Our results expand on the fundamentals of dopamine transport and prompt a reevaluation of the impact of K⁺ on other transporters in this pharmacologically important family.

62. Trafficking, localization and interaction partners of Na⁺/HCO₃⁻ cotransporter NBCn1

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Finely tuned regulation of acid-base transporter localization is vital for cell and tissue pH homeostasis. Sodium-bicarbonate co-transporter NBCn1 (SLC4A7) is a key contributor to pH homeostasis, yet little is known about the regulation of its localization.

We previously showed that the scaffold protein Rack1 is required for NBCn1 plasma membrane localization through interaction with the proximal NBCn1 C-terminal¹. Here, we investigated which other mechanisms control NBCn1 localization, and mapped its subcellular localization. We show that N-terminal residues 98-148, part of a predicted N-terminal β -sheet, and a short α -helical domain of the C-terminal (1133-39) are all required for plasma membrane insertion of NBCn1 in epithelial cells. GST-pulldown, native co-immunoprecipitation and proximity ligation analyses (PLA) revealed interaction of NBCn1 with cell-cell adhesion- and polarity complex proteins, including α -catenin, claudin-3, occludin, DLG1 and LLGL1. In congruence with this, EGTA-induced disruption of cell-cell contacts abolished NBCn1 plasma membrane localization.

Our C-terminal pulldown indicated robust interaction of NBCn1 with Rho GTPases (confirmed by PLA), Arp2/3 proteins and VASP. Accordingly, NBCn1 localized highly specifically to lamellipodia and filopodial tips of migrating epithelial cells and primary cortical neurons.

Finally, using multiple lines of evidence in live and fixed cells, we found that NBCn1 localizes to the centrosome and primary cilium in non-dividing, polarized epithelial cells, and to the mitotic spindle, centrosome and midbodies in mitotic cells. Supporting this, NBCn1 was enriched in isolated spindle- and centrosomal fractions. We speculate that a yetto-be-identified function of NBCn1 in these structures may contribute to its role in cell cycle progression².

In conclusion, we mapped new molecular determinants of NBCn1 membrane localization in its N- and C-terminal regions and identified its unexpected localization in subcellular structures associated with cell cycle control. We present a model in which NBCn1 may be trafficked to centrosomes and mitotic spindle through Rab11 recycling vesicles.

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63. C@PA: Computer-aided Pattern Analysis for the Exploration of Undiscovered Polypharmacological Space in Novel Drug Discovery

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Computational approaches are useful tools to discover bioactive agents to address pharmacological targets of physiological and pathological relevance. By using the vastness of chemical space, optimal filter techniques applying molecular descriptors inhere invaluable potential for the virtual screening of potentially very potent and effective drug candidates. However, the pharmacological space is covered by 'black-spots' of pharmacological targets that cannot be addressed by small-molecule modulators – hindering the targeted development of the drugs of the future. The exploration and exploitation of these so-called 'under-studied' pharmacological targets are the major obstacles in medicinal chemistry.

The proposed design and development of polypharmacological agents provide a high potential to address under-studied pharmacological targets, and hence, explore these as pharmacological drug targets of the future. Conserved substructural features such as (non-)aromatic poly(hetero)cycles and functional groups have very recently been identified as superior molecular descriptors as demonstrated by the novel drug discovery tool 'Computer-aided Pattern Analysis' ('C@PA').¹

Using the well-established model protein family of ABC transporters, a binary pattern distribution scheme of substructural elements of statistical significance was established for a multitarget dataset correlating the entire bioactivity space of compounds addressing the wellstudied ABC transporters ABCB1, ABCC1, and ABCG2.² By classification according to the individual pharmacological profile of the compounds (inactivity, selective, dual, and triple inhibition), substructural components were identified that impede or promote multitargeting of ABC transporters.¹ These substructural components were used as molecular descriptors in a virtual screening approach, which was able to predict potent multitarget ABC transporter inhibitors by an outstanding 21.7%.¹ The extension of this computational model to overcome the molecular structure and bioactivity limitations set by the statistical thresholds increased the biological hit rate to 40.0%.^{3,4} These discoveries opened up a new perspective in the development of structurally truly novel bioactive agents addressing a yet completely uncharted territory of pharmacological space.

