# 47TH SANDBJERG MEETING ON MEMBRANE TRANSPORT

**Monday May 18th - Wednesday May 20th 2015**

**Sandbjerg Estate**
**Sønderborg**
**Denmark**

# Scientific program

**MONDAY MAY 18TH**

**13.00-13.40 Arrival and coffee**

**13.40-13.45 Welcome and introductory remarks**

## SESSION 1

### $NA^+/K^+$ ATPASE ISOFORMS IN HEALTH AND DISEASE

*Coordinator: Karin Lykke-Hartmann and Bente Vilsen, Biomedicine, Aarhus, Denmark*

**13.45-14.30 Steven Karlish**, Weizmann Institute, Dept. of Biological Chemistry, Rhehovot, Israel
*Development of isoform selective cardiac glycosides as agents to reduce intraocular pressure*

**14.30-15.00 Jan Koenderink**, Radboud University Medical Center, Nijmegen, Holland
*$Na^+,K^+$-ATPase isoforms in disease*

**15.00-15.30 Rikke Holm**, Aarhus University, Biomedicine, Physiology, Denmark
*Rescue of $Na^+$ affinity in aspartate-928 mutants of $Na^+,K^+$-ATPase by secondary mutation of glutamate-314*

**15.30-16.00 Coffee**

**16:00-16.30 Steven Clapcote**, Faculty of Biological Sciences, Leeds University, Leeds, UK
*Characterization of cognitive deficits in mice with an alternating hemiplegia of childhood-linked mutation*

**16.30-17.00 Thomas Holm**, Aarhus University, Biomedicine, Biochemistry, Aarhus, Denmark
*A mouse model for Alternating Hemiplegia in Childhood*

**17.00-17.30 Karin Lykke-Hartmann**, Aarhus University, Biomedicine, Biochemistry, Denmark
*Translational studies of sodium pump knock-in mouse models*

**17.30-18.00 Vladimir Matchkov**, Aarhus University, Biomedicine, Physiology, Denmark
*The α2 isoform of $Na^+,K^+$-ATPase modulates vascular tone via activation of Src kinase (Src) signaling pathway: a lesson from mouse model for Familial Hemiplegic Migraine Type 2 (FHM2)*

**18.00-19.00 Dinner**

**Evening free**

**22.00 Midnight snack (Natmad)**

**TUESDAY MAY 19TH**

## SESSION 2:

### SENSING THROUGH CHANNELS

*Coordinator: Helle Praetorius, Biomedicine, Aarhus, Denmark*

**9.00-9.30** **Kate Poole,** Department of Neuroscience, Max-Delbrück Center for Molecular Medicine, Berlin, Germany
*Tuning Piezo ion channels to detect molecular-scale movements relevant for fine touch*

**9.30-10.00** **Stuart Johnson,** Department of Biomedical Science, University of Sheffield, UK Calcium entry into stereocilia drives adaptation of the mechanoelectrical transducer current of mammalian cochlear hair cells

**10.00-10.30** **Coffee**

**10.30-11.00** **Sarah Falk,** Department of Drug Design and Pharmacology, University of Copenhagen, Denmark
Pain without Nociceptors? Nav1.7-Independent Pain Mechanisms

**11.00.11.30** **Peter Zygmunt,** Clinical Chemistry and Pharmacology, Department of Laboratory Medicine, Lund University, Sweden
Human TRPA1 is intrinsically cold- and chemosensitive with and without its N-terminal ankyrin repeat domain

**11.30-12.00** **Arend Vogt,** Humboldt University zu Berlin, Biology, Germany
*The diversity of light-driven proton pumps and their conversion into proton channels*

**12.00 - 13.00** **Lunch**

## SESSION 3:

### STRUCTURE AND FUNCTION OF MEMBRANE RECEPTORS

*Coordinator: Christian Brix Folsted Andersen, Aarhus, Denmark*

**13.30-14.00** **Kristian Stødkilde-Jørgensen,** Aarhus University, Biomedicine, Biochemistry, Denmark
*Hemoglobin Piracy by Trypanosome parasites*

**14.00-14.30** **Jan Terje Andersen**, Oslo University Hospital, Centre for Immune Regulation and Depart. of Immunology, Norway

*The role of albumin receptors in regulation of albumin homeostasis: implications for drug delivery*

**14.30-15.00** **Gregers Rom Andersen**, Aarhus University, Department of Molecular Biology and Genetics, Denmark
*The structure of the RAGE:S100A6 complex reveals a new mode of homodimerization for S100 proteins*

**15.00-15.30** **Coffee**

**15.30-16.00** **Mette Madsen**, Aarhus University, Biomedicine, Biochemistry, Denmark
*The membrane receptor megalin modulates proliferation and survival rates in melanoma cells – could it be a novel biomarker and therapeutic target in melanoma cancer?*

**16.00-16.30** **Tina Storm,** Aarhus University, Biomedicine, Anatomy Denmark
*Megalin in ocular health and disease*

**16.30–17.00** **Free time**

**17.00-18.00** **Keynote speaker:**

**Prof. Christoph Korbmacher**, Friedrich Alexander University Erlangen Nürnberg, Germany
*The epithelial $Na^+$ channel ENaC*

**18.00-19.00** **Dinner**

## SESSION 4:
## Posters and wine: 19.00 -21.00

*Poster contributions are listed alphabetically, by first author´s last name.*

1. **Renée Brekelmans**, Aarhus University, Biomedicine, Physiology

   *Does hydochlorothiazide acidify the urine?*

2. **Henriette Christensen**, Aarhus University, Biomedicine, Anatomy
   *Luminal acid/base transporting proteins in the choroid plexus epithelium*

3. **Steen Fagerberg**, Aarhus University, Biomedicine, Physiology
   *P2X receptor desensitization by high concentrations of ATP changes the survival rate of THP-1 attacked by RTX toxins Hlya and LTxA.*

4. **Anne-Sofie Greve Christensen**, Aarhus University, Biomedicine, Physiology

   *The effect of P2-receptor inhibition on the cytotoxic effects of α-haemolysin from E. coli – a murine sepsis model*

5. **Gitte Tindbæk Nielsen**, Aarhus University, Biomedicine, Biochemistry

   *Is megalin involved in regulating the apoptotic/anti-apoptotic apparatus of melanoma cells?*

6. **Jette Skov Alstrøm**, Copenhagen University, Institute for Cellular and Molecular Medicine

   *Isoform-specific phosphorylation-dependent regulation of connexin hemichannels*

7. ***Annette Buur Steffensen,*** Copenhagen University, Institute for Cellular and Molecular Medicine

   *Cotransporter-mediated cerebrospinal fluid formation*

8. ***Anna Thorsø Larsen,*** University of Copenhagen, Biology, Molecular Integrative Physiology

   *The purinergic P2X7receptor is involved in glucose-stimulated ATP release and ß-cell proliferation*

9. ***Mai-Britt Thomsen,*** Aarhus University, Biomedicine, Biochemistry

   *Does the cytosolic adaptor protein Dab2 assist melanoma megalin during endocytosis?*

*Please hang up your poster in the morning, and let it remain up all of Tuesday.*

**22.00- Natmad (Traditional late-night open sandwiches snack)**

## WEDNESDAY MAY 20TH

# SESSION 5:

### FREE COMMUNICATIONS IN MEMBRANE TRANSPORT

*Coordinators: Markus Bleich, Kiel, Germany and Ivana Novak, Copenhagen, Denmark*

**9.00-9.20** **Brian Roland Larsen**, University of Copenhagen, Institute for Cellular and Molecular Medicine, Denmark
*GIutamate-induced astrocytic $[Na^+]_i$ elevation – a mechanism to increase $K^+$ clearance via the $Na^+/K^+$-ATPase?*

**9.20-9.40** **Inga Christensen**, Aarhus University, Biomedicine, Anatomy, Denmark
*Choroid plexus epithelial cells are polarized normally - but contain unusually located proteins*

**9.40-10.00** **Annette Buur Steffensens**, University of Copenhagen, Institute for Cellular and Molecular Medicine, Denmark
*Mechanisms underlying spreading depolarization-induced dendritic beading*

**10.00-10.20** **Marco Tozzi**, University of Copenhagen, Biology, Molecular Integrative Physiology, Denmark
*Expression and Function of $H^+/K^+$-ATPases in Pancreatic Ductal Adenocarcinoma*

**10.20-10.40** **Coffee**

**10.40-11.00** **Åsa Jönsson**, Aarhus University, Biomedicine, Pharmacology, Molecular Integrative Physiology, Denmark
*Characterization and effect on phosphate transport of mutations in the human SLC34A2 gene in pulmonary alveolar microlithiasis*

