From the Department of Women's and Children's Health
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A SPECIAL ROLE OF Na,K-ATPase AND ITS MOLECULAR PARTNERS FOR ASTROCYTE FUNCTION

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'Did you ever look at the stars?' he asked, pointing upwards.

'Often and often,' answered Will.

'And do you know what they are?'

'I have fancied many things.'

'They are worlds like ours,' said the young man. 'Some of them less: many of them a million times greater: and some of the least sparkles that you see are not only worlds, but whole clusters of worlds turning about each other in the midst of space. I do not know what there may be in any of them: perhaps the answer to all our difficulties or the cure of all our sufferings.'

"Will O' the Mill" 1887 Robert Louis Stevenson

To my family

A special role of Na,K-ATPase and its molecular partners for astrocyte function

THESIS FOR DOCTORAL DEGREE (Ph.D.)

by

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ABSTRACT

Astrocytes are glial cells that express several specific transporters and channels with specialized functions to maintain water, ion and neurotransmitter concentrations in order to preserve normal neuronal function. Astrocytes express two catalytic isoforms of plasma membrane enzyme Sodium Potassium-ATPase (Na,K-ATPase), that are essential for several of astrocyte's functions. In each cycle Na,K-ATPase actively transports 3 Na^+ ions out of the cell and 2 K^+ ions into the cell, using the energy of one ATP molecule. Na,K-ATPase establishes transmembrane Na^+ gradient that allows for efficient Na^+-coupled transport, including glutamate uptake. Na,K-ATPase is also responsible for active K^+ uptake from extracellular space and therefore maintenance of extracellular K^+ homeostasis. The overall goal of this thesis was to study the molecular and functional interactions between the Na,K-ATPase and other proteins responsible for astrocyte function.

The water channel aquaporin 4 (AQP4) is abundantly expressed in astrocytes. Emerging evidence suggests that AQP4 facilitates extracellular K^+ clearance by astrocytes and contributes to recovery of neuronal excitability. We found that AQP4 can assemble with its regulator metabotropic glutamate receptor 5 (mGluR5) and with Na,K-ATPase. The AQP4 NH_2-terminus was shown to interact with Na,K-ATPase catalytic α subunit and with mGluR5. Föster resonance energy transfer (FRET) studies in primary astrocytes derived from rat striatum showed that interaction occurs in intact cells. Thus AQP4/Na,K-ATPase/mGluR5 can form a macromolecular complex in astrocytes, that may be of functional importance for the regulation of water and K^+ homeostasis in the brain.

Astrocytes express two isoforms of the Na,K-ATPase catalytic α subunit: the ubiquitous α1 and the α2, which in the brain is predominantly expressed in astrocytes. The α2 isoform has lower Na^+ affinity than α1. We explored the relative roles of the α1 and α2 isoforms for the support of Na^+-coupled glutamate uptake in primary astrocytes. We found that the α2 isoform contributes to a more efficient restoration of increases in intracellular sodium concentration ($[Na^+]_i$) evoked by the Na^+-coupled glutamate uptake. Both α1 and α2 interacted molecularly with glutamate transporters via the 1st intracellular loop, but the interaction with α2 appeared stronger. The study points to a specific role for α2 in the handling of $[Na^+]_i$ transients in astrocytes and suggests that α1 and α2 may differ with regard to capacity to interact with the glutamate transporters.

Mutations in the Na,K-ATPase α2 subunit are associated with the neurological disease familial hemiplegic migraine type 2 (FHM2). In this study we

determined α1 and α2 abundance and glutamate uptake in primary cultures from heterozygous and homozygous α2 mutant mice carrying the human knock-in FHM2-mutation G301R. Both α2 abundance and glutamate uptake were significantly reduced in astrocytes expressing the mutant α2. The plasma membrane expression of mutant Venus-tagged α2 was also reduced in comparison to wild type α2. The results suggest that reduced capacity of astrocytes expressing α2 G301R mutant to take up glutamate, may lead to temporary increases in ambient glutamate concentration that, at least to some extent, may contribute to the symptoms in FHM2.

LIST OF SCIENTIFIC PAPERS

I. **Nina B. Illarionova**, Eli Gunnarson, Yanhong Li, Hjalmar Brismar, Alexander Bondar, Sergey Zelenin, Anita Aperia
Functional and molecular interactions between aquaporins and Na,K-ATPase
Neuroscience 168 (2010) 915–925

II. **Nina B. Illarionova**, Hjalmar Brismar, Anita Aperia, Eli Gunnarson
Role of Na,K-ATPase α1 and α2 Isoforms in the Support of Astrocyte Glutamate Uptake
Accepted for publication in *PloS ONE* (2014)

III. **Nina B. Illarionova**, Pernille Bøttger, Yutong Song, Eli Gunnarson, Karin Lykke-Hartmann and Anita Aperia
Reduced glutamate uptake in astrocytes expressing the Na,K-ATPase α2 mutation G301R. Implications for Familial Hemiplegic Migraine type 2.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ALS	amyotrophic lateral sclerosis
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AM-SBFI	acetoxymethyl Na^+-binding benzofuran isophthalate
AQP	aquaporin
BiP	Binding immunoglobulin protein
CNS	central nervous system
CT	cytosolic –COOH terminus of protein
D-Asp	D-aspartate
EAAT	excitatory amino acid transporter
ER	endoplasmic reticulum
FHM2	familial hemiplegic migraine type 2
FRET	Föster resonance energy transfer
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
GLAST	glutamate-aspartate transporter
GLT-1	glutamate transporter 1
GPCR	G-protein coupled receptor
HEZ	heterozygous

HOZ	homozygous
IP3R	Inositol triphosphate receptor
KBBP	kappa-B motif-binding phosphoprotein
MAGI-1	membrane-associated guanylate kinase with inverted orientation
mGluR	metabotropic glutamate receptor
MP	membrane potential
Na,K-ATPase	sodium potassium-ATPase
NCX	Na^+,Ca^{2+} exchanger
NHE	Na^+/H^+ exchanger
NHERF-2	Na^+/H^+ exchanger regulatory factors 2
NMDAR	The N-methyl-D-aspartate receptor
NT	NH_2^+ cytosolic terminus
PDZ	PSD95-Discs large-ZO1
PKC	phospho kinase C
PM	plasma membrane
ROS	reactive oxygen species
SBFI	Na^+-binding benzofuran isophthalate
WT	wild type

1 INTRODUCTION

1.1 ASTROCYTES

Brain function has long been attributed solely to the connectivity of neurons. However, new emerging evidence implicates glial cells in the majority of brain functions, including signal processing and output regulation. There are several types of CNS glia: oligodendrocytes – myelinating cells, microglia – immune defense cells and astrocytes – having diverse functions for neuronal support and recently NG2 glia was recognized as a CNS glia type - glial cell progenitors. Astrocytes express a large variety of transmembrane proteins, including metabotropic and ionotropic receptors, which allow them to sense neuronal activity and respond accordingly. In fact, astrocytes express almost the same set of receptors and ion channels as neurons, yet with K^+ channels dominating over Na^+ channels, preventing glial cells from generating action potentials. In different brain areas and even within the same brain area, the astrocyte population is heterogeneous both by morphology and astrocyte markers, as well as by expression of specialized plasma membrane (PM) proteins; probably to support the function of particular brain areas (Zhou and Kimelberg 2001; Matyash and Kettenmann 2010; Oberheim, Goldman et al. 2012).

1.2 ASTROCYTE FUNCTION

Astrocyte functions are numerous and diverse. The polarized morphology of astrocytes make them a key part in the blood-brain barrier, also being responsible for maintaining the homeostasis of the extracellular milieu, where they sense pH, ions, metabolites and other chemicals in the extracellular compartments and interstitial spaces (Nedergaard, Ransom et al. 2003; Kimelberg and Nedergaard 2010). Astrocytes play an important role for K^+ and neurotransmitter re-uptake

following neuronal activity, preventing neuronal excitotoxicity and neurodegeneration. Lately, astrocyte roles in modulating neuronal activity have been recognized. Astrocytes can modulate neurotransmission by releasing gliotransmitters into synaptic cleft, which include glutamate, ATP, D-Serine, GABA, and others (for review see Volterra and Meldolesi 2005; Angulo, Le Meur et al. 2008; Perea, Navarrete et al. 2009). One astrocyte may connect to 2 million synapses, which is believed to contribute significantly to synchronized neuronal activity by mediating signals via astrocyte processes locally to synapses. Astrocyte leaflets, or as they are also called peripheral astrocyte processes, experience vast motility. Astrocyte coverage of synapses varies between brain regions and is temporally dependent on neuronal activity (for review see Bernardinelli, Muller et al. 2014). The coverage of the synapse may play a role in synaptic plasticity as well as synaptogenesis.

In addition astrocytes have been shown to modulate local blood flow which is dependent on brain metabolism (Gordon, Choi et al. 2008). Also, astrocytes provide neurons with metabolic substrates via the proposed "glucose-lactate shuttle" process (for review see Magistretti 2006). Astrocytes can sense activity within the synapses and increase lactate production. This pathway starts with the uptake of neurotransmitter into astrocytes via the Na^+-coupled transporter. The local rise of intracellular sodium concentration ($[Na^+]_i$) activates Na,K-ATPase – an ATP dependent enzyme. It has been proposed that the associated increase in energy demand of the astrocyte will enhance its anaerobic glycolysis and, as a result, more lactate will be released for the neighboring neurons. Signaling events that occur between the Na,K-ATPase activation and increased glucose uptake by astrocytes, remain to be understood.

1.3 ASTROCYTES IN DISEASE

Several pathological conditions are associated with astrocyte dysfunction. For example, in mesial temporal lobe epilepsy, astrocyte K^+ and water channels as well as glutamate transporter functions have been found to be altered, leading to overexcitation and seizures (for review see Seifert and Steinhauser 2013). Also, other pathologies are attributed to mutations in genes expressed in astrocytes. Mutations in the Na,K-ATPase α2 are associated with hemiplegic migraine; mutations in GFAP cause Alexander disease; and the mutated form of the antioxidant enzyme superoxide dismutase 1 (SOD1) leads to familial amyotrophic lateral sclerosis (ALS) (for review see Li, Messing et al. 2002; Nagai, Re et al. 2007; Bottger, Doganli et al. 2012).