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64. Disruptive mutations in the serotonin transporter associate serotonin dysfunction with treatment-resistant affective disorder

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Background

Affective or mood disorders are a leading cause of disability worldwide. The serotonergic system has been heavily implicated in the complex etiology and serves as a therapeutic target. The serotonin transporter (SERT) is a major regulator of serotonin neurotransmission, but the disease-relevance of genetically impaired SERT function is unknown.

Methods

We obtained sequencing data for coding SERT variants from a cohort of 144 patients with treatment-resistant chronic affective disorders with a lifetime history of electroconvulsive therapy. Association analysis was employed to compare the allele frequency of coding SERT variants in the cohort, with that in the Genome Aggregation Database (GnomAD) and an ethnicity-matched healthy control population (N = 4554). To uncover potential disease-relevant mechanisms, we used uptake experiments, western blotting and fluorescence imaging to investigate the molecular phenotypes of disease-associated coding SERT variants.

Results

We identified two previously uncharacterized SERT coding variants, SERT-N217S and SERT-A500T, in three unrelated patients. Both variants were enriched in the patient cohort compared to GnomAD (SERT-N217S: OR = 151, $P = 0.0001$ and SERT-A500T: OR = 1348, $P = 0.0022$) and ethnicity-matched healthy controls (SERT-N217S: OR ≥ 17.7 , $P \leq 0.013$ and SERT-A500T: OR = ∞ , $P = 0.029$). Functional investigations revealed that the mutations exert distinct perturbations to SERT function, but their overall effects converge on a partial loss-of-function molecular phenotype. Thus, the SERT-A500T variant compromises the catalytic activity, while SERT-N217S disrupts proper glycosylation of SERT with a resulting dominant-negative trafficking deficiency. Moreover, we demonstrate that the trafficking deficiency of SERT-N217S is amenable to pharmacochaperoning by noribogaine.

Conclusion

We have identified and functionally characterized the first disease-associated SERT loss-of-function variants. The findings support SERT dysfunction as a risk factor for chronic affective disorders.

65. Cryo-EM structures of the caspase-activated protein XKR9 involved in apoptotic lipid scrambling

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The exposure of the negatively charged lipid phosphatidylserine on the cell surface, catalyzed by lipid scramblases, is an important signal for the clearance of apoptotic cells by macrophages. The protein XKR9 is a member of a conserved family that has been associated with apoptotic lipid scrambling. Here, we describe structures of full-length and caspase-treated XKR9 from *Rattus norvegicus* in complex with a synthetic nanobody determined by cryo-EM. The 43 kDa monomeric membrane protein can be divided into two structurally related repeats, each containing four membrane-spanning segments and a helix that is partly inserted into the lipid bilayer. In the full-length protein, the C-terminus interacts with a hydrophobic pocket located at the intracellular side acting as an inhibitor of protein function. Cleavage by caspase-3 at a specific site releases 16 residues of the C-terminus, thus making the pocket accessible to the cytoplasm. Collectively, the work has revealed the unknown architecture of the XKR family and has provided initial insight into its activation by caspases.

66. Membrane fluidity is critical for sugar GLUT transport

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Glucose (GLUT) transporters are essential for organism-wide glucose homeostasis in mammals. Due to their critical role in cellular growth and metabolism, GLUT dysfunction is associated with numerous diseases, such as diabetes and cancer. Structures of GLUT proteins and related proteins have enabled the construction of a detailed transport cycle. Despite these structural advances, transport assays using purified GLUT components have proven to be difficult to implement, hampering deeper mechanistic studies. Here, we have optimized a transport assay in liposomes for the fructose-specific isoform *rat* GLUT5. We demonstrate the importance of measuring sugar uptake with different crude-lipid compositions, and how inaccuracies can be made when analyzing the impact of mutations and inhibitors with suboptimal GLUT5 activity. By combining lipidomic analysis with native MS and thermal-shift assays, we replicate the GLUT5 transport activities seen in crude lipids with a small number of synthetic lipids. Subsequently, we show how GLUT5 is only active under a narrow-range of membrane fluidity, and that *human* GLUT1-4 prefers a similar lipid composition. Overall, our study provides a much-needed transport assay for analyzing GLUT activities *in vitro*, and illuminates why increased free fatty acids, as found in those suffering from metabolic disorders, impair GLUT transport.