**11.00-11.20** **Silke Härteis**, Friedrich Alexander University Erlangen Nürnberg, Germany
Prostate specific antigen (PSA)/Kallikrein related peptidase 3 (KLK3) regulates TRPV4 cation channel function by activating proteinase-activated receptor-2 (PAR2)

**11.20-11.40** **Mette Christensen**, Aarhus University, Biomedicine, Physiology, Denmark
*$[Ca^{2+}]_i$ oscillations and Il-6 release induced by α-haemolysin from Escherichia coli require P2 receptor activation in renal epithelia*

**11.40-12.00** **Casper K. Larsen**, Aarhus University, Biomedicine, Physiology, Denmark
*Reduced renal $K^+$ excretion with compensatory hyperaldosteronism in KCa1.1 channel ß2-subunit KO mice*

**12.00…** **Final remarks**

**12.10 - 13.00** **Lunch**

## Departure

### Sponsors

**We are very grateful for generous financial support for this year's Sandbjerg meeting from:**

- Aarhus Universitets Forskningsfond (AUFF)
- Aarhus University Graduate School of Health Sciences
- MEMBRANES

## Development of isoform selective cardiac glycosides as agents to reduce intraocular pressure

Steven J.D.Karlish
Weizmann Institute, Dept. of Biological Chemistry, Rhehovot, Israel

The ciliary epithelium in the eye consists of pigmented cells (PE), that express the α1ß1 isoform of Na,K-ATPase, and non-pigmented (NPE) cells that express mainly the α2ß3 isoform. In principle, a Na,K-ATPase inhibitor with selectivity for the α2ß3 isoform that penetrates the cornea, could effectively reduce intra-ocular pressure, with minimal systemic or local toxicity. We have recently synthesized a series of perhydro-1-4-oxazepine derivatives of digoxin (by HIO4 oxidation of the third digitoxose and reductive amination with various R-NH2 substituents) and showed that several derivatives have significant selectivity for human α2ß1 over α1ß1 isoform complexes (up to 8-fold). Furthermore, when applied topically, the most α2-selective derivatives effectively prevented or reversed pharmacologically raised intraocular pressure in rabbits (Katz etal J.Biol.Chem 289, 21153, 2014). A recent structure of Na,K-ATPase, with bound digoxin, shows the third digitoxose moiety in proximity to one residue in the ß1 subunit. As a test of the hypothesis that sugars of digoxin might interact with the ß subunit, we synthesized a new series of perhydro-1-4-oxazepine derivatives of digoxin with diverse substituents. Indeed several derivatives have enhanced selectivity for α2ß3 over α1ß1 (_33-fold in the most optimal case, respectively). When applied topically these derivatives potently reduce either pharmacologically raised or basal intraocular pressure in rabbits. The isoform-selective digoxin derivatives must effectively penetrate the cornea and inhibit α2ß3, so reducing aqueous humour production and intraocular pressure. The experiments demonstrate the central role of the NPE sodium pump in production of aqueous humour. The isoform selective digoxin derivatives may become interesting drug leads for treatment of ocular hypertension or glaucoma.

## $Na^+/K^+$-ATPase isoforms in disease

Jan B. Koenderink, Weigand KM, Swarts HGP
Radboud University Medical Center, Nijmegen, Holland

Sporadic and familial hemiplegic migraine type 2 are rare forms of hemiplegic migraine caused by mutations in the Na,K-ATPase α2 gene. Mutations in ATP1A3, the gene encoding the α3-subunit of Na,K-ATPase, are associated with the neurodevelopmental disorder Alternating Hemiplegia of Childhood and Rapid-onset Dystonia-Parkinsonism. Although knowledge about the affected genes is paramount, the next step in understanding the mechanism-of-disease of these mutations requires biochemical characterization of Na,K-ATPase containing these mutations. We have studied the functional consequences of many disease causing ATP1A2 and ATP1A3 mutations. We have looked at ATPase activity, phosphorylation, and ouabain binding, using purified membrane fractions from baculovirus-infected insect cells expressing the above-mentioned mutant enzymes. The tested mutants can be divided into different groups that might help understand how the variation in symptoms observed within patients is explained at the molecular level.

**Rescue of $Na^+$ Affinity in Aspartate-928 Mutants of $Na^+$,$K^+$-ATPase by Secondary Mutation of Glutamate-314**

Rikke Holm, Einholm AP, Andersen JP and Vilsen B
Aarhus University, Biomedicine, Physiology, Denmark

The $Na^+$,$K^+$-ATPase binds $Na^+$ at three transport sites denoted I, II, and III, of which site III is $Na^+$ specific and suggested to be the first occupied in the cooperative binding process activating phosphorylation from ATP. Here we demonstrate that the asparagine substitution of the aspartate associated with site III found in patients with rapid-onset dystonia parkinsonism or alternating hemiplegia of childhood causes a dramatic reduction of Na+ affinity in the α1-, α2-, and α3-isoforms of $Na^+$,$K^+$-ATPase, whereas other substitutions of this aspartate are much less disruptive. This is likely due to interference by the amide function of the asparagine side chain with $Na^+$ coordinating residues in site III. Remarkably, the $Na^+$ affinity of site III aspartate to asparagine and alanine mutants is rescued by second-site mutation of a glutamate in the extracellular part of the fourth transmembrane helix, distant to site III. This gain-of-function mutation works without recovery of the lost cooperativity and selectivity of $Na^+$ binding and does not affect the E1-E2 conformational equilibrium or the maximum phosphorylation rate. Hence, the rescue of $Na^+$ affinity is likely intrinsic to the $Na^+$ binding pocket, and the underlying mechanism could be a tightening of Na+ binding at $Na^+$ site II, possibly via movement of transmembrane helix four. The second-site mutation also improves $Na^+$,$K^+$-pump function in intact cells. Rescue of $Na^+$ affinity and $Na^+$ and $K^+$ transport by second-site mutation is unique in the history of $Na^+$,$K^+$-ATPase and points to new possibilities for treatment of neurological patients carrying $Na^+$,$K^+$-ATPase mutations.

**A mouse model for Alternating Hemiplegia in Childhood**

Thomas Holm, Isaksen TJ, Glerup S, Füchtbauer EM, Bøttger P, Heuck A, Nissen P, Lykke-Hartmann K
Aarhus University, Biomedicine, Biochemistry, Aarhus, Denmark

Specific mutations in the $Na^+/K^+$ ATPase α3 isoform cause the rare neurodevelopmental disease, Alternating Hemiplegia of Childhood (AHC). Children with AHC exhibit a wide range of neurological symptoms including hemiplegia, dystonia, ataxia, nystagmus, hyperactivity, seizures and developmental delays. Often, episodes are triggered by stressful events. We will present some of our latest data from our AHC mouse model, including pharmacological approaches to rescue several disease phenotypes.

**Translational studies of sodium pump knock-in mouse models**

Bøttger P, Glerup S, Gesslein B, Illarionova NB, Isaksen TJ, Heuck A, Clausen BH, Füchtbauer EH, Gramsbergen JB, Gunnarson E, Aperia A, Lauritzen M, Lambertsen KL, Nissen P, Karin Lykke-Hartmann
Aarhus University, Biomedicine, Biochemistry, Denmark

Migraine is a complex brain disorder, and understanding the complexity of this prevalent disease could improve quality of life for millions of people. Migraine co-morbidity involves depression, and anxiety. Familial Hemiplegic Migraine type 2 (FHM2) is a subtype of migraine with aura and co-morbidities like epilepsy/seizures, cognitive impairments and psychiatric manifestations. FHM2 disease-mutations locate to the ATP1A2 gene encoding the astrocyte-located α2-isoform of the sodium-potassium pump (α2$Na^+/K^+$-ATPase). We show that knock-in mice heterozygous for the FHM2-associated G301R-mutation (α2+/G301R) phenocopy FHM2 by mimicking migraine symptoms. In vitro studies showed impaired glutamate uptake in hippocampal mixed astrocyte-neuron cultures from α2G301R/G301R E17 embryonic mice, and moreover, induction of cortical spreading depression (CSD) resulted in reduced recovery in α2+/G301R mice. NMDA-type glutamate receptor antagonists or progestin-only treatment reverted specific α2+/G301R behavioral phenotypes, and our findings support that haploinsufficiency of the α2-isoform encoding gene impairs $K^+$ clearance and glutamate uptake. This study demonstrate that psychiatric manifestations are part of the FHM2-pathology and link the α2$Na^+/K^+$-ATPase to the glutamate system, with the female sex hormone cycle exerting aggravating effects hereon.