Astrocyte dysfunction is also associated with several neurodegenerative diseases, such as Alzheimer (AD), Parkinson, ALS and others (for review see Verkhratsky, Olabarria et al. 2010). Astrocytes are affected at a very early stage of neurodegenerative pathology, when neuronal connectivity starts to change. Atrophic changes, such as a reduction in the number of primary and secondary branches in astrocytes have been noticed early in disease development and may contribute to a disrupted metabolic neuron-astrocyte connection, excitotoxicity and neuronal death.

1.4 ASTROCYTE MICRODOMAINS

Astrocytes have an irregular shape with many processes extending from the cell soma and numerous endpoint leaflets (in humans more than 2 million, in rodents around 120 000), enabling one single astrocyte to have numerous connections with thousands of synapses, capillaries, arterioles and other glial cells. Astrocytes also provide connectivity between each other by gap junctions creating an astrocyte *syncytium*, allowing spreading of molecules as big as 1

kDa. For instance, Na^+ and glucose can spread via the astrocyte *syncytium* over several hundred micrometers, which may trigger signaling and metabolic changes in connected cells (Bernardinelli, Magistretti et al. 2004; Rouach, Koulakoff et al. 2008). The *syncytium* can have local, anatomical restrictions of connectivity, only allowing for molecular spread within a certain area, such as in the somatosensory cortical barrels and in olfactory bulbs glomeruli (Houades, Koulakoff et al. 2008; Roux, Benchenane et al. 2011). Astrocytes have a localized area of influence, as noticed for protoplasmic astrocytes in the hippocampus, that occupy defined units, that do not overlap with neighboring astrocytes (Bushong, Martone et al. 2002). This may allow each single astrocyte to have local sensitivity and modulate activity in corresponding area.

Modulations and influences in local and restricted areas are also achieved by the PM of any cell exhibiting a varying composition of microdomains, which serves particular cellular functions. Since astrocytes are polarized cells with diverse inter-cellular contacts, the targeting of proteins to a particular astrocytic unit as well as protein-protein co-assemblies, may be critical for generating functional microdomains. The extracellular space in the brain is also divided into microdomains of restricted volumes. As an example, the tripartite synapse, where a neuronal synapse is enveloped by astrocyte leaflets, allows for diffusion-limited space restriction and neuron-astrocyte communication. The diffusion-limited spaces allow little spillover of released ions and neurotransmitters, and thus efficient maintenance of homeostasis.

1.5 ION AND WATER BALANCE IN THE BRAIN

Ion and water homeostasis in the brain is of outmost importance to ensure normal neurological function. The narrow intracellular and extracellular spaces create diffusion-limited spaces for ions, neurotransmitters and

metabolites. The concentrations of these affect the function of pumps, ion exchangers, channels and other ion-coupled transports. A well-tuned water balance is one of the determinants of molecular concentrations, especially in diffusion-limited spaces. Extracellular and intracellular ion concentrations both influence resting membrane potential, which partially affects the direction of transport through channels and transporters.

1.5.1 Extracellular potassium concentration

One function of astrocytes is to maintain the extracellular water and ion milieu in order to preserve neuronal excitability. During neuronal activity, extracellular K^+ changes from basal values of around 3-4 mM up to 10 mM. However, in pathology, such as ischemia and migraine, this limit can be exceeded and extracellular K^+ concentration can increase to around 60 mM. In physiologic conditions, K^+ is returned to basal levels by local glial and neuronal uptake: passive uptake through channels such as NKCC1 and Kir4.1 and active uptake via the Na,K-ATPase enzyme. It is still debated which uptake mechanism that is most important for restoring extracellular K^+ concentrations, as well as the temporal and spatial involvement of these mechanisms (Macaulay and Zeuthen 2012). After K^+ enters the astrocyte *syncytium* it is believed to move down the concentration gradient via gap junctions and eventually exit into the blood stream (for review see Kofuji and Newman 2004). Accompanying water facilitates K^+ clearance, which has been supported by genetic knock-out studies of the major water channel in the brain – AQP4. Genetic knock-out of the AQP4 channel shows slower recovery of extracellular K^+ concentration (Amiry-Moghaddam, Williamson et al. 2003; Binder, Yao et al. 2006; Strohschein, Huttmann et al. 2011). Increases in extracellular K^+ concentrations have also

been shown to dynamically affect astrocyte AQP4 function by regulation of AQP4 water permeability (Song and Gunnarson 2012).

1.5.2 Intracellular sodium concentration

The astrocyte transmembrane Na^+ gradient (around 130-140 mM extracellularly and 3-15 mM intracellularly) allows for a range of functions, including: Na^+-coupled transport of various molecules down the Na^+ concentration gradient, which is essential for the membrane potential (MP) and recently has been suggested to be involved in astrocyte metabolic signaling; as well as clearance of extracellular K^+ ions (for review see Kirischuk, Parpura et al. 2012). Studies have demonstrated that in astrocyte primary and secondary processes, a local rise in $[Na^+]_i$ may occur, mainly resulting from glutamate Na^+-coupled uptake (Langer and Rose 2009). Sodium signaling has also been proposed to pass through the astrocytic network via gap junctions, possibly affecting the function of neighboring astrocytes, transmitting signals for metabolic changes including glucose uptake and the production of lactate, - resulting in modulations of neuronal activity (Bernardinelli, Magistretti et al. 2004; Magistretti 2006; Langer, Stephan et al. 2012).

Astrocyte $[Na^+]_i$ fluctuations are due to work of several Na^+ channels and transporters: Na,K-ATPase; NKCC1; glutamate transporters; GABA transporters; AMPARs; NMDARs; purinoreceptors; sodium/calcium exchanger (NCX); Sodium Hydrogen Exchanger (NHE); sodium/bicarbonate co-transporter NBC; monocarboxylate transporter (MCT1); glucose transporter (GLUT); mitochondrial sodium/calcium exchanger (NCLX), and others. The direction of the Na^+ current is mostly dependent on the MP and the ligands concentrations. Sodium transporters and channels are tightly coupled; hence they can affect each

other's function via their common Na^+ ligand, regulating major functions of the cell.

1.6 PROTEIN FUNCTIONAL DOMAINS

Most cellular functions are defined by proteins functions. Protein complexes often include proteins that serve the same function in the cell, presumably because it is "energy and time efficient" for the cell. The close proximity of functionally coupled proteins often affects activity of the complex by conformational changes and influence on the local ligand gradients. It is a current opinion in cell biology, that functionally coupled proteins commonly form domains in the subcellular compartment with a particular function (Hartwell, Hopfield et al. 1999).

Protein-protein interactions are often mediated via specific interaction domains, such as Src homology 2 and 3 (SH2 and SH3); phosphotyrosine-binding domain (PTB); PDZ domain; PH domain; and many others that recognize particular amino acid sequence on the partner protein (for review see Pawson and Nash 2003). Scaffolding proteins and scaffold-associated proteins may mediate assembly of the network of functionally coupled protein complexes, and target them to a particular cellular domain. In astrocytes, the polarized targeting of proteins is largely unexplored, probably because the astrocyte specialized domains - for instance the perivascular or perisynaptic domains - are formed in the 3D structure of the brain. However, for several proteins of interest in this thesis some scaffolding proteins allowing for polarized expression were detected. For instance, the water channel AQP4 binds via its C-terminal SSV amino acid motif to a PDZ domain of α-syntrophin of the dystrophin protein complex, which targets AQP4 to the astrocyte perivascular endfeet (Amiry-Moghaddam, Xue et al. 2004; Bragg, Amiry-Moghaddam et al. 2006). Whilst the astrocyte

glutamate transporter GLAST C-terminus binds to the PDZ domain of the NHERF1 scaffolding protein, which probably allows the targeted expression in astrocyte (Lee, Rayfield et al. 2007).

1.7 NA,K-ATPASE

The key protein for Na^+ and K^+ homeostasis, as well as for the transmembrane ion gradients, is the Na,K-ATPase. Na,K-ATPase is the most energy consuming enzyme in the brain, consuming around 50 % of all the energy used by the brain (Ames 2000). In each cycle, the Na,K-ATPase pumps 3 Na^+ out of the cell and 2 K^+ into the cell, while hydrolyzing 1 ATP molecule. The Na^+ and K^+ gradients across the PM, generated by the Na,K-ATPase, are of vital importance for cellular function. These include transport of nutrients and neurotransmitters, maintenance of cellular excitability, cell volume stabilization and retention of the resting membrane potential.

The Na,K-ATPase complex consists of three subunits: α, β and FXYD.

1.7.1 Na,K-ATPase α– catalytic subunit

In mammals, there are four known isoforms of the Na,K-ATPase α-subunit. Alpha 1 is ubiquitously expressed throughout all the cells in the body and is thought to be the "housekeeping" isoform that establishes the transmembrane Na^+ and K^+ gradients. Alpha 2 has a more restricted expression and is expressed together with α1 in glial cells, smooth muscle cells, cardiac myocytes, and adipocytes. Several studies also indicate that α2 is expressed in neurons. During development, using immunohistochemistry and *in situ* mRNA hybridization, α2 was shown to be expressed in neurons throughout the brain (Moseley, Lieske et al. 2003). During maturation, the expression of α2 becomes limited to glial cells and a few types of neurons, for example to the neurons of the respiratory center

(Ikeda, Onimaru et al. 2004). Alpha 3 is predominantly neuronal with some also expressed in the ovaries, while α4 is only expressed in male gonadal tissues.

Alpha 1 – 3 share great amino acid homology (in humans there is 87 % homology and in rodents 86 %), but this is less so with α4. In spite of sharing more than 80 % amino acid homology, α1, α2 and α3 are different with regards to their ligand-binding affinities (see Table 1). Neurons express both α1 and α3, which is essential for recovery of the $[Na^+]_i$ following neuronal activity (Azarias, Kruusmagi et al. 2012). Yet glial cells express both α1 and α2, which may be associated with pulsatile $[Na^+]_i$ changes occurring in astrocytes during Na^+-coupled glutamate uptake. The catalytic α subunit consists of 10 transmembrane domains (TM). Three major cytosolic domains are recognized: the actuator domain, the nucleotide binding domain and the phosphorylation domain. The actuator domain includes the NH^{2+} cytosolic terminus (NT) and the second cytoplasmic domain (CD2) connecting TM2 and TM3.