67. Structural and Dynamical Investigation of the Multidrug Resistance-Associated Protein 1 (MRP1) in Different Lipid Bilayers by Means of Molecular Dynamics

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Xenobiotic membrane crossing events are essential in pharmacological processes, especially in pharmacokinetics (*i.e.*, Absorption, Distribution, Metabolism, and Elimination). Membrane transporters located in liver and kidneys are particularly relevant since these organs are involved in the metabolism and elimination of most of the xenobiotics. ATP-binding cassette transporters including C-family multidrug-resistance associated proteins (ABCC/MRPs) have been stressed out as "emerging clinical importance" by the pharmacologist community. Owing to the absence of resolved human MRP structures, the cryo-EM resolved bovine MRP1 (*bMRP1*) structures might be used as a prototype for ABCC family, especially because several conformations were resolved along the transport cycle. However, the interplay between lipid bilayer membrane and protein dynamics remains unclear.

μ s-Scale molecular dynamics (MD) simulations were performed considering the different conformations of *bMRP1* in different lipid bilayers with different ratios of POPC, POPE and cholesterol. Different bound states were also investigated, considering, ATP-, substrate- and ATP/substrate-bound states for inward facing conformation as well as ATP-bound and ADP-bound states for the outward-facing conformation. The timescale of the MRP1 transport cycle is far beyond the range of unbiased MD simulations. Therefore, several flavours of biased MD techniques were considered (*e.g.*, steered MD and metadynamics) using machine learning based collective variable to address this challenge.

Dynamic investigations of structural descriptors reveal relatively weak dependence on lipid bilayer membrane composition. However, cholesterol binding hot-spots were observed

suggesting an important role for *bMRP1* function. Regarding the inherent protein dynamics, MD simulations suggests that wide open IFapo conformation is very unlikely given the systematic spontaneous closing of nucleotide-binding domains regardless of the boundstate. This is in line with the literature for NBD degenerated ABC transporters. Internal structural variabilities of *bMRP1* domains is significantly modified upon ATP and/or substrate binding. Key words: molecular dynamics, ABCC family, MRP1

68. Insights on the conformational dynamics of three LeuT cysteine mutants using pulsed EPR spectroscopy

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LeuT (Leucine Transporter) is a small amino-acid transporter from the hyperthermophilic bacterium *Aquifex aeolicus* and a well-studied bacterial homolog of neurotransmitter:sodium symporters (NSS), especially the solute carrier 6 (SLC6) family. Within the nervous system, SLC6 transporters play a vital role in the termination of synaptic transmission and their dysfunction leads to severe neurological conditions rendering them key pharmacological targets.

LeuT was also the first homolog of the SLC6 transporters to be crystallised and remains the main reference transporter for creating models of the transport cycle of eukaryotic NSS transporters¹. Large conformational changes have been proposed previously^{2,3} which we aim to probe using a combination of site-directed spin labelling (SDSL) and advanced electron paramagnetic resonance (EPR) spectroscopy.

In the EPR work presented here the data analysis indicates statistically significant distance distribution profiles that provide dynamic insights on three of the main conformational states of LeuT observed previously using structural biology methods which are also consistent with the existing functional models in the literature³. As an aside we have also observed evidence for the presence of dimeric LeuT complexes in detergent micelles. Although these results reveal a robust link between expected conformational changes derived from the proposed models and the coarse-grained EPR distance information derived from coupled exogenous spin labels, further experiments must be undertaken in more native lipid environments before conclusions can be drawn on the potential physiological relevance.

We also present preliminary studies using divalent metal ions as exogenous spin probes where expected distances (and distributions) should be more accurate than those achieved when using an exogenous (and floppy) nitroxide spin label. In addition, the combination of such engineered divalent metal ion coordination sites together with cysteine variants will provide further opportunities to triangulate distances and conformational changes during the dynamic transport cycle itself.