**The α2 Isoform of $Na^+,K^+$-ATPase Modulates Vascular Tone via Activation of Src kinase (Src) Signaling Pathway: a Lesson from Mouse Model for Familial Hemiplegic Migraine Type 2 (FHM2)**

Hangaard L, Staehr C, Bouzinova E, Lykke-Hartmann K, Xie Z, Sandow S, Aalkjaer C, Vladimir Matchkov
Aarhus Univesity, Biomedicine, Physiology, Denmark

The vasogenic theory of migraine suggests that the aura is associated with vasoconstriction-induced hypoxemia in the brain, while the subsequent headache is caused by a rebound vasodilation. The alternative theory views migraine as a neurologic disorder. Neither of the theories can alone account for the pathophysiology of migraine.
Familial hemiplegic migraine type 2 (FHM2) has been shown associated with point mutations in the a2 isoform of Na,K-ATPase, including G301R mutation. The a2 isoform expresses and plays an important role in both neuronal and vascular tissues, making it difficult to prioritize these tissues for the pathology of migraine.
Homozygotes mice bearing G301R die very early but heterozygotes (+/-G301R) are viable. Vascular functions of middle cerebral (MCA) and mesenteric small arteries (MSA) from wild type (WT) and +/-G301R mice were compared. A peptide inhibiting the Na,K-ATPase-dependent Src kinase activation, pNaKtide, was used to analyze the potential role of this signaling pathway.
There was no difference in blood pressure (telemetry) between WT and +/-G301R mice. MCA from +/-G301R mice showed a significant reduction in the a2 isoform expression (to 53±12%, n=5; whole-mount immunohistochemistry). Accordingly, ouabain in the concentrations up to 3•10-5 M constricted MCA from +/-G301R significantly less than WT. MCA diameter was larger in +/-G301R compared with WT. MCA constricted stronger to U46619, endothelin and K+-depolarization. This was associated with sensitization to $[Ca^{2+}]_i$. pNaKtide significantly suppressed contraction and abolished differences between groups. In contrast, there was no difference in the contractile responses for MSA from +/-G301R and WT. Moreover, pNaKtide significantly suppressed the contraction only of WT MSA.
Elevated $[K^+]_{out}$ hyperpolarized and relaxed MCAs from +/-G301R mice more than from WT. These responses were $Ba^{2+}$ sensitive. Accordingly, arteries from +/- G301R had elevated mRNA for inward rectifying $K^+$ channels; 190±32% of the WT.
FHM2-associated mutation leads to elevated contractility and relaxation to metabolically elevated $K^+$ of cerebral arteries. MCA hypercontractility is mediated via Src activation. We suggest these changes are involved in the vasoconstriction-induced hypoxemia in the brain during the aura and in the rebound vasodilation during subsequent headache. This signaling is prominent in cerebral but not in peripheral circulation.

# Abstracts Orals Tuesday the 19th May 2015

**Tuning Piezo ion channels to detect molecular-scale movements relevant for fine touch**

Wetzel C, Goek C, Herget R, Lapatsina L, Ngo HD, Lewin G, Kate Poole
Department of Neuroscience, Max-Delbrück Center for Molecular Medicine, Berlin, Germany

In sensory neurons, mechano-transduction is sensitive, fast and requires mechanosensitive ion channels. In order to quantitatively study this mechanosensitive channel activity we have developed a new method to directly monitor mechanotransduction at defined regions of the cell-substrate interface. We have found that molecular-scale (approx. 13 nm) displacements are sufficient to gate mechano-sensitive currents in mouse touch receptors. Using neurons from knockout mice, we found that displacement thresholds increase by one order of magnitude in the absence of the membrane scaffolding protein, stomatin-like protein 3 (STOML3). Piezo1 is the founding member of a class of mammalian stretch-activated ion channels, and we have shown that STOML3, but not other stomatin-domain proteins, reduces the activation threshold for Piezo1 and Piezo2 currents in a heterologous system to approx 10 nm. Structure–function experiments localize the Piezo modulatory activity of STOML3 to the stomatin domain, and higher-order scaffolds are a prerequisite for function. As such, we have shown that STOML3 is the first potent modulator of Piezo channels that tunes the sensitivity of mechanically gated channels to detect molecular-scale stimuli relevant for fine touch. The dependence of STOML3 function on its oligomerisation allowed us to design and execute a small molecule screen for compounds that alter STOML3 self-association; we identified small molecules that, when exogenously applied, can modulate the mechano-sensitivity of sensory neurons via changes in STOML3 scaffolds.

**Calcium entry into stereocilia drives adaptation of the mechanoelectrical transducer current of mammalian cochlear hair cells**

Stuart L. Johnson, Corns LF, Marcotti W
Department of Biomedical Science, University of Sheffield, UK

Mammalian cochlear hair cells transduce sound waves into electrical signals which are then relayed to the auditory centres of the brain. Sound waves displace the stereociliary bundles which project from the apical surface of hair cells. Mechanotransducer (MT) channels located at the tips of these stereocilia are opened by the movement of the hair bundle towards the taller stereocilia. In lower vertebrate hair cells, calcium entry through open MT channels causes channel adaptation, resulting in a lower channel open probability and a resetting of their operating range. This ensures that the hair cells always operate around their maximum sensitivity. It remains uncertain whether calcium-dependent adaptation is also present in mammalian cochlear hair cells.

We mechanically deflected the hair bundles of both outer and inner hair cells of mice using a piezo-driven fluid jet. We found that when the calcium influx into hair cell stereocilia was abolished/decreased by either depolarising the cells to near the calcium reversal potential or exposing the cells to the in vivo endolymphatic calcium concentration (40 microM), the adaptation of the MT current was abolished. The resting open probability, which is dependent on the degree of adaptation, was also increased under these conditions. Decreasing the amount of free calcium within the cell by increasing the concentration of the intracellular calcium buffer BAPTA also abolished all manifestations of adaptation. These findings show that MT current adaptation in mouse auditory hair cells is directly modulated by calcium.

**Pain without nociceptors? Nav1.7-independent pain mechanisms**

Minett MS, Sarah Falk, Santana-Varela S, Bogdanov YD, Nassar MA, Heegaard AM, Wood JN
Department of Drug Design and Pharmacology, University of Copenhagen, Denmark

Nav1.7, a peripheral neuron voltage-gated sodium channel, is essential for pain and olfaction in mice and humans. We examined the role of Nav1.7 as well as Nav1.3, Nav1.8, and Nav1.9 in different mouse models of chronic pain. Constriction-injury-dependent neuropathic pain is abolished when Nav1.7 is deleted in sensory neurons, unlike nerve-transection-related pain, which requires the deletion of Nav1.7 in sensory and sympathetic neurons for pain relief. Sympathetic sprouting that develops in parallel with nerve-transection pain depends on the presence of Nav1.7 in sympathetic neurons. Mechanical and cold allodynia required distinct sets of neurons and different repertoires of sodium channels depending on the nerve injury model. Surprisingly, pain induced by the chemotherapeutic agent oxaliplatin and cancer-induced bone pain do not require the presence of Nav1.7 sodium channels or Nav1.8-positive nociceptors. Thus, similar pain phenotypes arise through distinct cellular and molecular mechanisms. Therefore, rational analgesic drug therapy requires patient stratification in terms of mechanisms and not just phenotype.

**Human TRPA1 is intrinsically cold- and chemosensitive with and without its N-terminal ankyrin repeat domain**

Moparthia L, Survery S, Kreir M, Simonsen C, Kjellbom P, Högestätt ED, Johanson U and Peter M. Zygmunt
Clinical Chemistry and Pharmacology, Department of Laboratory Medicine, Lund University, Sweden

The discovery of transient receptor potential (TRP) ion channels, including the TRP subtype A1 (TRPA1), as molecular chemo- and thermosensors has opened-up new avenues for understanding how organisms monitor the physicochemical environment.
As discovered by us, the mammalian TRPA1 is activated by plant-derived thiol-reactive electrophilic compounds and oxidants, including isothiocyanates and diallyl disulfide from mustard and garlic. This original finding and those of numerous subsequent studies have consolidated mammalian TRPA1 as a prime detector of tissue damaging environmental chemicals and pro-inflammatory compounds. Several TRPA1 homologues exist in the animal kingdom, and the ability of TRPA1 to sense potentially harmful electrophilic compounds has been conserved for ~500 millions of years, whereas the thermosensitive properties of TRPA1 have diverged later.
As thermosensors, mammalian and insect TRPA1 look like yin yang. Rat and mouse TRPA1 has been suggested to respond to cold temperatures below 20°C, whereas fruit fly (Drosophila melanogaster) and mosquito (Anopheles gambiae) TRPA1 seem to respond to warm temperatures above 25°C. Ever since the identification of mouse, rat and human TRPA1 in sensory neurons, a role for TRPA1 as a noxious cold sensor has been controversial. Here. we have cloned and purified TRPA1 from Homo sapiens and the malaria mosquito Anopheles gambiae and provide evidence that these TRPA1 ion channels are indeed intrinsically cold and heat sensitive proteins, respectively.
Chemical control of TRPA1 activity may provide novel drug treatments of pain and strategies for developing insect and arachnid repellents for prevention of human diseases, such as malaria and tick-born encephalitis.