Table 1. Na,K-ATPase ligand binding

	$K_{m(Na+)}$ mM	$K_{m(K+)}$ mM	$K_{m(ATP)}$ mM	IC50, ouabain nM
Alpha 1	12	0.83	3.5	48000
Alpha 2	22	0.79	3.3	58.5
Alpha 3	33	0.55	7.8	6.7

Data reviewed from following papers (Jewell and Lingrel 1991; Munzer, Daly et al. 1994; O'Brien, Lingrel et al. 1994; Zahler, Zhang et al. 1997)

Genetic knock-out homozygous mutants of the α1, α2 or α3 subunits, respectively, die in early embryonic development or at birth (James, Grupp et al.

1999; Barcroft, Moseley et al. 2004; Ikeda, Onimaru et al. 2004). While heterozygous α1 knock-out animals have been found to have increased locomotor response to methamphetamine and increased corticosterone in plasma (Moseley, Williams et al. 2007). Heterozygous α2 animals were reported to have increased anxiety, reduced spatial learning ability and late onset obesity (Kawakami, Onaka et al. 2005; Moseley, Williams et al. 2007). Heterozygous α3 mice have decreased spatial learning, and increased locomotor activity (Moseley, Williams et al. 2007).

1.7.2 Na,K-ATPase β subunit – chaperon protein and activity modulator

There are three isoforms of the β subunit. The β subunits show great diversity in amino acid sequence. The homology between the human beta subunits is about 35 %. The activity of the α catalytic subunit was shown to be differently modulated by the different β subunit isoforms, probably because the β subunit is important for occlusion of the K^+ ions (Morth, Pedersen et al. 2007). The β subunit also plays a major role in targeting the Na,K-ATPase to the PM, hence tightly regulating expression levels of the catalytic α subunit (Ackermann and Geering 1990; McDonough, Geering et al. 1990; Geering 2001). In the *Xenopus* oocytes expression system, all β subunit isoforms could bind to all isoforms of α subunit in a ratio 1:1 (Crambert, Hasler et al. 2000). However, co-immunoprecipitation studies showed that binding specificity was greater between α1 and β1 and between α2 and β2, respectively (Tokhtaeva, Clifford et al. 2012). The α and β subunits assemble readily in the ER via the help of the chaperone immunoglobulin binding protein BiP (Beggah, Mathews et al. 1996). Thus any misfolded α catalytic subunits cannot leave the ER. In the absence of the β subunit the α subunit interacts with β-COP, which also will result in retention of α in the ER (Morton, Farr et al. 2010).

1.7.3 Na,K-ATPase FXYD subunit – regulator of activity

In mammals there are 7 FXYD subunits. These subunits are rather small transmembrane proteins, most of which consist of only one transmembrane domain. The different isoforms of FXYD have tissue-specific expressions. They are not necessary for the catalytic function of Na,K-ATPase, but can modulate the enzyme by changing its binding affinities for ligands (for review see Geering 2006).

The tissue-specific and developmental expression patterns of the different α, β, and FXYD isoforms may regulate the activity of the Na,K-ATPase, supporting Na^+ and K^+ transmembrane gradients and thus cellular functions via ion homeostasis, co-transport, excitability and other functions.

1.7.4 Na,K-ATPase interactions

Na,K-ATPase is known to interact with numerous proteins in the PM, the sarcoplasmic reticulum, the Golgi, and caveolar - all allowing for regulated PM targeting and Na,K-ATPase function (Wang, Haas et al. 2004; Alves, Farr et al. 2010; Reinhard, Tidow et al. 2013).

Several functional transporting protein domains have been described for Na,K-ATPase α2, signifying the role of this subunit in influencing the function of transporters via alterations in the local ion balance. For example, a series of works describe a transporting protein complex consisting of the Na,K-ATPase α2 and the Na^+, Ca^{2+} exchanger (NCX) in cardiac myocytes, skeletal muscle and astrocytes (Juhaszova and Blaustein 1997; Lencesova, O'Neill et al. 2004). This protein complex was proposed to overlay the endoplasmic reticulum (ER) with its sarcoendoplasmic reticulum Ca^{2+}-ATPase pump (SERCA). Changes in α2

activity alter local Na^+ concentration and influence local Ca^+ concentration via the NCX exchanger, which may trigger calcium dependent signaling and global calcium events (Golovina, Song et al. 2003). The diffusion-limited spaces between the PM and the ER may be small and may be a significant co-factor in regulating Na^+ and Ca^{2+} concentrations and thus the Na,K-ATPase α2, NCX and SERCA transporting protein complexes.

Also, interaction between the Na,K-ATPase α2 and the neuron-specific K^+ and Cl^- co-transporter KCC2 was shown in neurons in the brainstem respiratory center (Ikeda, Onimaru et al. 2004). In this complex, α2 was proposed to influence the local K^+ gradient around the KCC2 transporter, thus supporting the extrusion of Cl^- from the cytosol of neurons. This transporting protein domain function may be crucial for generating the breathing rhythm.

In neurons, the Na,K-ATPase enzyme was shown to interact with the scaffolding protein PSD-95 via its NH_2-terminus (NT) (Blom, Ronnlund et al. 2011). PSD-95 is one of the main organizer proteins in the synapse, interacting with multiple receptors and ion channels in the postsynaptic membrane.

By interacting with IP3R, Na,K-ATPase is believed to trigger signaling cascades in the cell, among others leading to anti-apoptotic signaling (Zhang, Malmersjo et al. 2006; Liu, Spicarova et al. 2008; Liu and Xie 2010; Aperia 2012).

1.7.5 Na,K-ATPase mutations

Several mutations in Na,K-ATPase genes has been shown to be associated with neurological diseases. Mutations in the gene coding Na,K-ATPase α2 are associated with familial hemiplegic migraine type 2 (FHM2) (Bottger, Doganli et al. 2012). Mutations in Na,K-ATPase α3 are associated with rapid-onset dystonia parkinsonism (Bottger, Doganli et al. 2012), alternating hemiplegia of

childhood and CAPOS syndrome (cerebellar ataxia, areflexia, pes cavus, optic atrophy and sensorineural hearing loss) (Demos, van Karnebeek et al. 2014). Some of the mutations in Na,K-ATPase has been found to lead to a decrease in Na,K-ATPase α activity or inability to be expressed in the PM. For many of the mutations the biochemical consequences are yet unknown. There is no known disease associated to mutations in the Na,K-ATPase α1 isoform, probably due to its ubiquitous expression and vital importance for viability of any cell.

1.8 GLUTAMATE TRANSPORTERS

1.8.1 Glutamate transporter isoforms and expression

Upon excitatory neuronal activity neurons release the neurotransmitter glutamate into the synaptic cleft, where its concentration can rise from 25 nM up to 1 mM (Dzubay and Jahr 1999; Herman and Jahr 2007). Glutamate has to be rapidly removed from the extracellular space, mainly by uptake into surrounding astrocytes to avoid excitotoxicity (Rothstein, Dykes-Hoberg et al. 1996). Upon this uptake the $[Na^+]_i$ concentration in the astrocyte will rapidly increase, as glutamate is co-transported with Na^+, which in turn will activate the Na,K-ATPase.

To date five excitatory amino acid transporters (EAAT) are discovered. EAAT3, EAAT4, EAAT5 are expressed mainly in neurons and EAAT1 and EAAT2 in glial cells (corresponding to GLAST and GLT-1 in rodents). The expression of glial glutamate transporters is developmentally and brain region specifically regulated (Regan, Huang et al. 2007) Further, the expression is upregulated in cell cultures in the presence of neurons and neuron-released factors (Chaudhry, Lehre et al. 1995; Swanson, Liu et al. 1997; Perego, Vanoni et al. 2000; Yang, Gozen et al. 2009). Recent findings also indicate that neuronal activity influences

the expression density and the subcellular localization of glutamate transporters in the PM (Benediktsson, Marrs et al. 2012). In astrocytes glutamate transporters are targeted to the sites adjacent to neurons (Chaudhry, Lehre et al. 1995) and, specifically, cluster opposing synapsin-1 positive synapses (Benediktsson, Marrs et al. 2012).

Each glutamate is co-transported with 3 Na^+ ions and one H^+ ion and counter-transported with one K^+ ion (Levy, Warr et al. 1998; Owe, Marcaggi et al. 2006). Astrocytes respond with a large $[Na^+]_i$ increase during glutamate uptake, which is especially pronounced in the astrocyte processes (Langer and Rose 2009), indicating site-specific expression of glutamate transporters. This $[Na^+]_i$ signal may be a key to enhanced metabolism in the astrocyte following excitatory neuronal activity (Bernardinelli, Magistretti et al. 2004).

The direction of transport depends on the ligands concentrations as well as the MP (Szatkowski, Barbour et al. 1990; Billups and Attwell 1996). Thus glutamate transport may also have a reversed direction and transport glutamate out of the cell, if K^+ and Na^+ transmembrane concentration gradients change significantly, as may be the case in severe pathological conditions such as ischaemia (for review see Grewer, Gameiro et al. 2008). Reversal of the glutamate transporter direction is believed to contribute to the neuronal excitotoxicity by excessive release of glutamate into the extracellular space (Rothstein, Dykes-Hoberg et al. 1996; Rossi, Brady et al. 2007).

1.8.2 Glutamate transporters in disease

Excitotoxicity in the brain, resulting from excessive extracellular glutamate concentration contributes largely to neuronal degeneration. One of the suggested pathways for neurodegeneration, would be activation of glutamate receptor

NMDA on the neuron PM, and intracellular Ca^{2+} overload; and increase in NO and ROS, depolarization of mitochondrial membrane and decrease in ATP production (Yamagata, Andreasson et al. 1993; Bezzi, Carmignoto et al. 1998; Pepicelli, Fedele et al. 2002; Gu, Nakamura et al. 2010). Reduced expression or function of glutamate transporters is observed in several neurodegenerative conditions, such as ALS, AD, PD and Huntington's (for review see Seifert, Schilling et al. 2006; Foran and Trotti 2009; Estrada-Sanchez and Rebec 2012). Knock-down of the glial GLT-1 transporter in a mouse model results in elevated extracellular glutamate levels, spontaneous seizures, neurodegeneration and progressive paralysis (Tanaka, Watase et al. 1997).