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69. Spectral shift technology to measure transporter-ligand interaction.

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The techniques for analysing transporter-ligand interactions provide insights into the functioning of transporter proteins, help in drug screening against diseases of the CNS and enhance our understanding of the underlying pathological processes. Also, these methods can help find conditions for protein purification, Cryo-EM and X-ray experiments, thereby giving structural information about transporter proteins. Scintillation proximity assay (SPA) is the most common technique for assessing ligand binding in neurotransmitter transporter research. When a radioligand binds bead-immobilized proteins, SPA beads emit light. Ideally, in drug screenings, a potential ligand displaces the radioligand and causes a decrease in detected radioactivity. However, if the potential ligand has an alternative binding mode, it may result in low radioligand displacement, even with high-affinity binding. Also, the beads can interact with the radioligands, resulting in high background signal.

Here, we present a spectral shift-based method to measure thermodynamic parameters of the transporter-ligand interactions. In these experiments, we can detect slight shifts in the fluorescent peak positions of a labelled protein when it binds to a ligand. We measured dissociation constants of LeuT (bacterial leucine transporter) interactions with its ligands and ions, enthalpy and entropy of the binding, and screened for potential detergents we can use with LeuT. The method provides a high signal-to-noise ratio, dissociation constants similar to those previously reported, demands low amounts of the protein and is fast to perform.

70. Structures and mechanism of the plant PIN-FORMED auxin transporter.

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Auxins are an essential class of hormones that orchestrates a manifold of plant growth and development processes. Most auxin effects are triggered by an auxin gradient across the plant organ, created by polar auxin transport. The PIN-FORMED (PIN) protein family is the heart of this process generating auxin export from the cytosol to the extracellular space. For many decades, the lack of structural and biochemical data has impeded a detailed comprehension of the molecular mechanism behind PIN-mediated auxin transport. Here we present biophysical analysis and three structures of *Arabidopsis thaliana* PIN8 at 2.9-3.4 Å resolution; two outward facing conformations with and without auxin bound, and one inward facing conformation with the known inhibitor and herbicide naphthylphthalamic acid (NPA) bound. Our results provide the first comprehensive molecular model for auxin recognition and transport by PINs, and explains a central mechanism of polar auxin transport, a core feature of plant physiology, growth and development.

71. A Fluorescence-based Competitive Counterflow Assay to Distinguish Between Substrates and Inhibitors of Multispecific Organic Anion Transporting Polypeptides

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Organic anion transporting polypeptides (OATPs) encoded by the SLCO genes are transmembrane proteins expressed in the epithelial and endothelial cells of the human body. OATPs mediate the sodium- and ATP-independent cellular uptake of large (> 300 Da), negatively charged or amphipathic organic molecules. Multispecific OATPs (OATP1A2, OATP1B1, OATP1B3 and OATP2B1) - besides their physiological substrates (steroid and thyroid hormones, bile acids, prostaglandins and bilirubin) – also recognize various kinds of drugs, food components and toxins. Owing to their key role in the hepatic clearance of their drug substrates, investigation of the interaction with OATP1B1 and OATP1B3 during drug development is recommended by international regulatory agencies (EMA, FDA, and PMDA). In addition, investigation of the interaction with OATP1A2 and OATP2B1, influencing intestinal, hepatic or central nervous system uptake of drugs may also be justified. Recently, a novel assay termed as Competitive Counterflow has been developed. The method is based on the exchanger function of OATPs and is able to distinguish between transported substrates and inhibitors. In our work, we have developed fluorescence-based competitive counterflow assays for OATPs, 1A2, 1B1, 1B3 and 2B1. With these assays, we provide simpler methods to investigate the nature of the interaction between new molecular entities and multispecific OATPs.