**The diversity of light-driven proton pumps and their conversion into proton channels**

Arend Vogt, Hegemann P
Humboldt University zu Berlin, Biology, Germany

Microbial rhodopsins are integral seven-transmembrane helix proteins which bind covalently all-trans-retinal as light sensitive chromophore. They are subdivided into sensory rhodopsins, ion channels and ion pumps. Light-driven ion pumps transport protons, sodium or chloride across the plasma membrane against their electrochemical gradient. Bacteriorhodopsin (BR) from Halobacterium salinarum is the most notable proton pump and transports protons out of the cell. More recently proton pumps have been employed in neuroscience as optogenetical tools for silencing of neuronal activity by hyperpolarization or as voltage sensors.
It was generally assumed that all light-driven microbial proton pumps behave basically in the same matter like bacteriorhodopsin. We analyzed a variety of proton pumps using two-microelectrode voltage-clamp measurements (TEVC) of Xenopus leavis oocytes. We have found that the naturally occurring proton pumps show different behaviors at high electrochemical load, i.e. low extracellular pH and negative voltage. Photocurrents of Bacteriorhodopsin and the rhodopsin from the eukaryotic microalga Coccomyxa subellipsoidea (CsR) are always outward directed and inactivate at high load. In contrast, the rhodopsins from Exiguobacterium sibiricum (ESR) and from the cyanobacterium Gloeobacter violaceus (GR) show inward directed photocurrents at high load. The rhodopsin Arch3 from the archaeon Halorubrum sodomense is well established as optogentical tool and shows weak inward directed photocurrents at high load.
We have used CsR for an efficient mutagenesis study and identified key determinants for the directivity and the power of the pumps. Mutations at position R83 and Y57 (BR numbering) converted CsR into an operational proton channel with inward or outward rectification depending on the replacement. Such light-activated proton pumps will be of great interest for optogenetic applications in which a specific proton release is required (e.g., in lysozymes or neuronal vesicles).

**Hemoglobin Piracy by Trypanosome parasites**

Kristian Stødkilde-Jørgensen, Andersen CBF
Aarhus University, Biomedicine, Biochemistry, Denmark

Human African Trypanosomiasis, also known as sleeping sickness, is a disease caused by protozoan parasites of the Trypanosoma genus. The drugs available for treating an infection are all associated with severe adverse effects and thus new ways to treat the disease are desperately needed. In this regard, understanding how the parasites take up exogenous ligands is interesting. The parasites cannot synthesize heme themselves, yet require this prosthetic group for incorporation into hemoproteins. The need for exogenous heme is accommodated by a haptoglobin-hemoglobin receptor (HpHbR). This receptor enables uptake of hemoglobin (Hb) bound by the plasma protein haptoglobin (Hp). Interestingly, HpHbR is essential for human immunity against all but two trypanosome subspecies. The immunity arises as the parasites are tricked into taking up a trypanolytic compound that mimics the Hp-Hb com-plex.
To understand the underlying mechanisms of Hb uptake and its role in human innate immunity, we crystallized and obtained the structure of HpHbR while binding the Hp-Hb complex. In the present talk, I will show how the structure not only answers key questions regarding Hb uptake, but how it also showcases an evolutionary arms race between humans and trypanosomes.

**The role of albumin receptors in regulation of albumin homeostasis: implications for drug delivery**

Jan Terje Andersen
Oslo University Hospital, Centre for Immune Regulation and Department of Immunology, Norway

Albumin is the most abundant protein in blood and acts as a molecular taxi for a plethora of small insoluble substances such as nutrients, hormones, metals and toxins. In addition, it binds a range of medical drugs. It has an unusually long serum half-life of almost 3 weeks, and although the structure and function of albumin has been studied for decades, a biological explanation for the long half-life has been lacking. Now, recent research has unravelled that albumin-binding cellular receptors play key roles in homeostatic regulation of albumin. Here, I will discuss our current understanding of albumin homeostasis with a particular focus on the impact of the cellular receptors, namely the neonatal Fc receptor (FcRn) and the cubilin-megalin complex, and their importance on uses of albumin in drug delivery.

**The structure of the RAGE: S100A6 complex reveals a new mode of homodimerization for S100 proteins**

Yatime L[1], Betzer C[2], Mortensen S[1], Jensen PH[2] and Gregers Rom Andersen[1]
1. Department of Molecular Biology and Genetics, Aarhus University, Denmark.
2. Danish Research Institute of Translational Neuroscience - DANDRITE, Department of Biomedicine, Aarhus University, Denmark.

S100 proteins constitute a large family of $Ca^{2+}$-dependent regulators of homeostatic processes. Besides functioning intracellularly in calcium homeostasis, cell growth and differentiation, cytoskeleton dynamics, and energy metabolism, S100 proteins can be relocalized to the extracellular compartment where they act as damage-associated molecular patterns by becoming ligands of the receptor for advanced glycation end-products (RAGE), a pattern recognition receptor sensing endogenous stress signals associated with inflammation. Many S100 proteins are overexpressed in tumors and, through their signaling network, facilitate the communication between cancer and stromal cells, thereby maintaining an inflammatory microenvironment favorable to tumor growth and metastasis. Despite the importance of RAGE-S100 crosstalk in sustaining inflammation, the structural basis for S100 proteins interaction and signal transduction through RAGE is unknown. To gain insights into S100 proteins recognition mode by RAGE, we have undertaken the biochemical and structural characterization of various RAGE:S100 complexes, using analytical size exclusion chromatography and X-ray crystallography. The structure of the isolated human RAGE ectodomain was determined and revealed that the unliganded receptor is prone to homodimerization via its highly basic V domain. We also managed to obtain the first crystallographic structures of a RAGE:S100 complex between the human RAGE ectodomain and both human and mouse S100A6. The structures revealed a binding site for S100A6 located primarily in the RAGE C1 domain, thus being quite different from previous propositions, and suggested that zinc can further stabilize the RAGE:S100A6 interaction. Cell-based assays confirmed that this binding mode also occurs with the endogenous, cell-bound receptor. Furthermore, the structures revealed that S100A6 binding induces a novel dimeric conformation of RAGE that appears suited for signal transduction and intracellular effector recruitment. Surprisingly, the S100A6 ligand adopts a $Zn^{2+}$-stabilized dimeric conformation radically different from known S100 dimer structures. Sequence analysis and modeling suggest that this conformation may be adopted by a variety of S100 proteins in the presence of oxidative conditions and/or extracellular divalent cations. These results provide valuable insights on S100 ligand recognition mode by RAGE and have implications for the targeting of S100 proteins in anti-cancer therapies.

**The membrane receptor megalin modulates proliferation and survival rates in melanoma cells – could it be a novel biomarker and therapeutic target in melanoma cancer?**

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If melanoma cancer is detected at an early stage, where it is still localized to the skin, the patient holds a good prognosis. Patients with early stage melanoma cancer are usually cured by surgical resection of the melanocytic lesion. However, if melanoma cancer spreads beyond the regional lymph node, the prognosis is poor and only about 10 % of the patients diagnosed with metastatic melanoma with distant metastases survive. Unfortunately, no biomarkers have been established than can identify the most aggressive primary melanoma tumors, predict metastasation, and point towards the need of adjuvant treatment. Aggressive melanoma cells are characterized by increased proliferative activity, improved anti-apoptotic machinery, as well as enhanced metastatic potential. We recently hypothesized that acquired expression of megalin by melanocytic lesions/melanoma tumors improves cancerous characteristics. Megalin is an endocytic receptor known to bind an extensive number of different ligands. Megalin either mediates their uptake from the extracellular environment or facilitates their intracellular trafficking. Megalin has been widely studied in relation to embryonic development; especially of the brain, where it is known to play a fundamental role in modulating sonic hedgehog signaling events.
Our initial study has revealed for the first time that megalin is frequently expressed in melanomas and melanoma metastases. Megalin is only rarely expressed in benign nevi. Our functional analyses have indicated that melanoma megalin associates with the endocytic apparatus and that it can be internalized from the cell surface to intracellular vesicles. Groundbreaking, our results indicate that sustained megalin expression in melanoma cells is crucial for cell maintenance. We observed that siRNA-mediated reduction of melanoma cell expression of megalin significantly decreased melanoma cell proliferation and in particular survival rates.
Our study has thus established a platform for acknowledging megalin as a potential new biomarker of aggressive melanoma cells. It might furthermore be addressed as a future therapeutic cancer target, specifically in melanoma.