1.8.3 Glutamate transporter interactions

In the PM the glutamate transporters are assembled into trimers, with three independent binding sites for glutamate. The glial glutamate transporters have been shown to interact with Na,K-ATPase (this thesis Paper II and Rose, Koo et al. 2009; Genda, Jackson et al. 2011). As mentioned above, the Na,K-ATPase establishes the required transmembrane Na^+ gradient for Na^+-coupled transport. It may be speculated that the expression of Na,K-ATPase isoform with relatively low Na^+ affinity is beneficial to govern the local $[Na^+]_i$ in the same transporting protein domain as the glutamate transporter. Several scaffolding proteins, e.g. NHERF-2, GFAP, Sept2, are known to interact with the C-terminal (CT) of the glial glutamate transporters to stabilize it in the PM and by this they may have a regulatory role upon glutamate uptake (Kinoshita, Kimura et al. 2004; Sullivan, Lee et al. 2007; Ritter, Asay et al. 2011)

1.9 FAMILIAL HEMIPLEGIC MIGRAINE

1.9.1 Familial Hemiplegic Migraine overview

Familial Hemiplegic Migraine (FHM) is a subtype of migraines inherited by an autosomal dominant mode. The first attacks occur in the first and second decade of life and are generally triggered by emotional stress, physical exertion or head trauma (Jurkat-Rott, Freilinger et al. 2004). The attacks are often associated with motor weakness (hemiplegic) and can be accompanied by aura – visual, motor and auditory disturbances. Aura is thought to arise from cortical spreading depression - a wave of neuronal and glial cells depolarization (Lauritzen, Dreier et al. 2011). The aura may last for 20 min, after which the headache occurs and may last for up to 72 h.

In some migraine patients severe conditions such as coma and cerebral edema, cerebellar ataxia, epilepsy and mental retardation have been described (Jurkat-Rott, Freilinger et al. 2004; Spadaro, Ursu et al. 2004; Vanmolkot, Stroink et al. 2006; Deprez, Weckhuysen et al. 2008).

Several genes are linked to the FHM: *CACNA1A*, which codes for the neuronal voltage gated Ca^{2+} channel $Ca_{V2.1}$ (leading to FHM type 1), *ATP1A2*, which codes for Na,K-ATPase α2 (leading to FHM type 2), and *SCN1A*, which codes for the neuronal voltage gated Na^{+} channel $Na_{v1.1}$ (leading to FHM type 3) (for review see Russell and Ducros 2011).

1.9.2 Molecular mechanisms for the Familial Hemiplegic Migraine type 2

FHM2 has been linked to more than 65 mutations in *ATP1A2*, the gene encoding Na,K-ATPase α2. These mutations are spread over the whole Na,K-ATPase molecule. The molecular mechanisms leading to FHM2 have not yet been

identified. It has been proposed that mutations in the Na,K-ATPase α2 subunit result in increased concentrations of extracellular glutamate and/or K^+, making the brain more susceptible to cortical spreading depression (Segall, Mezzetti et al. 2005; Russell and Ducros 2011). Bente Vilsen's group has extensively studied 9 different Na,K-ATPase α2 mutations and their affinities for external K^+ (Schack, Holm et al. 2012). Eight out of the 9 mutants showed higher affinities for K^+ than the wild type Na,K-ATPase α2 enzyme. All the mutants displayed a reduced turnover rate at saturating K^+ and Na^+ concentrations, suggesting that in a situation of cellular stress (i.e. epilepsy in which extracellular K^+ is high), FHM2 mutants may have reduced Na,K-ATPase activity. These findings correlate well with the onset of the FHM2 attack when overexcitation is followed by depolarization of neurons and glial cells.

One of the first FHM2 families described carried a Na,K-ATPase α2 missense mutation in the third transmembrane domain - G301R (Spadaro, Ursu et al. 2004). Patients' symptoms included hemiplegic migraine, seizures, prolonged coma, elevated temperature, sensory deficits, and transient or permanent cerebellar signs, such as ataxia, nystagmus, and dysarthria. Our collaborators in Århus, Denmark, designed a knock-in mouse model carrying the Na,K-ATPase α2 G301R mutation. We have used this model to test whether the Na,K-ATPase α2 FHM2 mutation displays alterations in cellular glutamate uptake. A reduced glutamate uptake may be predicted to increase the ambient glutamate concentration and thus contribute to neuronal overexcitation.

1.10 AQUAPORIN 4

1.10.1 Aquaporin 4 isoforms and expression

Aquaporins (AQPs) are PM protein channels permeable to water according to the transmembrane osmotic gradient. AQP4 is expressed in CNS, lung, kidney,

gastrointestinal system, skeletal muscle, salivary glands, adrenals, anterior pituitary, prostate and seminal vesicles (Mobasheri, Marples et al. 2007). In the brain AQP4 is expressed mainly in astrocytes (Nielsen, Nagelhus et al. 1997), but also in other type of glia, such as reactive microglia (Tomas-Camardiel, Venero et al. 2004). AQP4 has two main protein isomers which differ with regards to 23 amino acids at the NT and named according to their starting methionine: M1 and M23 AQP4 isoforms. In the PM AQP4 assembles into heterotetramers consisting of the M1 and/or M23 isoforms. An increased ratio of the M23 isoform promotes the formation of large orthogonal arrays from the tetramers (Furman, Gorelick-Feldman et al. 2003). The AQP4 amino acids V24, A25 and F26 located in NT, are responsible for the formation of large orthogonal arrays, by providing interactions between the tetramers, while the NT of the M1 isoform disrupts the orthogonal array formation (Suzuki, Nishikawa et al. 2008; Crane and Verkman 2009). It has also been observed that the M1 isoform surrounds the core of M23 built orthogonal arrays (Rossi, Moritz et al. 2012). In astrocytes the large orthogonal arrays are often found in the perivascular endfeet of the astrocyte PM (Nielsen, Nagelhus et al. 1997; Rash, Yasumura et al. 1998) and are thought to be important for water balance at the blood-brain barrier.

The water permeability of aquaporins can be dynamically regulated by exogenous and endogenous factors via mechanisms involving intracellular signaling and probably protein phosphorylation (for review see Gunnarson, Zelenina et al. 2004; Zelenina 2010). Astrocyte AQP4 water permeability has been found to be dynamically regulated by glutamate and K^+ (Gunnarson, Zelenina et al. 2008; Gunnarson, Song et al. 2009; Song and Gunnarson 2012).

Reduced astrocyte AQP4 expression has an expected effect of reduced transmembrane water permeability (Solenov, Watanabe et al. 2004), but studies

have also reported delayed K^+ and glutamate clearance (Binder, Yao et al. 2006; Zeng, Sun et al. 2007), enlarged extracellular space (Yao, Hrabetova et al. 2008), increased water content in the brain (Haj-Yasein, Vindedal et al. 2011), increased coupling between the astrocytes (Strohschein, Huttmann et al. 2011) as well as changes in synaptic plasticity, impaired hearing and smell (for review see Papadopoulos and Verkman 2013). Altered PM water permeability will likely influence neurotransmitter and ion homeostasis, which may lead to the described pathologies. Compensatory mechanisms may however facilitate molecular diffusion by increased astrocyte *syncytium* connectivity and enlarged extracellular space.

Nevertheless, AQPs are not the only channels that can permeate water through the PM. In fact, most transmembrane transport is accompanied by water molecules, and each ion transported can be surrounded by around 120-180 molecules of water. The glutamate transporter, has been shown to increase its permeability for water by 40 % during glutamate treatment (MacAulay, Gether et al. 2002), which could be one of contributors to astrocyte swelling observed during glutamate uptake.

1.10.2 AQP4 in disease

Many studies have focused on the role of astrocyte AQP4 in brain edema and brain injury. During brain edema AQP4 seems to play opposing roles, as it facilitates astrocyte swelling and cytotoxic brain edema formation, but may also be important in the resolution of brain edema and reduce swelling in the brain parenchyma (for review see Papadopoulos and Verkman 2007). AQP4 is known to be the target in the neurological disease neuromyelitis optica, an autoimmune disorder which affects the optic nerve and spinal cord, leading to edema and inflammation, myelin loss and neurodegeneration (for review see Saadoun and

Papadopoulos 2010; Ratelade and Verkman 2012). In a few pathological conditions the expression and polarity of AQP4 in astrocytes is altered. In an ALS model decreased expression of AQP4 and increased expression of Kir4.1 in astrocytes of $SOD1^{G93A}$ mice was detected, which may alter K^+ homeostasis and lead to neurodegeneration (Bataveljic, Nikolic et al. 2012). For glioma – an aggressive tumor, loss of cell polarization is accompanied by AQP4 orthogonal arrays degradation and AQP4 tetramers re-distribution (Neuhaus 1990). Interestingly, glioma cells often express both AQP4 and AQP1. Their expression is thought to enhance glioma migration (McCoy, Haas et al. 2010).

1.11 METABOTROPIC GLUTAMATE RECEPTORS GROUP I

During neuronal activity, several astrocyte G-protein coupled receptors are activated: metabotropic glutamate receptors (mGluRs), purinergic and adenosine receptors, GABAB receptors, which trigger signaling events to modulate astrocyte function (Kang, Jiang et al. 1998; Wang, Lou et al. 2006; Cunha 2008). Here I will focus on the group I mGluRs receptors – mGluR1 and mGluR5, and their known regulatory function in astrocytes. Group I mGluRs are a $G\alpha_{q/11}$ bound receptors and their signaling cascade includes PLC activation and IP3R mediated Ca^{2+} signaling cascades as well as activation of PKC via diacyl-glycerol and phosphorylation of downstream protein targets. The signaling cascades started by group I mGluRs activation have well described long-term effects in neurons, regulating mRNA transcription and thus synaptic plasticity. Increasing data implicate group I mGluRs in the regulation of astrocyte function and in the cross-talk between astrocytes and neurons. Activation of group I mGluRs in astrocytes in a hippocampal slice preparation enhances astrocyte K^+ and glutamate uptake within minutes via a PKC dependent pathway (Devaraju, Fiacco 2013). Glutamate uptake is also acutely upregulated in reactive

astrocytes, mediated by mGluR5 activation (Vermeiren, Najimi et al. 2005). AQP4 water permeability is increased in primary astrocytes and hippocampal slice preparation upon group I mGluRs agonist treatment (Gunnarson, Zelenina et al. 2008). Group I mGluR induced Ca^{2+} responses in astrocytes were found to be increased in relation to decreased neuronal activity, while increased neuronal firing rates depressed mGluR-triggered Ca^{2+} signaling (Xie, Sun et al. 2012). One can speculate that astrocyte group I mGluRs can modulate several astrocyte functions and that its signaling is finely tuned in response to changes in neuronal activity.