72. The targeted metabolome of the solute carrier family

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RESOLUTE, a public-private partnership funded by the Innovative Medicine Initiative of the European Union and the European Federation of Pharmaceutical Industries and Associations, aims to advance knowledge and research on solute carrier transporters (SLCs) by generating reagents and tools as well as performing diverse functional analyses for the family of 446 proteins. The SLC family remains understudied both on the level of individual members and their functional relationships, with a third of them having unknown substrates. Using a targeted metabolomics approach covering 200 metabolites, we analyzed cell lines with inducible overexpression of individual human SLCs either in wildtype or cognate knock-out cell lines. To date we have acquired metabolic profiles for more than 300 human SLCs and observe statistically significant changes of metabolites covering a diverse set of metabolic pathways for roughly half of the SLCs. While we observe the accumulation of known substrates for some SLCs, we mainly investigate downstream metabolic consequences of SLC expression. Similarity clustering of metabolic signatures indicates functional relationships between SLCs and contributes to their deorphanization. Following the open access ethos of the RESOLUTE

consortium, targeted metabolomics data sets for at least 80% of the SLC family members will be publicly released towards the end of the year.

Keywords: solute carrier, SLC, transporter, metabolomics

73. An open-access collection of reagents and datasets for SLC research

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The solute carrier (SLC) superfamily consists of more than 450 proteins that mediate transmembrane transport of a wide variety of substrates. Despite their frequent association with disease and importance as drug transporters and drug targets, a large proportion of the SLC family remains poorly characterized. The *Research Empowerment on Solute Carriers* (RESOLUTE) consortium (<https://re-solute.eu>) set out to study the SLC superfamily in a systematic and holistic approach to fill the knowledge gaps and provide reagents, data and protocols that will aid further research to unlock the full therapeutic potential of these transporters. Funded by the *Innovative Medicines Initiative* (IMI), EU and *European Federation of Pharmaceutical Industries and Associations* (EFPIA), one of our main goals is to make our reagents and datasets available to the scientific community at the best possible conditions. Among others, we generated over 3000 plasmid vectors for SLC overexpression and CRIPSR/Cas9 KO, 600 isogenic HEK293 cell lines conditionally expressing epitope tagged SLCs, 1000 monoclonal SLC KO cell lines for 315 SLCs, and 450 KO clones in which corresponding SLC transgenes were introduced to rescue SLC expression in an inducible manner. Some of these reagents are already available at public repositories, others can be requested directly from the consortium. Using this toolset, we generated comprehensive datasets such as transcriptomics, intracellular localization, protein-protein interactions, metabolomics, Ionomics and genetic interaction maps for the majority of SLCs. Moreover, we established functional assays for about 70 SLCs using our cell models and generated high-affinity binders for a small but increasing number of targets. Here, we want to provide an overview of the reagents, datasets and assays we created and highlight a few examples of insightful data that demonstrate the potential of our SLC toolset.

74. Individual charge translocations upon GABA, Na⁺ and Cl⁻ binding to human GAT-1 indicate conformational flexibility

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Charge translocation across the cell membrane generate currents that can be studied for the functional characterization of a transporter. Solid-supported membrane electrophysiology (SSME) utilizes membrane vesicles to measure electrogenic steps within the reaction cycle, triggered by substrates, at 0 mV. The solution exchange with a time resolution of up to 3 ms allows detection of pre-steady state (PSS) currents.

Here we present a SSME-based study on the human Na⁺/Cl⁻/γ-aminobutyric acid (GABA) co-transporter GAT-1, that reveals individual electrogenic events triggered by substrate binding. The well-known Na⁺-induced PSS current shows a time constant of 13 ms, and an EC₅₀ of 32

mM, in good agreement with the estimated $\text{Na}^+ K_d$ of 20 mM, concluding that this electrogenic event may be directly associated with Na^+ binding. We also detected PSS currents that have not been observed before with conventional electrophysiology. One is induced by GABA, but only in presence of Na^+ , consistent with current models in literature that assume Na^+ binding generates a state with high affinity for GABA. This PSS current EC_{50} is 65 μM , in good agreement with the estimated K_d for GABA of 40-100 μM , indicating that this PSS current is directly associated with GABA binding. Finally, we detected another PSS current induced by Cl^- . The Cl^- dependence of this current is well described by a double Michaelis-Menten plot, with a first EC_{50} of 9 mM and a second EC_{50} of 215 mM. The PSS currents induced by Na^+ and Cl^- are independent from the presence of other substrates, thus suggesting a random binding order for these two substrates in the transport cycle. The presence of three distinct substrate-induced electrogenic events can be the result of substrate binding or conformational rearrangements, such as alternating access or local conformational transitions, thus potentially indicating a high conformational flexibility of GAT-1.