**Megalin in ocular health and disease**

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Mutation of the megalin-encoding gene (LRP2) causes the rare Donnai-Barrow/ Facio-Oculo-Acoustico-Renal Syndrome which is partially characterized by high-grade myopia. Previous studies of renal megalin function have established that megalin is crucial for conservation of renal filtered nutrients and plasma proteins but the role of megalin in ocular physiology and pathology is unknown. To elucidate this, we have investigated ocular megalin expression and the ocular phenotype of megalin-deficient mice. Topographical and subcellular localization of megalin as well as the ocular phenotype of megalin-deficient mice were examined with immunological techniques using light-, confocal-, and electron microscopy. In normal mice, megalin was identified in vesicular structures in the retinal pigment epithelium (RPE) and non-pigmented ciliary body epithelium (NPCBE). Histological investigations of ocular mouse tissue from megalin-deficient mice revealed a severe high myopia phenotype with grossly enlarged RPE melanosomes as well as abnormal ciliary body development. In conclusion, the complex and severe ocular phenotype observed in the megalin-deficient mice suggests that developmental abnormalities may play a key role in the the high myopia observed in all Donnai-Barrow Syndrome patients and that megalin harbors important roles in ocular development and/or physiology. Finally, our data shows that megalin-deficient mice may provide an invaluable model for future studies of megalin in ocular physiology and pathology.

**The epithelial sodium channel (ENaC)**

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The epithelial sodium channel (ENaC) is a member of the ENaC/degenerin family of ion channels. ENaC is localized in the apical membrane of epithelial cells and is the rate limiting step for sodium absorption in epithelial tissues including the aldosterone-sensitive distal nephron (ASDN) and respiratory epithelia. Abnormal ENaC activation in the ASDN may cause sodium retention and arterial hypertension. ENaC regulation in the ASDN is highly complex with aldosterone-dependent and independent mechanisms. ENaC activity can be stimulated by norepinephrine which may contribute to the hypertensive effect of increased renal sympathetic activity. A unique feature of ENaC is its proteolytic activation which involves specific cleavage sites and the release of inhibitory peptide fragments. However, the physiologically relevant proteases involved in ENaC regulation remain to be identified. In nephrotic syndrome filtered plasminogen is converted to plasmin by tubular urokinase. ENaC activation by tubular plasmin may contribute to sodium retention in nephrotic syndrome. ENaC mutations identified in patients with atypical cystic fibrosis (CF) mimic proteolytic channel activation and may play a role in the pathophysiology of pulmonary symptoms in CF. Interestingly these latter mutations do not seem to cause arterial hypertension unlike the gain-of-function mutations identified in patients with Liddle's syndrome (pseudohyperaldosteronism).

# Abstracts Orals Wednesday 20th May 2015

## Glutamate-induced astrocytic $[Na^+]_i$ elevation – a mechanism to increase $K^+$ clearance via the $Na^+/K^+$-ATPase?

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Neuronal activity in the brain is associated with a transient increase in the extracellular $K^+$ concentration. Accumulated $K^+$ would result in a depolarization of neurons and glia and disturbance of neuronal signaling. The excess $K^+$ is initially cleared by surrounding astrocytes by mechanisms involving the $Na^+/K^+$-ATPase. During the majority of neuronal activity, glutamate increases in the synaptic cleft along with $K^+$. Glutamate is swiftly re-absorbed by astrocytic $Na^+$-coupled glutamate transporters, thereby elevating the intracellular $Na^+$ concentration. The $Na^+/K^+$-ATPase consists of an α- and a ß-subunit, with several isoforms of each subunit present in the mammalian brain. The combination of isoforms control the functional characteristics of the $Na^+/K^+$-ATPase, e.g. its response to changes in extracellular $K^+$ or intracellular $Na^+$. Though the astrocytic α2ß2 isoform constellation responds directly to extracellular $K^+$ above basal levels, it has been suggested that the intracellular astrocytic $Na^+$ concentration may govern $Na^+/K^+$-ATPase activity and consequently control its ability to clear $K^+$ from the extracellular space. It thus remains unresolved whether the $Na^+/K^+$-ATPase-mediated clearance of $K^+$ is driven by the elevation of $[K^+]_o$ or $[Na^+]_i$. The apparent intracellular $Na^+$ affinity of isoform constellations involving ß2 has remained elusive due to inherent expression of ß1 in most expression systems as well as the technical challenges in measuring an intracellular affinity in an intact system. We therefore expressed the different astrocytic isoform constellations in Xenopus oocytes (α1ß1, α2ß1, α1ß2, α2ß2) and determined the apparent $Na^+$ affinity by two different methods: An enzymatic phosphorylation assay on harvested membranes and two-electrode voltage clamp on intact cells gradually loaded with $Na^+$. The obtained $Na^+$ affinities indicated that the $Na^+/K^+$-ATPase was near saturation at basal astrocytic $[Na^+]_i$, irrespective of isoform constellation, and was not stimulated by parallel glutamate transporter activity. Ongoing extracellular hippocampal slice recordings of stimulus-induced $[K^+]$ transients, using ion-sensitive microelectrodes, will reveal whether a glutamate transporter-induced increase in $[Na^+]_i$ contributes to $Na^+/K^+$-ATPase-mediated $K^+$ clearance in a setting approximating native conditions.

**Choroid plexus epithelial cells are polarized normally - but contain unusually located proteins**

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The choroid plexus epithelium secretes the majority of the cerebrospinal fluid. This important brain-supporting function is enabled by the composition of transporter proteins in the plasma membrane of the epithelial cells. The choroid plexus cells are unique in that certain transporter proteins are located in different cellular domains when compared to most other polarized epithelial cells. One example is the ubiquitously expressed $Na^+$,$K^+$-ATPase, which is found in the luminal membrane domain of choroid plexus cells - opposite its typical basolateral localization. The polarity of epithelial cells is established on the basis of extracellular clues. Polarity proteins inside the cells translate these clues into a specific orientation, and this determines the organization of all intracellular components. From vast knowledge of protein trafficking, it is believed that a given transporter protein is always inserted into a specific cellular domain of a polarized epithelial cell. The deviations from this in the choroid plexus epithelium continue an important cell biological question; what determines the cellular localization of transporter proteins?
As a first step to elucidate this question, thus clarify the mechanism behind atypical localization of certain proteins in the choroid plexus cells, we utilized immunohistochemistry. By this approach, we wanted to clarify whether deviations in the localizations of polarity proteins form the basis for the unusual localization of membrane proteins in the choroid plexus cells. So far, the immunohistochemical stainings of choroid plexus cells showed three different polarity proteins, PAR-1, PAR-3 and aPKC, located in each their cellular domain. PAR-1 is found near the basal plasma membrane, PAR-3 at the tight junctions, and aPKC beneath the luminal membrane. All three were located as expected for normally polarized cells. We conclude that these central polarity proteins are distributed normally in the choroid plexus cells, and that they do not seem to be involved in the unusual localizations of key transport proteins in these cells. More work in the research topic is required to clarify what enables unusual localization of certain transporter proteins in the otherwise normal choroid plexus cells, and furthermore to answer what determines the localization of transporter proteins in all epithelial cell types.

**Mechanisms underlying spreading depolarization-induced dendritic beading**

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Spreading depolarizations (SDs) are waves of sustained neuronal and glial depolarization that propagate massive disruptions of ion gradients through the brain. SD is associated with migraine aura and recently recognized as a novel mechanism of injury in stroke and brain trauma patients. SD leads to neuronal swelling as assessed in real time with 2-photon laser scanning microscopy (2PLSM). Pyramidal neurons do not express aquaporins and thus display low inherent water permeability. Yet, SD rapidly induces focal swelling (beading) along the dendritic shaft by unidentified molecular mechanisms. To address this issue, we induced SD in hippocampal slices by focal KCl-microinjection and visualized the ensuing dendritic beading by 2PLSM.
We confirmed that dendritic beading failed to arise during large (100 mOsm) hyposmotic challenges, underscoring that neuronal swelling does not occur as a simple osmotic event. Dendritic beading was strictly dependent on the presence of $Cl^-$ and accordingly, combined blockade of $Cl^-$-coupled transporters, in addition to lactate transporters, led to a significant reduction in dendritic beading without interfering with SD. Furthermore, our in vivo data showed a strong inhibition of dendritic beading upon pharmacological blockage of these cotransporters. We propose that SD-induced dendritic beading takes place as a consequence of the altered driving forces and thus activity for these cotransporters, which by transport of water during their translocation mechanism may generate dendritic beading independently of osmotic forces.

## Expression and Function of $H^+/K^+$-ATPases in Pancreatic Ductal Adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) continues to be one of the most lethal malignancies, with median survival of less than one year and overall 5-year survival less than 5% (1). Potential targets of cancer therapy are ion channels and transporters involved in acid/base transports which are deregulated in cancers and contribute to create a reversed pH gradient (intracellular pH higher than extracellular). This favors cellular proliferation, invasion, metastasis and resistance to chemotherapy in in a variety of cancers (3) and this can be true also for PDAC. Recent studies have shown that pancreatic ducts express gastric and non-gastric $H^+/K^+$-ATPases and they have a physiological role in pH regulation and bicarbonate secretion (2).