The scaffold associated proteins for anchoring of the mGluR5 in neurons are well explored; among them are Homer, NHERF2, Shank and syntaxin (for review see Hall and Lefkowitz 2002). Whether similar scaffolding proteins also anchor mGluRs in the astrocyte PM remains to be identified. Via scaffolding proteins mGluRs may be linked to functional protein complexes, allowing for rapid, local signaling events involving phosphorylation and Ca^{2+} modulating events.

2 METHODOLOGICAL CONSIDERATIONS

2.1 ETHICAL APPROVAL

Animal care and experimental procedures were conducted in accordance with Swedish animal protection legislation and experimental protocols approved by the Northern Stockholm Laboratory Animal Review Board.

2.2 PRIMARY ASTROCYTE AND NEURON CELL CULTURES

In this thesis astrocyte enriched culture or mixed astrocyte-neuron primary culture derived from either striatum or hippocampus was used. The selection for culture with either astrocytes enriched culture or astrocyte and neurons mixed culture was achieved with growth medium. It has become clear that astrocytes are most developed morphologically and functionally in the presence of neurons or neuron conditioned medium, see for example Paper III Fig. 2. To date the majority of astrocyte primary culture studies are performed on cultures where neurons were deliberately eliminated, leaving the astrocytes without their functional counterparts – neurons. With advancing imaging technologies and increased number of astrocyte and neuronal markers it is of benefit to study differentiated astrocytes function in the environment with neurons or neuron conditioned medium.

For the Paper II neuron conditioned medium (NCM) was applied to astrocyte culture to achieve higher differentiation of astrocytes. Their morphology changed into cells with longer processes. In agreement with previously published studies NCM addition for 24 h had increased the expression of glutamate transporter GLAST (Drejer, Meier et al. 1983; Schlag, Vondrasek et al. 1998; Figiel and Engele 2000). The NCM addition had no effect on the expression of Na,K-ATPase. It has been proposed that Na,K-ATPase α2 is upregulated in glial

cell in presence of neurons (Knapp, Itkis et al. 2000). I didn't observe any change in expression of either α1 or α2 after addition of NCM for 24-48 h. Possibly, the up-regulation of α2 requires longer time or other factors.

2.3 OVEREXPRESSION OF EXOGENOUS PROTEINS

For all studies in this thesis an approach of exogenous protein overexpression was used to visualize and functionally study the proteins of interest. For the Paper II several fluorescently tagged Na,K-ATPase proteins were expressed. The fluorescent tag was placed at the NT of α subunits, to allow for unaltered α subunits targeting to the PM. The codons of the first 5 amino acids in the NT of the Na,K-ATPase were removed in the DNA construct, as these amino acids are cleaved in the posttranslational modifications in the cell (Feschenko and Sweadner 1995). The function of Na,K-ATPase α1 and α2 subunits in the dynamic regulation of $[Na^+]_i$ was studied by overexpressing either α1 or α2 in primary astrocytes. Red fluorescent protein mCherry was co-expressed with either α1 or α2, however was not directly tagged to the α subunit. This ensured, that the α activity wasn't biased by any tag, but allowed identification of the transfected cells by red fluorescent signal in the cytosol. In the field of view cells lacking mCherry signal were identified as non-transfected cells. Often, however the mCherry signal was dim, possibly because mCherry was placed after the Na,K-ATPase α sequence in the IRES plasmid which may have resulted in a weaker expression.

2.4 PLASMA MEMBRANE PROTEIN CO-EXPRESSIONS

In the second study (Paper II), co-expression of Na,K-ATPase α1 and α2 isoforms with glutamate transporter GLAST was measured. Previous studies showed co-distribution of Na,K-ATPase α2 and glutamate transporter

predominantly in perisynaptic astrocyte processes using immunostaining and electron microscopy (Cholet, Pellerin et al. 2002). Here I used a different approach to study co-localization of Na,K-ATPase isoforms with GLAST, which does not rely on antibody staining specificity. I expressed Venus tagged α1 or α2 together with mTurquoise-tagged GLAST in primary astrocyte culture from striatum. Overexpression of the protein of interest with a fluorescent tag allows for live cell imaging and avoidance of unspecific antibody staining as well as preservation of cell morphology. However, it places other questions, for instance, the relevance of overexpression versus endogenous protein expression levels.

2.5 IMAGING OF INTRACELLULAR SODIUM

Several fluorescent Na^+-selective indicators are suitable for measurements of $[Na^+]_i$: Na^+-binding benzofuran isophthalate (SBFI), CoronaGreen, CoronaRed, Asante Natrium. They functionally differ by their K_d for Na^+, their ion selectivity, sensitivity to pH and temperature, the linear range of measurement, and quantum yield of the fluorescent indicator. All of these factors must be considered while choosing the appropriate $[Na^+]_i$ measurement tool and performing the experiments.

SBFI was developed in 1989 in the laboratory of professor Roger Tsien (Harootunian, Kao et al. 1989), it has since been widely used for measurement of $[Na^+]_i$ concentration in various cell types. It is determined to have a high resolution fluorescence response to the $[Na^+]_i$ concentrations at the range of 0-40 mM (Fig. 1A), which is within the physiological astrocyte $[Na^+]_i$ concentrations. The dissociation constant of SBFI for Na^+ is 11.3 mM.

We chose SBFI over other Na^+ indicators for its ratiometric properties. Upon Na^+ binding the excitation of SBFI shifts to shorter wavelengths. It allows comparing emissions at both excitation wavelengths 340 nm and 380 nm and to calculate a ratio as an internal normalization control of fluorescent indicator leakage and bleaching, as well as minor focus changes. Also, we chose this indicator for suitable range of $[Na^+]_i$ sensitivity and well established procedures for calibration *in situ*. Focus stability of recording is important for monitoring dynamic changes in $[Na^+]_i$ and a ratiometric indicator is beneficial for increased stability of the measurements.

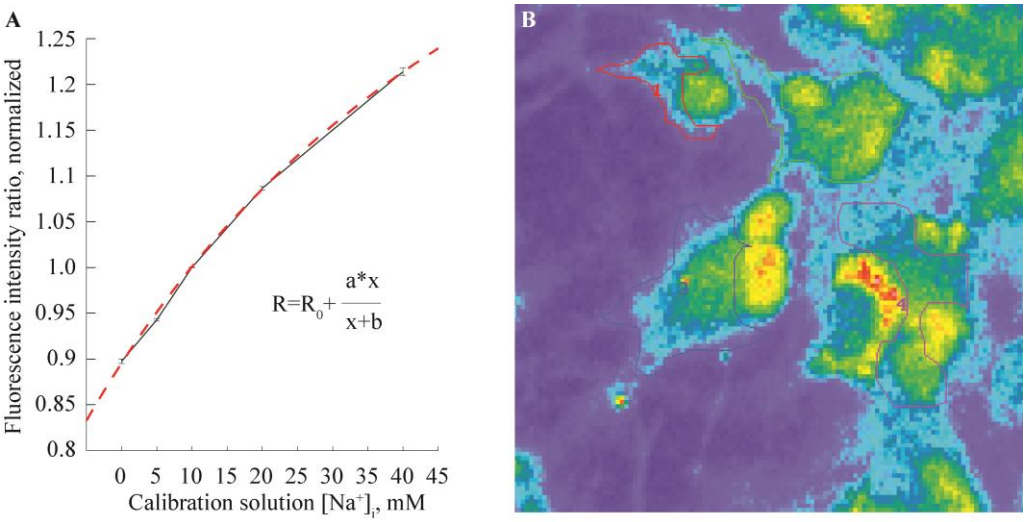


Figure 1. Intracellular sodium concentration measurement using SBFI. A. Fluorescence intensity ratio 340/380 nm was fit (R^2>0.95) into right rectangular hyperbola function (the formula insert). **B.** For SBFI fluorescence intensity measurement, regions of interest were selected for each cell in the field of the view. Region of interest was selected to avoid the nucleus as shown with color outline.

To load the SBFI into the astrocytes, its acetoxymethyl ester modification (AM-SBFI) was used, which allows for transmembrane transfer of Na^+ indicator. One

of the draw-backs with AM indicators is that though they are strongly loading in the soma of the astrocyte, they often have weaker loading in the astrocyte processes. Therefore the intracellular ion measurements using the acetoxymethyl ester SBFI (AM-SBFI) are mostly possible in the cell soma. To measure the $[Na^+]_i$ in the astrocyte processes PM an impermeable Na^+ indicator may be loaded with a patch pipette, filling also the distant processes and providing sufficient fluorescent intensity for measurement.

New generation $[Na^+]_i$ indicators have wider $[Na^+]_i$ range and sensitivity as well as usually higher quantum yield, resulting in a better signal/noise ratio. For example, Asante NaTRIUM Green 2 with $K_{d(Na+)}$=20 mM, allows for linear $[Na^+]_i$ changes in the range 0-60 mM.

2.5.1 Cytosolic SBFI calibration

SBFI calibration is best performed *in situ*, since this indicator is sensitive to its environment, such as pH and K^+ concentration. Therefore *in situ* calibration was performed after each recording with 0, 5, 10, 15, 20, 40 and when necessary 90 mM Na^+ solutions plus ionophores and 2 mM ouabain.

Several publications indicate linear change of SBFI fluorescence intensity in the 0-50 mM $[Na^+]_i$ range. In my recordings the calibration curve was better fit into a right rectangular hyperbola function: $R = R_0 + ((a*x)/(x+b))$, where R_0, a and b are coefficients obtained from the fit (adapted from Zahler, Zhang et al. 1997). $[Na^+]_i$ was calculated by a formula $[Na^+]_i = (R - a)/(b+a - R)*R_0$ (Fig. 1A).

The calibration was performed for each recording, rather than using a master calibration curve, which allowed for better sensitivity of the assays.

2.6 OUABAIN: AN ISOFORM SPECIFIC INHIBITOR OF NA,K-ATPASE

Cardiotonic steroids (CTS), such as ouabain, are specific inhibitors of Na,K-ATPase, binding to an extracellular pocket and blocking the pumping activity of the enzyme. Cardiotonic steroids extracted from plants have since long been used to treat congestive heart failure. In the human body also low amounts of several endogenous CTS are identified, for example marinobufagenin and digoxin, and there is growing evidence for their involvement in regulation of blood pressure, cell differentiation and apoptosis and other novel functions (for review see Bagrov, Shapiro et al. 2009). In rodents Na,K-ATPase α1 has relatively low affinity for ouabain, unlike in all other mammals, while α2 and α3 have relatively high ouabain affinities, approximately 1000 fold greater than α1. The IC50 value for ouabain is 48 μM for rat α1 and 58 nM for rat α2 (O'Brien, Lingrel et al. 1994). Therefore, in several experimental setups using rodent primary cultures ouabain was used to selectively block α2 and to separate its function from total Na,K-ATPase pool.