75. Sonidegib sensitizes cancer cells to cytotoxic agents by inhibiting drug efflux functions of ABCB1 and ABCG2 in vitro

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The functional overexpression of ATP-binding cassette (ABC) transporters is considered one of the essential mechanisms causing multidrug resistance (MDR) in cancer cells. A novel Hedgehog pathway inhibitor, sonidegib, was approved for the therapy of basal cell carcinoma and has been clinically evaluated for the treatment of several types of solid tumors, including non-small cell lung cancer (NSCLC). In this study, we aimed to investigate the potential of sonidegib to act as a chemosensitizer through its inhibitory effects on human ABC transporters. First, we found that sonidegib can effectively decrease the accumulation of both cytostatic and non-cytostatic probe substrates of ABCB1 and ABCG2 in MDCKII cells. Second, our drug combination MTT assays revealed that sonidegib is able to synergistically enhance the cytotoxicity of daunorubicin and mitoxantrone in ABCB1- and ABCG2-overexpressing MDCKII/A431 cells, respectively. Furthermore, sonidegib did not alter the mRNA levels of *ABCB1*, *ABCG2* and *ABCC1* in various NSCLC cell lines. Finally, we also demonstrated that overexpression of ABCB1, ABCG2 and ABCC1 did not establish the drug resistance to sonidegib. To sum up, sonidegib was characterized as a dual ABCB1/ABCG2 inhibitor with potential MDR-reversal properties. Importantly, MDR-combating potential of examined drug was not compromised by strengthening of MDR phenotype or efflux. Our findings can serve as a valuable basis for future preclinical/clinical studies evaluating the sonidegib's efficacy in the combined treatment of ABCB1/ABCG2 overexpressing tumors. *The study was supported by the Czech Science Foundation (grant No. 20-20414Y).*

76. Probing the substrate-to-protein proton transfer mechanism of the serotonin transporter

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The neurotransmitter serotonin (5-HT) has been linked to a plethora of regulatory functions, including gut homeostasis, cognition, mood, and sleep. While present in the synaptic cleft, 5-HT transfers the neuronal signal by binding to the receptors on the postsynaptic membrane. The termination of 5-HT signalling is carried out by a serotonin transporter (SERT), a transmembrane protein which uptakes the 5-HT from a synaptic cleft into a presynaptic neuron against its concentration gradient and by utilising sodium ion symport. To satisfy the condition of an electroneutral transport cycle, the uptake of positively charged 5-HT and Na⁺ needs to be counteracted by either symport of negatively charged ions, antiport of positively charged particles, or by transporting 5-HT in its neutral form. We tested a hypothesis by Rudnick and Sandtner in which a proposed mechanism of electroneutral transport relies on a symport of Na⁺ and uncharged 5-HT coupled with K⁺ antiport. Under this premise, a proton from an amino group of 5-HT is transferred to Asp98 in the central binding site of SERT, thus rendering both 5-HT and Asp98 neutral. We performed molecular dynamics simulations of SERT in inward occluded and inward open conformations with 5-HT and Asp98 in both charged and uncharged states, reflecting the proposed mechanism of substrate-to-protein proton transfer. We observed enhanced 5-HT unbinding from the central site under the conditions of neutral 5-HT and Asp98. Additionally, we tested the feasibility of the proton transfer by using alchemical free energy calculations for the outward open and inward open conformations with differing starting protonation states. Although the calculations show inconclusive results regarding the proton dependent mechanism of 5-HT transfer, we report a careful approach to hypothesis testing by utilising unbiased simulation methods paired with more detailed assessment of energetic terms that presumably dictate the mechanism of transfer.