The aim of the present study was to investigate whether these proton pumps are expressed and functional in PDAC cell lines and whether proton pump inhibitors (PPIs) can affect cancer progression.

Data from western blot quantification analysis and quantitative PCR revealed different expression of the gastric HKα1, non-gastric HKα2 and HKß subunits (ATP4A; ATP12A; ATP4B) of $H^+/K^+$-ATPases in the pancreatic cancer cell lines compared with the normal one. The protein expression was also verified with immunocytochemical analyses. In BxPC-3, Capan-1 and HPDE cells omeprazole and SCH28080 inhibited cell proliferation up to 50% in a dose-dependent manner and the migration rate up to 70%, particularly on cancer cells.

In conclusion, these data provide for the first time evidence that the $H^+/K^+$-ATPases are involved in PDAC progression and that PPIs can attenuate proliferation and migration of pancreatic cancer cells. This could suggest a possible use of PPIs in cancer therapy.

**Characterization and effect on phosphate transport of mutations in the human Slc34a2 gene in pulmonary alveolar microlithiasis**

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The sodium phosphate co-transporter (NaPi-IIb) is a member of the sodium-phosphate co-transporter family SLC34A. These co-transporters are expressed in several tissues and play a major role in the homeostasis of inorganic phosphate. Mutations in the SLC34A2 gene, that encodes NaPi-IIb, cause defect cell-uptake of phosphate, which leads to formation of calcium-phosphate concretions in the lungs, as seen in pulmonary alveolar microlithiasis (PAM). PAM is a very rare disease with less than 600 patients reported worldwide. The clinical course varies, from asymptomatic to severe disease with progression into lung fibrosis, respiratory failure, and cor pulmonale. There is no known effective treatment of PAM, with the exception of lung transplantation. We have earlier described a new mutation in the SLC34A2 gene in two patients with PAM from Aarhus University Hospital (1).
cDNA, encoding the wild type human NaPi-IIb and four mutants, were subcloned into a vector optimized for expression in Xenopus oocytes. Mutations were introduced by site-directed mutagenesis, the plasmids were linearized with NotI and cRNA was synthesized using a T3 Message Machine kit. Oocytes from X. laevis frogs were injected with the cRNA and subsequently radioisotope uptake assays were performed. In addition, direct sequencing of SLC34A2 was performed on blood samples from three PAM patients.
Preliminary results: The wild type SLC34A2 transported phosphate in the presence of sodium, whereas the mutants showed similar uptake levels as the non-injected oocytes. In addition, the expression levels of the wild type and the mutants, investigated by Western Blot, showed the expected molecular weights. Furthermore, three new mutations were found in three patients with PAM.
These preliminary findings show, as expected, a reduced phosphate uptake in four mutants compared to the wild type SLC34A2. In addition, three novel mutations were found in three patients with PAM.
This project will provide important new knowledge about the function of different mutations in the SLC34A2 gene. The only currently existing treatment of the PAM disease is lung transplantation. Hopefully, these findings may help to develop a specific pharmacological treatment for the disease.

**Prostate specific antigen (PSA)/Kallikrein related peptidase 3 (KLK3) regulates TRPV4 cation channel function by activating proteinase-activated receptor-2 (PAR2)**

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Transient receptor potential vanilloid 4 (TRPV4), a member of the TRP channel superfamily, is a non-selective cation channel. It has a broad spectrum of physiological functions and is expressed in a wide range of tissues including the prostate. However, the functional significance of TRPV4 in the prostate is still unclear. KLK3 (kallikrein-related peptidase 3), also known as PSA (prostate-specific antigen), is a member of the family of kallikrein-related peptidases and has been linked to cancer-associated pathophysiology in particular in the prostate. Although the proteinase targets for PSA in the prostate are not established, other members of the KLK family of enzymes can signal by cleaving and activating proteinase-activated receptors (PARs), including PAR2. Of note, proteases can regulate TRPV4 via activating proteinase-activated receptor-2 (PAR2). Therefore, the aim of this study was to investigate whether PSA 1) is as a potential regulatory protease of TRPV4 affecting its activity and 2) cleaves PARs.
We determined TRPV4 activity by measuring whole-cell currents elicited by the selective TRPV4 agonist GSK1016790A in Xenopus laevis oocytes heterologously expressing human TRPV4 using the two-electrode voltage-clamp technique. Active PSA was purified from pooled human seminal fluid by immunoaffinity chromatography followed by anion exchange chromatography. To test the effect of proteases on TRPV4 channel activity, oocytes were pre-incubated for 30 min in the presence or absence of PSA. To characterize the effect of PSA we used the PSA-inhibiting antibody or two PSA-stimulating peptides.
Our data demonstrate that by activating PAR2, PSA can regulate the activity of TRPV4 by showing a stimulatory effect on GSK1016790A-induced TRPV4 currents of four- to fivefold. The stimulatory effect of PSA on TRPV4 currents was enhanced by PSA-stimulating peptides and was prevented by the PSA-inhibiting antibody. Interestingly, the increase of TRPV4 currents by PSA was essentially abolished by two inhibitors of protein kinase C (Go6983, GF109203X) and by pre-incubation with calcium chelator BAPTA-AM indicating that the stimulatory effect involves calcium signaling (Gq-coupled signaling pathway).
In conclusion, our work reveals a novel action of PSA in conjunction with PAR2 that may be relevant for prostate physiology and pathophysiology.

**$[Ca2^{+}]_i$ oscillations and Il-6 release induced by alpha-haemolysin from Escherichia coli require P2 receptor activation in renal epithelia**

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Urinary tract infections are commonly caused by a-hemolysin (HlyA)-producing Escherichia coli. In erythrocytes, the cytotoxic effect of HlyA is strongly amplified by P2X receptors, which are activated by extracellular ATP, released from the cytosol directly through the HlyA pore. In renal epithelia, HlyA causes reversible $[Ca^{2+}]_i$ oscillations, which trigger interleukin-6 (Il-6) and Il-8 release. We speculate that this effect is caused by HlyA-induced ATP release from the epithelial cells and successive P2 receptor activation.
Here, we demonstrate that HlyA-induced $[Ca^{2+}]_i$ oscillations in renal epithelia were completely prevented by scavenging extracellular ATP. In accordance, HlyA was unable to inflict any $[Ca^{2+}]_i$ oscillations in 132-1N1 cells, which lack P2R completely. After transfecting these cells with the hP2Y2 receptor, HlyA readily triggered $[Ca^{2+}]_i$ oscillations, which were abolished by P2 receptor antagonists. Moreover, HlyA-induced $[Ca^{2+}]_i$ oscillations were markedly reduced in medullary thick ascending limbs isolated from P2Y2 receptor deficient mice compared to wild type. Interestingly, the following HlyA-induced Il-6 release was absent in P2Y2 receptor deficient mice. This suggest that HlyA induces ATP release from renal epithelia, which via P2Y2 receptors is the main mediator of HlyA-induced $[Ca^{2+}]_i$ oscillations and Il-6 release. This supports the notion that ATP-signaling occurs early during bacterial infection and is key player in the further inflammatory response.

**Reduced renal $K^+$ excretion with compensatory hyperaldosteronism in $K_{Ca}1.1$ channel ß2-subunit KO mice**

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The kidney is the primary organ responsible for excreting $K^+$, ensuring whole body $K^+$ homeostasis by precisely matching $K^+$ excretion to dietary $K^+$ intake. $K^+$ is secreted into the urine in the collecting ducts, and two distinct mechanisms for $K^+$ secretion exist; a constitutive mechanism mediated by ROMK (Kir1.1) in principal cells and a flow-induced mechanism mediated by BK channels (KCa1.1) in intercalated cells. Both mechanisms are up-regulated by aldosterone. Here we studied renal $K^+$ excretion in KO mice for the ß2-subunit of the BK channel.
The ß2 KO mice have increased plasma aldosterone, low renin expression and normal plasma [$K^+$]. The low renin in ß2 KO mice indicates that hyperaldosteronism was triggered by a $K^+$ handling deficiency, rather than hypotension and activation of the renin-angiotensin-aldosterone system. We hypothesize that ß2 KO mice have decreased BK channel-mediated renal $K^+$ secretion, which is compensated by hyperaldosteronism and up-regulation of ROMK-mediated $K^+$ secretion, allowing ß2 KO mice to maintain normal plasma [$K^+$]. In fact, when treated with eplerenone (mineralocorticoid receptor antagonist) for 4 days, ß2 KO mice develop hyperkalemia (4.15 mM ± 0.13 in WT vs. 4.60 mM ± 0.10 in KO, P = 0.013).
Urinary $K^+$ excretion following oral $K^+$ load (15% of normal daily intake) was not different between WT and KO mice under control conditions. However, when treated with eplerenone, ß2 KO mice had a significantly lower urinary $K^+$ excretion rate (P=0.044) and significantly higher plasma [$K^+$] 3 hours after oral $K^+$ load (10.0 mM ± 0.4 in WT vs. 11.4 mM ± 0.5 in KO, P = 0.044). Our data support that hyperaldosteronism in ß2 KO mice is part of a chronic compensation to a decreased BK channel-mediated renal $K^+$ secretion.