2.7 PROTEIN-PROTEIN INTERACTION ASSAYS

To test for protein-protein interaction several biochemical and imaging assays can be employed. Several of these assays are used in this thesis: Co-IP (suitable for whole protein-protein interactions); Pull-down (suitable for defining the motif of interaction); FRET (suitable for *in situ* whole protein-protein interaction). There are other methods, that are not used in this thesis, such as Yeast two hybrid assay (suitable for large screenings for interaction partners in yeast libraries) or Co-IP combined with mass spectrometry analysis (suitable for protein-protein interaction screening), as well as surface plasmon resonance (suitable for characterizing interaction kinetics of direct protein-protein

interactions) All of these methods have their strong and weak sides, which will be discussed below. Thus it is important to use a combination of methods when identifying and characterizing a protein-protein interaction.

2.7.1 Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) assay allows for full size protein-protein interactions to be tested for. The assay relies on specificity of antibody used for immunoprecipitation of protein of interest - "bait" protein. To control for specificity of antibody interaction a control assay is performed in the same conditions, but instead of primary IP antibody, an IgG antibody is used raised in the same species as the IP antibody. As protein source I used total rat brain lysate in a RIPA buffer with mild detergents, such as Na^+-deoxycholate and Triton-X 100. Mild detergents allowed for optimal PM solubility and preservation of protein structure, allowing for protein-protein interactions. A similar buffer was also used in the pull-down assays (see below). Using brain lysate as a protein source may allow for protein complexes to be pulled out including associated proteins, such as scaffolding proteins and other members of the complex. In this setup Co-IP cannot give an answer to whether the interaction is direct or indirect. The Co-IP is a useful tool when testing for a predicted protein-protein interaction. When a screening for unknown/novel interaction partners is done, then Co-IP may be followed up by mass spectrometry analysis of the Co-IP sample.

2.7.2 Pull-down assay

Pull-down assay allows further to characterize interaction between proteins and to map the interacting domains. In this assay whole "bait" proteins or protein parts are amplified in E.coli, purified and set on the beads to pull out interacting

proteins from total protein lysate. Since the “bait” protein is synthesized from a construct expressed in E.coli, it allows modifying “bait” protein sequence and deducing the potential binding site between the interacting partners. When total protein lysate is used as a source of interacting proteins, the interaction may also be mediated via other proteins, thus pulling out a whole complex. However, when the “bait” protein is purified and mixed with purified potential partner, the direct protein-protein interactions can be established.

Often a 3D structure of a protein is important for mediating protein-protein interactions. If only part of protein was used as “bait”, the interaction should be confirmed by full-length protein interaction assays, such as Co-Immunoprecipitation or *in situ* Förster resonance energy transfer (FRET).

2.7.3 Förster resonance energy transfer

One of the techniques used to study protein-protein interactions in a cell PM is FRET. This technique is based on a process by which one fluorophore (the donor) while excited can transfer energy, without emitting a photon, to its neighboring fluorophore (the acceptor) and bring it to the excited state to emit light. The donor emission and acceptor excitation spectra should overlap for the energy transfer to occur. Also, two fluorophores should be in a close proximity to each other (~10 nm) and the spatial orientation of the donor and acceptor against each other is important for the energy transfer to occur. For classical FRET, several controls and system preparations should be performed to control for biases to the accuracy of measurements. Unwanted direct excitation of the acceptor while exciting the donor and signal “bleed-through” of the emission spectra of donor into the emission spectra of the acceptor may give false positive results. To tackle these issues in this study we have used a modified FRET technique, acceptor photo bleaching FRET, which helped us resolve some of

these biases due to the internal normalization procedure. Upon recording the donor emission, the acceptor fluorescence is irreversibly photo bleached, which if FRET was present increases emission efficiency of donor. By this technique the bleed-through problem is partially managed. It also eliminates the need for pre-experimental calibrations of the donor and acceptor spectra. For our study of AQP4 and Na,K-ATPase expression in the PM we tagged AQP4 with Venus and Na,K-ATPase was stained with antibodies tagged with Cy3.

2.8 CELLULAR GLUTAMATE UPTAKE

To measure glutamate uptake in primary cultures Tritium labeled D-Aspartate (^{3}H D-Asp) uptake was used. D-Asp is transported into the cell by the same EAAT/glutamate transporters as glutamate. To separate the uptake function from other glutamate triggered events in the cell the glutamate is commonly substituted with D-Asp. EAATs Km value for glutamate is around 50-100 μM, and is similar to the Km for D-Asp (Arriza, Fairman et al. 1994; Swanson, Liu et al. 1997). When relative comparisons are made between the groups, it is possible to use this indicator of glutamate uptake. To date the radioactively labeled D-Asp assay is probably one of the most sensitive ways to measure D-Asp uptake in a biochemical method. There are several FRET based indicators for measurement of extracellular glutamate concentrations (Hires, Zhu et al. 2008; Marvin, Borghuis et al. 2013). For intracellular glutamate there are yet no fluorescent indicators.

2.9 PLASMA MEMBRANE WATER PERMEABILITY ASSAY

For this thesis we have used live cell imaging recordings for PM water permeability measurements, previously established in our laboratory (Zelenina and Brismar 2000; Zelenina, Zelenin et al. 2002). This method is based on

imaging of a confocal cross-section of a cell filled with the fluorescent indicator – calcein. By quickly changing the extracellular solution osmolality from 300 mOsm to 200 mOsm water enters the cells and dilutes the calcein and its fluorescence intensity decreases accordingly in the selected intracellular section of the cell. The initial rate of fluorescence decrease is proportional to the water permeability of the PM. Primary astrocytes have a high water permeability of their PM, due to the endogenous expression of AQP4.

3 AIMS OF THE THESIS

The overall aim of this thesis was to investigate the molecular and functional protein-protein interactions of Na,K-ATPase in astrocytes.

Specific aims:

- To explore the interaction between the putative members of transporting protein domain in astrocytes: Na,K-ATPase, AQP4 and mGluR5 and to investigate its functional regulation in intact astrocytes.
- To investigate the molecular and functional interactions between the Na,K-ATPase α subunits and glutamate transporters (GLT-1 and GLAST) in astrocytes and to characterize dynamic changes in $[Na^+]_i$ in response to glutamate exposure.
- To characterize glutamate uptake in astrocytes expressing mutant Na,K-ATPase α2 - a model of familial hemiplegic migraine type 2.

4 SUMMARY, RESULTS AND BRIEF DISCUSSION

In current work we identified several molecular and functional interaction partners for the Na,K-ATPase in astrocytes. Our findings indicate that the astrocyte Na,K-ATPase is implicated in functional complexes of importance for the brain function, including water, ion and glutamate homeostasis. The data support that the Na,K-ATPase can form macromolecular complexes that may function as transporting protein domains in astrocyte PM.

4.1 PAPER I

In the first study we tested for protein-protein interaction between the Na,K-ATPase and AQP4. The rationale for the study was based on the previous studies indicating a supportive role of the AQP4 water channel in K^+ homeostasis associated with neuronal activity (Amiry-Moghaddam, Williamson et al. 2003) and the important role of Na,K-ATPase in K^+ uptake (D'Ambrosio, Gordon et al. 2002). We hypothesized that protein interaction between the Na,K-ATPase and AQP4 may exist in the astrocyte PM, maintaining local osmotic balance during transmembrane K^+ currents.

4.1.1 AQP4 interacts with Na,K-ATPase via its NH_2- cytosolic terminus

The full-length AQP4 was found to co-immunoprecipitate with full-length Na,K-ATPase α1 and mGluR5, a previously known regulator of AQP4 water permeability (Gunnarson, Zelenina et al. 2008). Using pull-down assays we identified the site of the AQP4 protein that interacts with the Na,K-ATPase α1 subunit. We localized that the binding site between the AQP4 and the Na,K-ATPase was in the NH_2- cytosolic terminus (NT) of the AQP4 (Paper I Fig. 2). NT of AQP4 protein differs between the M1 and M23 isoforms of AQP4, however the critical amino acids for this interaction found to be in the common

part of the M1 and M23 isoforms. Similarly, the Na,K-ATPase α2 and the mGluR5 receptor were also found to interact with AQP4 NT. The findings suggest the existence of a common, intermediate scaffolding protein bound to the NT of AQP4, which mediates the assembly of this complex. To date however, no scaffolding proteins binding to the NT of AQP4 are identified.

4.1.2 AQP4 interaction with Na,K-ATPase *in situ*

To confirm that this interaction also occurs *in situ*, Föster resonance energy transfer (FRET) studies were performed. Full-length AQP4 tagged with the fluorescent protein Venus (FRET Donor) was overexpressed in primary astrocytes. Endogenous Na,K-ATPase was immunolabeled with antibodies tagged with fluorochrome Cy3 (FRET Acceptor). The FRET efficiency, as discussed in section 2, is detectable when donor and acceptor are within ~10 nm distance. Mutagenesis studies directed to the identified key amino acids for interaction on AQP4 reduced the FRET efficiency by half, confirming the protein-protein interaction between AQP4 and Na,K-ATPase. Since the acceptor was tagged indirectly to Na,K-ATPase, via primary and secondary antibodies, this would imply that the actual interacting partners (AQP4 and Na,K-ATPase) could be further than 10 nm apart. It should be noted that we, by these methods, could not rule out the presence of an intermediate, scaffolding protein mediating this interaction.

The full-length AQP4 subunit was found to co-immunoprecipitate with the full-length Na,K-ATPase α1 and α2 and the mGluR5, but not with the GLT-1 glutamate transporter. In the Paper II in this thesis the Na,K-ATPase and glutamate transporter GLT-1 were found to co-immunoprecipitate. This may indicate different protein compositions in the macromolecular complexes containing Na,K-ATPase, suggesting that AQP4 and GLT-1 may interact with

different pools of Na,K-ATPase in the PM. Functionally though, AQP4 and GLT-1 may still be linked in a macromolecular complex, as supported by the findings that GLT-1 expression was reduced upon exposure to antibodies in neuromyelitis optica targeting AQP4 (Hinson, Roemer et al. 2008) as well as the reduction of GLAST expression found in an AQP4 knock-out model (Zeng, Sun et al. 2007).