# POSTERS

# 1

**Isoform-specific phosphorylation-dependent regulation of connexin hemichannels**

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Connexins (Cx) form gap junction channels which are made up of two connexons (hemichannels) expressed in the cell membrane of adjacent cells. Unopposed hemichannels may open towards the extracellular matrix upon stimulation by e.g. removal of divalent cations from the extracellular solution and allow transmembrane flux of isoform-specific fluorescent dyes and physiologically relevant molecules, such as ATP, and current (ions). Cx43 and Cx30 are the major astrocytic connexins. Protein kinase C (PKC) regulates Cx43 in their gap junction configuration and may also act to keep Cx43 hemichannels closed. In contrast, the regulation of Cx30 hemichannels by PKC is unexplored. To determine phosphorylation-dependent regulation of Cx30 and Cx43 hemichannels, these were heterologously expressed in Xenopus laevis oocytes and their opening was induced with divalent cation free solution (DCFS). Connexin dephosphorylation was promoted with PKC inhibitors which did not affect hemichannel opening of either connexin. PKC activation had no effect on Cx43-mediated hemichannel activity whereas both dye uptake and current through Cx30 hemichannels were reduced. We detected no PKC-induced Cx30 or Cx43 internalization from the plasma membrane indicating that reduced Cx30 hemichannel activity occurred via a PKC-dependent gating event closing the channel. In an attempt to resolve the PKC phosphorylation site on Cx30, alanine mutations of seven putative cytoplasmic PKC consensus sites were created to prevent phosphorylation (T5A, T8A, T102, S222A, S225A, S239A, and S258A). All seven Cx30 mutations responded to PKC activation suggesting that Cx30, in its hemichannel configuration, is not regulated by phosphorylation of a single site. In conclusion, Cx30 – but not Cx43 – hemichannels close upon PKC activation, illustrating that connexin hemichannels display not only isoform-specific permeability profiles but also isoform-specific phosphorylation-dependent regulation.

# 2

**Does hydrochlorothiazide acidify urine?**

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The loop-diuretic furosemide inhibits NaCl reabsorption in the thick ascending limb of Henle's loop (TAL) in the kidney, by blocking the $Na^+$ Cl- $K^+$ cotransporter, NKCC2. In addition to its diuretic effect, furosemide also provokes an acute and pronounced acidification of the urine. This is traditionally explained by an increased $Na^+$ delivery to the connecting tubule (CNT) and cortical collecting duct (CCD), which is believed to stimulate $H^+$ secretion via the apical $H^+$-ATPase in a-intercalated cells. However, we have recently demonstrated that the TAL itself plays an important role in the acidifying effect of loop diuretics by activation of the apical $Na^+/H^+$ exchanger 3 (NHE3).
The reabsorption of NaCl in the distal convoluted tubule (DCT), located upstream of the CNT/CCD, can be inhibited with thiazide diuretics. Similarly to furosemide, thiazides also increase the delivery of $Na^+$ to the CNT/CCD, but an acute effect on urine acidification has to our knowledge never been reported. This study intends to investigate the acute effect of NCC inhibition with hydrochlorothiazide on urine pH and electrolytes in mice in an in vivo experimental setup. Preliminary data suggests that hydrochlorothiazide does not acidify the urine. This suggests that the current mechanism explaining furosemide-induced urine acidification may not be correct, because other methods for increasing $Na^+$ delivery to the distal nephron do not acidify urine. Thus, this study solidifies our previous finding that $H^+$ secretion by furosemide takes place already in the TAL and will contribute to a better understanding of how the kidneys secrete acid.

# 3

**The effect of P2-receptor inhibition on the cytotoxic effects of a-haemolysin from E. coli – a murine sepsis model**

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Haemolytic bacterial toxins cause cell lysis by forming pores in plasma membranes. However, we demonstrated that a-haemolysin (HlyA) from E. coli requires extracellular ATP and P2X receptor activation to cause lysis. Murine erythrocytes express both P2X1 and P2X7 and inhibition of both receptor subtypes completely prevent the HlyA-induced haemolysis. Interestingly, free plasma haemoglobin is partly responsible for the symptoms during sepsis and is associated with a poorer outcome of sepsis both in mice and in patients. Inhibition of P2X receptors will improve the outcome of experimental induced sepsis with HlyA producing E. coli in a murine model.
Iv-injection of HlyA producing E. coli or vehicle in anaesthetized mice for determination of the role of P2X-receptors on the outcome of sepsis. Mice are pre-treated with subcutaneous injection of P2X-receptor antagonists. Body temperature and blood pressure are measured continuously. The mice are either terminated after 2.5 hours, where blood is collected for determination of haemolysis and plasma levels of IL1ß, TNFa, IL-6 and KC (murine equivalent of IL-8) or allowed to die spontaneously of the bacteraemia (under anaesthesia). Mice subjected to iv-injection of HlyA producing E. coli show an increased body temperature and pulse pressure. Mice subjected to bacteria showed a massive increase in the plasma levels of IL1ß (~23 times increase), TNFa (459 times increase), IL-6 (229 times increase) and IL-8 (~48 times increase). Neither haemolysis nor the cytokine release was statistically significantly different in mice pre-treated with non-selective P2X receptor antagonist BBG (50 mg/kg, resulting in a plasma level of ~3 µM) compared to vehicle. Notably, 3 µM BBG is sufficient to completely prevent HlyA-induced haemolysis in murine erythrocytes. The survival was also comparable between BBG injected and control, with a small trend towards longer survival of the BBG treated mice. Surprisingly, the haemolysis is massively increased in P2X7 knockout mice compared to wild type, p=0.0062. Moreover, our preliminary results show a markedly lower survival of the P2X7 receptor knockout mice.
There is an apparent susceptibility of P2X7 receptor knockout mice for acute bacteraemia. Since non-specific inhibition of P2X receptors show a different profile from the P2X7 knock out mice, it is interesting to investigate specific outcome in the absence of functional P2X1 receptors. These data may have important clinical perspective, since loss of function mutations of the P2X7 receptor are relatively common in humans.

# 4

**Luminal acid/base transporting proteins in the choroid plexus epithelium**

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Cerebrospinal fluid (CSF) pH most likely has a direct effect on brain extracellular fluid pH, and disturbances in brain extracellular fluid pH have been shown to affect neuronal excitability and cause symptoms such as seizures and panic attacks. Little is known about the mechanisms behind CSF pH control. The CSF is produced by the choroid plexus epithelium (CPE), located in the ventricular system of the brain. It is believed that CSF pH is regulated by the CPE but the mechanism is not yet known.
We detect V-ATPase-encoding mRNAs in fluorescence-activated cell sorted (FACS) CPE samples. Immunofluorescence staining shows the protein to be evenly dispersed across the cytoplasm in CPE cells and mass spectrometry on surface-biotinylated CPE cells shows the presence of several V-ATPase subunits in the luminal membrane. However, in vitro intracellular pH measurements on freshly isolated CPE cells show no effect of the V-ATPase inhibitor Concanamycin A on acid extrusion following an acid load. The alpha chain of the non-gastric $H^+/K^+$-ATPase (HK α2) is also detected by mass spectrometry on surface-biotinylated CPE cells, and immunohistochemistry supports these data by locating the protein to the luminal CPE membrane. However, HK α2-encoding mRNA cannot be detected in FACS CPE samples and no beta chain has been identified at this site. We detect NHE6 and NHE1 mRNA by RT-PCR on FACS CPE samples and both proteins have been detected by mass spectrometry on surface-biotinylated CPE cells, indicating their presence in the luminal CPE membrane. Previous functional studies on freshly isolated CPE cells from NHE1 knockout mice have shown that sodium-dependent, and bicarbonate-independent acid extrusion was almost completely abolished in these mice as compared to wild type mice. NBCe2-encoding mRNA is readily detected in FACS CPE samples as well as by mass spectrometry on surface-biotinylated CPE cells, and previous studies has shown the sodium-bicarbonate cotransporter to be located in the luminal CPE membrane, where it mediates the export of sodium and bicarbonate into the CSF. Thus, to date luminal NHE activity is the most probable mechanism for CSF acidification by the choroid plexus.