4.1.3 Astrocyte transporting microdomain

We proposed that the protein-protein complex with Na,K-ATPase, AQP4 and mGluR5 in astrocytes may be implicated in the recovery of extracellular K^+ concentrations following neuronal activity. The relative contributions of astrocyte K^+ channels and transporters (Na,K-ATPase, Kir4.1, NKCC1, KCC) in the K^+ uptake from the extrasynaptic space is currently still debated (for review see Macaulay and Zeuthen 2012). In physiological conditions the Na,K-ATPase may play the key role for the K^+ inward currents, while the Kir4.1 regulates the basal extracellular K^+ concentration (D'Ambrosio, Gordon et al. 2002). Findings in a recent paper support the importance of astrocyte Na,K-ATPase in the recovery of extracellular K^+ (Larsen, Assentoft et al. 2014). During neuronal activity astrocytes will take up not only K^+ but also neurotransmitters with co-transported and counter-transported ions. During this transport osmotic imbalance may occur, for which purpose water flow through AQP4 would be beneficial.

4.1.4 Regulation of Na,K-ATPase and AQP4 complex via mGluR5

In the described macromolecular complex, the water flow via AQP4 permeability may be regulated by glutamate via activation of the complex "partner" mGluR5. We found that short treatment with glutamate enhanced

AQP4 water permeability (Paper I Fig. 6A). This was in accordance with our previous finding that activation of group I mGluRs increase AQP4 water permeability in astrocytes, mediated by an intracellular signaling cascade and phosphorylation of AQP4 (Gunnarson, Zelenina et al. 2008). Interestingly, group I mGluRs activation has also been reported to enhance astrocyte glutamate and K^+ uptake (Devaraju, Sun et al. 2013). While the increased glutamate uptake was attributed to PKC phosphorylation of glutamate transporter, the mechanism for the observed enhancement of K^+ currents was unclear. We tested the short term effect of the group I mGluRs agonist DHPG on Na,K-ATPase activity, by measuring changes in $[Na^+]_i$ (Paper I Fig. 6B). We observed no change in $[Na^+]_i$ which may imply that group I mGluRs activation does not influence Na,K-ATPase activity. Group I mGluRs mediated increase in AQP4 water permeability may also indirectly facilitate K^+ uptake. The astrocyte transporting protein complex between the Na,K-ATPase, AQP4 and mGluR5 may serve efficient extracellular K^+ homeostasis and its function may be enhanced during neuronal activity via mGluR5.

4.2 PAPER II

4.2.1 Na^+-coupled glutamate uptake is dependent on α1 and α2 Na,K-ATPase

In the second study we investigated the role of Na,K-ATPase α1 and α2 in supporting astrocyte glutamate uptake. The α2 isoform only or α1+α2 were selectively inhibited by the use of isoform-specific ouabain concentrations. We then measured ^{3}H D-Aspartate (^{3}H D-Asp) uptake as an estimate for glutamate uptake in primary astrocytes. When the α2 isoform was selectively inhibited we observed a decrease in glutamate uptake that was almost equal in magnitude to the decrease found when both α1+α2 were inhibited: D-Asp uptake was

decreased by 23 % upon inhibition of α2 (using 1 μM ouabain for 15 min) and by 26 % upon inhibition of α1 and α2 together (using 2 mM ouabain for 15 min). These results indicated that the Na^+-coupled D-Asp uptake was equally dependent on the activity of both α1 and α2 pumps in primary striatum astrocyte culture. In cortical primary astrocytes as well as in cortical gliosomes short-term ouabain treatment (10 nM – 1 μM) caused increase of D-Asp uptake (Rose, Koo et al. 2009; Matos, Augusto et al. 2013). Previous findings indicated that glutamate uptake can be short-term modulated via signaling through mGluR5a receptor in reactive cortical astrocytes, which appears to lead to a higher number of mainly GLT-1 in the PM (Vermeiren, Najimi et al. 2005). The mechanism of short-term regulation of astrocyte glutamate uptake may be different between different brain regions, which could explain why we didn't observe this mechanism.

4.2.2 Na,K-ATPase α2 inhibition causes moderate $[Na^+]_i$ increase

All Na^+-coupled transport, such as glutamate uptake into astrocytes, is majorly dependent on the transmembrane Na^+ gradient. We therefore proceeded to measure changes in $[Na^+]_i$ upon α1 and α2 inhibition, respectively. Somewhat surprisingly, selective inhibition of α2 only caused a modest, 2 mM, increase in $[Na^+]_i$ (Paper II Fig. 1C), which was disproportional to the decrease in glutamate uptake (23 %) caused by α2 inhibition. There exists a Na^+ gradient between the soma and the astrocyte processes in response to synaptic stimulation (Langer and Rose 2009). The α2 subunit together with glutamate transporter can localize to astrocyte perisynaptic microdomains (Cholet, Pellerin et al. 2002) and may significantly contribute to Na^+ homeostasis in astrocyte processes and leaflets. In the current study $[Na^+]_i$ was measured in the astrocyte soma, due to limitations in the cell loading of the Na^+ sensitive indicator AM-SBFI. Thus it is possible, that

$[Na^+]_i$ actually was higher in the astrocytes processes. The somatic $[Na^+]_i$ increase corresponds with the data obtained on cortical primary astrocyte cultures, where 500 nM ouabain treatment resulted in 2 mM $[Na^+]_i$ increase (Golovina, Song et al. 2003). There could be also other explanation to the small increase in $[Na^+]_i$ that we observed here. At rest, when basal astrocyte $[Na^+]_i$ is around 10 mM, the Na,K-ATPase α2 will be operating at less than 10 % of its maximum rate (K_{mNa} = 22 mM). Therefore it may have a modest contribution in the regulation of basal $[Na^+]_i$ levels.

4.2.3 Dynamic changes in $[Na^+]_i$ upon glutamate treatment, effect of overexpression of α1 or α2

We proceeded to study how α1 and α2 maintain $[Na^+]_i$ homeostasis in astrocytes when exposed to glutamate. We recorded the changes in $[Na^+]_i$ in primary astrocytes overexpressing either the α1 or the α2 subunit. This allowed us to study the relative importance of each endogenous isoform as a driving force for glutamate uptake. Extrasynaptic glutamate concentrations can reach 1 mM upon vesicular glutamate release from firing neurons (Dzubay and Jahr 1999). We choose to use glutamate concentration of 200 µM, to saturate glutamate transport, as the $K_{m(glutamate)}$ is around 50 µM for glutamate transporters (Swanson, Liu et al. 1997). Exposure to glutamate 200 µM caused a larger increase in $[Na^+]_i$ in α1 than in α2 overexpressing cells and, as a consequence, the recovery time for $[Na^+]_i$ was shorter in cells overexpressing α2 (Paper II Fig. 3). The results suggest that α2 may be more efficient than α1 to rapidly restore a large transient increase in $[Na^+]_i$, which may be related to the relatively low Na^+ affinity of α2 (K_m for $[Na^+]_i$ is 12 mM for α1 and 22 mM for α2). Intracellular Na^+ dynamics was also recorded during treatment with lower glutamate concentration of 50 µM. We observed similar tendencies in results for 50 µM as

also for 200 µM glutamate treatment, i.e. lower maximum $[Na^+]_i$ increase and faster recovery for the α2 expressing astrocytes in comparison with the α1 expressing astrocytes (Fig. 2A and 2B).

The $[Na^+]_i$ reaches a plateau after glutamate treatment (Paper II Fig. 3A and other studies Chatton, Marquet et al. 2000; Barat, Boisseau et al. 2012). The intracellular sodium plateau probably reflects that an equilibrium for Na^+ uptake, mainly through glutamate transporters, and counteracting Na^+ extrusion by the Na,K-ATPase is achieved. Astrocytes can, as we showed, successfully sustain large $[Na^+]_i$ increase. The maximum top level, however, is lower for the α2, than the α1 expressing astrocytes, again probably due to a relatively low Na^+ affinity for the α2 isoform.

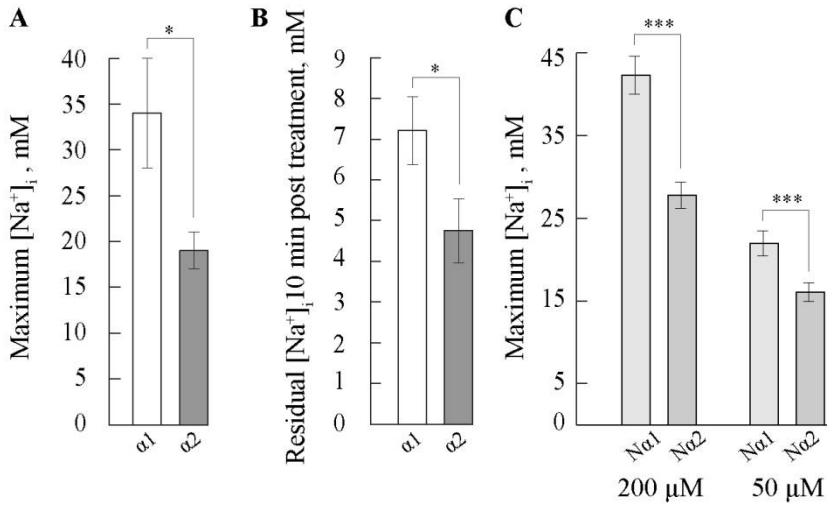


Figure 2. Transient changes in $[Na^+]_i$ following glutamate treatment in astrocytes expressing predominantly the Na,K-ATPase α1 or α2 isoforms. A. Maximum $[Na^+]_i$ after 5 min exposure to glutamate 50 µM in α1 (white) and α2 (grey) expressing astrocytes (Mann-Whitney U test, N=17, * P<0.05). **B.** Intracellular sodium concentration recovery after discontinuation of glutamate 50 µM treatment in α1 (white) and α2 (grey) expressing astrocytes (Mann-Whitney U test, N=17, * P<0.05). **C.** Maximum $[Na^+]_i$ after exposure to glutamate 200 or 50 µM in non-transfected astrocytes from the same field of view as α1 and α2 transfected cells - Nα1 and Nα2, respectively (One-way ANOVA, N=54-106, *** P<0.001). Data are presented as mean ± SEM.