# 5

**P2X receptor desensitization by high concentrations of ATP changes the survival rate of THP-1 monocytes attacked by RTX toxins HlyA and LtxA**

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The Repeats in Toxins (RTX) exotoxin alpha-haemolysin (HlyA) from Escherichia coli is an important virulence factor for ascending urinary tract infections. The RTX exotoxin LeukotoxinA from Actinobaccilus actinomycetemcomitans is known to be involved in aggressive forms of peridontitis, but is relevant for some systemic reactions as well.
The extracellular signalling molecule, adenosine triphosphate (ATP) has been shown to play an important role in the erythrocyte damage inflicted by HlyA and LtxA, and the subsequent recognition and phagocytosis of HlyA-attacked erythrocytes by the monocytic cell line THP-1.
It is, however, uncertain how HlyA and LtXA affect the monocytes themselves. Here we show that HlyA and LtxA initate two different patterns of $[Ca2^{+}]i$ signalling combined of both immediate influx of $Ca2^{+}$ trough the toxin pore and activation of P2X and P2Y receptors by released ATP. Moreover we investigate the THP-1 cell resistance to HlyA and LtxA and found that blockage of P2Xreceptors by either desensitization with high concentrations of ATP or blockage by oxATP resulted in increased survival of HlyA-attacked THP-1 cells and a decreased survival of LtxA-attacked THP-1 cells.
In combination with the optimizing effect on erythrocytes of HlyA, these new findings suggest an important interplay between monocytes, erythrocytes and HlyA in situations of high amount of extracellular ATP, which may be relevant for the development of cell lysis and cell recognition, at the place of injury.

# 6

**The purinergic P2X7receptor is involved in glucose stimulated ATP release and ß-cell proliferation**

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Adenosine triphosphate (ATP) is not only important as an intracellular energy source, but extracellularly it acts as a short-range signalling molecule that promotes a broad range of physiological responses by activating purinergic receptors on the cell membrane. Various subtypes of purinergic receptors are found in pancreatic ß-cells and ATP act on these receptors regulating insulin release, in some cases even in non-stimulating glucose concentration. In addition, a few studies indicate that P2 purinergic receptors may also regulate ß-cell survival, which is highly relevant to type 1 and 2 diabetes. One of the receptors that may be relevant to cell survival is the P2X7 receptor, which is expressed in ß-cells, but its detail role in ß-cell physiology is unclear. A recent study indicates that high glucose can lead to extracellular ATP release in mouse ß-cells. It is of utmost relevance to understand molecular mechanism regulating ATP release and ß-cell survival.
The aim of this study was to determine whether extracellular glucose load influences P2X7 receptor signalling in pancreatic ß-cell and determine how this signalling can modulate ß-cell function. For this purpose we used pancreatic ß-cell line INS-1e and in situ live cell luminometry to monitor ATP release and BrdU assay to monitor proliferation. We show INS-1e releases ATP in response increase in glucose (from 4-5mM to 16,7-25mM) and that this release is reduced both by the P2X7 receptor inhibitor AZ10606120 and an inhibitor of the transmembrane channel pannexin-1. Cell proliferation assays showed that stimulation with ATP or the specific P2X7 agonist BzATP resulted in increased cell proliferation. In addition, cell proliferation stimulated by high glucose was markedly reduced by P2X7 receptor inhibition.
In conclusion, our study shows that the P2X7 receptor might be an important regulator of ß-cell proliferation and ATP release.

# 7

**Is megalin involved in regulating the apoptotic/anti-apoptotic apparatus of melanoma cells?**

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The multiligand endocytic receptor megalin is a transmembrane protein known to mediate cellular uptake and trafficking of various ligands. Amongst the many established ligands for megalin is clusterin, suggested to play a role during cellular protection from programmed cell death (apoptosis), which is particularly relevant in cancer cells.
Interestingly, a recent study performed in our research group showed that megalin is frequently expressed in melanoma tumors and metastases here off. For comparison, megalin was only rarely observed in benign counterparts. In addition, the same study demonstrated that siRNA-mediated knockdown of megalin expression affects melanoma cell survival and induces apoptosis. Megalin knockdown results in a significantly increased number of apoptotic cells in megalin knockdown cultures compared to the number of cells in cultures treated with non-targeting siRNA. This points towards a role for megalin in regulating the apoptotic/anti-apoptotic apparatus of melanoma cells.
To further investigate the observed effect of megalin knockdown on melanoma cell culture survival rates, this study aims to investigate a potential relation between megalin expression and/or function and expression levels and phosphorylation status of the protein Bcl2; which is known to operate during cellular protection from apoptosis.
Preliminary results indicate that siRNA-mediated knockdown of megalin in melanoma cell cultures causes a decrease in the amount of Bcl2-encoding mRNA, which eventually can be speculated to lead to less Bcl2 protein and less protection from apoptosis. The phosphorylation status of the remaining Bcl2 protein is currently being investigated and compared to the phosphorylation status of Bcl2 protein in control cultures because phosphorylation of Bcl2 is known to regulate its anti-apoptotic activity.

# 8

## Cotransporter-mediated cerebrospinal fluid formation

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Cerebrospinal fluid (CSF) production takes place at around 500 ml/day in adult humans. The majority of the CSF is produced in choroid plexus (CP) which is a highly specialized cell layer with a vast variety of membrane transport proteins expressed in its cell membrane, one of which is the $K^+/Cl^-$ cotransporter (KCC). It has been of long-standing scientific interest to resolve the mechanisms of CSF production but the molecular players involved have remained elusive. As CSF production readily takes place against an osmotic gradient and is only slightly reduced by genetic deletion of AQP1, conventional osmotically driven water transport does not appear to underlie CSF production. This project aims to resolve the role of the KCCs and their ability to translocate water as the molecular mechanism responsible for cerebrospinal fluid formation. Localization and semi-quantification of the different KCC isoforms in mice choroid plexus is addressed by immunohistochemistry, Western Blotting of intact choroid plexus in addition to FACS-purified choroid plexus epithelial cells, and quantitative PCR of FACS-purified choroid plexus epithelial cells. The extent to which KCC activity participates in CSF production is assessed ex vivo by live cell imaging of calcein-loaded mice choroid plexus, while the ability of the KCCs to directly transport water will be quantified upon heterologous expression in the Xenopus laevis expression system. Activation of KCC in the luminal membrane of choroid plexus induced water transport against an imposed osmotic gradient which indicates that KCC would indeed be able to drive water transport, i.e. CSF production, independently of the osmolarity of the ventricular cerebrospinal fluid. Although this study is ongoing, our preliminary data suggests that CSF is produced by cotransport proteins, such as the $K^+/Cl^-$ cotransporter, that directly couple ion transport to water translocation.

# 9

**Does the cytosolic adaptor protein Dab2 assist melanoma megalin during endocytosis?**

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Our research group recently showed that malignant melanoma tumors and metastases hereof frequently express megalin, while benign counterparts only rarely express megalin. Our study demonstrated that melanoma megalin localizes partly to the plasma membrane and extensively to intracellular vesicular structures. Interestingly, we were able to show that melanoma megalin can be internalized from the cell surface to intracellular vesicles and it can colocalize with a marker of early endosomes; Rab5, indicating a role for melanoma megalin in endocytosis. A crucial role for melanoma megalin in uptake and/or trafficking of ligands was further suggested from the data we obtained when megalin expression was lowered by treatment with siRNA targeting the gene encoding megalin. This had severe effects and decreased both proliferation and survival rates dramatically. The cytosolic adaptor protein Disabled-2 (Dab2) is involved in clathrin-mediated endocytosis of specific cargo proteins. Dab2 has the ability to bind directly to clathrin and assist the assembly of the characteristic clathrin coat surrounding endocytic vesicles. Simultaneously, it can recruit selected cargo to these clathrin-coated vesicles thus; it functions as a clathrin-associated sorting protein. One of the cargo proteins binding to Dab2 is megalin. To further delineate the potential involvement of melanoma megalin in clathrin-mediated endocytosis and vesicular trafficking of ligands, this project aims to investigate the functional relationship between megalin and Dab2 in malignant melanoma cells. A protocol for effective siRNA-mediated knockdown of Dab2 in melanoma cells has already been established and our preliminary results indicate that successful knockdown of Dab2 can be achieved both at RNA and protein level within 48-72 hours. Most interestingly, Dab2 knockdown appears to have a similar negative effect on melanoma cell culture viability as megalin knockdown does indicating that these two proteins might be of similar importance to melanoma cell maintenance. We are currently investigating in further detail the effects of Dab2 knockdown on melanoma cell culture proliferation and survival rates for comparison with the pattern observed from megalin knockdown studies. Finally, the ultimate aim is to establish if Dab2 is essentially the sorting adaptor protein required for melanoma megalin function.