The non-transfected cells from α1 and α2 transfected plates (Nα1 and Nα2 correspondently) were also analyzed. Following glutamate treatment maximum $[Na^+]_i$ was lower in Nα2 cells, than in Nα1 cells (Fig. 2C). It may be that some cells were connected via gap junctions so that the transfected phenotype influenced $[Na^+]_i$ dynamics in neighboring cells. There may also have been more transfected cells in the optical field of view, when the expression of mCherry indicator was weak.

4.2.4 Isoform specificity of Na,K-ATPase α interactions with glutamate transporters

Molecular interactions between the Na,K-ATPase and glutamate transporter have been reported in a few studies using co-immunoprecipitation and immunoprecipitation of GLT-1 from rat cortex followed by liquid chromatography-coupled tandem mass spectrometry (Rose, Koo et al. 2009; Genda, Jackson et al. 2011). However, these studies report opposing results for Na,K-ATPase α subunit interaction specificity with the glutamate transporter. Genda and Jackson et al. found that in rat cortex GLT-1 immunoprecipitated with Na,K-ATPase α1, α3 and β1; but not α2 while Rose and Koo et al. showed in cerebellum co-immunoprecipitation of only α2 and α3 with glutamate transporters. We performed co-immunoprecipitation from the whole brain lysate and found interaction of glutamate transporters with both α1 and α2. It is possible that in different brain regions these interactions would appear differently and therefore may be region and isoform specific.

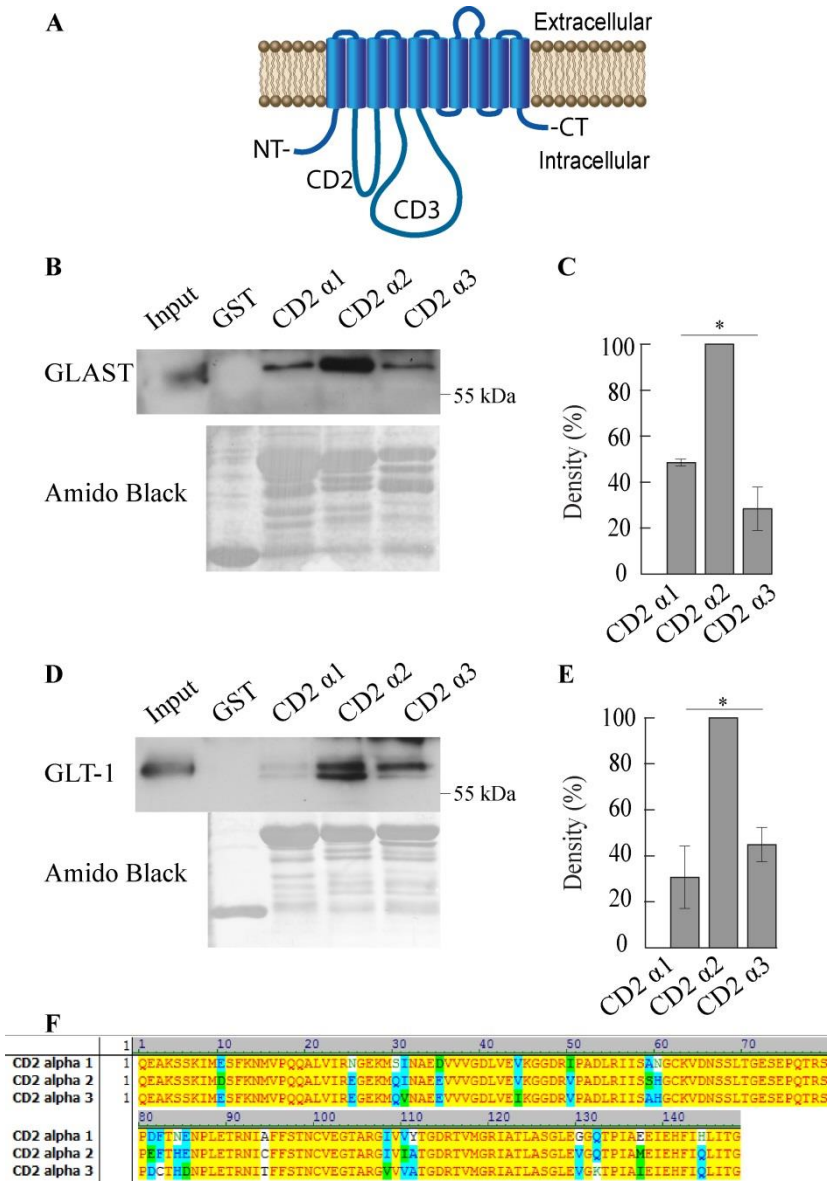


Figure 3. Na,K-ATPase α subunit CD2 domain was found to interact with both glial glutamate transporters – GLT-1 and GLAST. A. Schematic representation of Na,K-ATPase α subunit embedded into the PM; NT-, CD2, CD3 and -CT are selected domains used in pull-down assay. **B.** and **D.** The interaction between glutamate transporter and the CD2 α2 domain is stronger than the interaction with the CD2 domains of Na,K-ATPase α1 or α3. **C.** and **E.** Mean integrated densities for glutamate transporter immunostaining in GST pull-down assays of CD α1, CD α2 and CD α3, respectively, in percent of CD2 α2 staining density. (One-way ANOVA, N=3, * P<0.05). **F.** Homology alignment map between the CD2 domain of rat α1, α2 and α3 isoforms. Alpha 1 and α2 share 90 % homology, α1 and α3 share 88 % homology, while α2 and

α3 share 92 % homology. Identical amino acids marked yellow; conservative – blue; similar – green; non-similar – no color.

We continued to identify the binding domains for the glutamate transporter interaction with the Na,K-ATPase α subunits. Pull-down studies were performed using different parts of the Na,K-ATPase protein as "bait" to pull down Na,K-ATPase interacting protein partners. The Na,K-ATPase molecule was screened for potential binding sites, selecting all large loops of the molecule for "bait" (Fig. 3A): NT, CD2, CD3, CT. We found interaction between both glutamate transporters GLAST and GLT-1 and the CD2 domain of all three Na,K-ATPase α subunits: α1, α2 and α3 (Paper II Fig. 4 and Fig. 3B, 3D). This is probably, at least partly, attributed to the high homology that exists in the CD2 domain between these isoforms (Fig. 3F). There appeared to be a stronger binding between the α2 and both glutamate transporters, compared to the α1 and α3. The reason for this is unclear, however it may imply that several protein interaction domains involved in the α2 CD2 interaction with glutamate transporters, which could explain stronger binding of α2 to the glutamate transporter. The exact amino acids involved in the protein interactions still remain to be identified.

To evaluate the co-expression of Na,K-ATPase and the glutamate transporter GLAST in the PM we performed confocal imaging of co-expressed GLAST with either the α1 or the α2 subunit in primary astrocytes (Paper II Fig. 4I). Co-localization of both α1 and α2 with GLAST was observed. Cultured primary astrocytes are less polarized than in the intact brain, since endothelial cells and synaptic contacts are reduced or absent. Immunogold studies have indicated that glutamate transporters are concentrated in astrocyte leaflets, adjacent to neuronal PM (Chaudhry, Lehre et al. 1995), and α2 has been found to be mainly localized in leaflets facing glutamatergic synapses (Cholet, Pellerin et al. 2002). It is

possible that the interaction between the α2 and the glutamate transporters is relatively stronger than for the other α subunits, which in differentiated astrocytes in the brain may result in co-localization in the subcellular domains of leaflets facing glutamatergic synapses. The combined molecular and functional interactions between astrocyte α2 and the glutamate transporters, as shown in this thesis, may thus enable efficient regulation of $[Na^+]_i$ during glutamate uptake following neuronal activity.

4.3 PAPER III

4.3.1 Glutamate uptake in primary cultures derived from hippocampus of FHM2 knock-in mouse model

The molecular consequences of Na,K-ATPase α2 dysfunction in FHM2 are not well understood. Migraine attacks are usually preceded by a spreading wave of glial and neuronal depolarization, possibly caused by altered glutamate and/or K^+ uptake from the extracellular space (Leo, Gherardini et al. 2011). In this study D-Asp uptake (as a measure of glutamate uptake) was measured in primary cultures derived from hippocampus of the FHM2 knock-in G301R mutant mouse model. Two culture types were used; either astrocyte enriched cultures or mixed cultures of astrocytes and neurons. D-Asp uptake was reduced in the mixed astrocyte-neuron cultures derived from E17 G301R heterozygous (HEZ) (by 10 %) and homozygous (HOZ) (by 17 %) mouse brains as compared to WT (Paper III Fig. 3D). Interestingly, in the astrocyte enriched cultures, no significant change in D-Asp uptake was found between the genotypes (Paper III, Fig. 3C). It is difficult to ascertain the reasons for the difference between the two cultures. Immunolabeling against GFAP revealed morphological changes in astrocytes when co-cultured with neurons - the astrocytes were larger, with more extensive branching (Fig. 4). These astrocytes are possibly more differentiated, due to the

exposure to neuron-released factors as well as direct interactions with neurons in the mixed cultures. The absolute counts per minute of radioactively labeled ^{3}H D-Asp, normalized to protein concentration, was 2.9 times higher in the astrocyte and neuron mixed cultures, than in the astrocyte enriched cultures. This could probably reflect a higher expression level of Na,K-ATPase α2 in astrocytes cultured in the presence of neurons (Paper III Fig. 3A and 3B), but also likely a higher number of glutamate transporters participating in the uptake. Higher CPM value, increases signal to noise ratio and therefore the sensitivity of the assay may be higher for the mixed culture in comparison with enriched culture. It is also plausible that the neuronal glutamate transporter (EAAT3) contributes to the changes observed, due to secondary effects of the Na,K-ATPase α2 mutation on neuronal glutamate uptake.

Inefficient glutamate uptake may lead to overexcitability in the brain (Seifert and Steinhauser 2013). Apart from neurotoxic events it may lead to excess of excitatory neuronal activity in different areas of the brain. For instance, Na,K-ATPase α2 knock-out mice and W887R FHM2 mutant knock-in mice display greater anxiety levels than WT mice, which may be related to overexcitability in amygdala (Ikeda, Onaka et al. 2003; Leo, Gherardini et al. 2011). Na,K-ATPase α2 mutation may also result in insufficient K^{+} uptake and increased neuronal susceptibility to excitation.

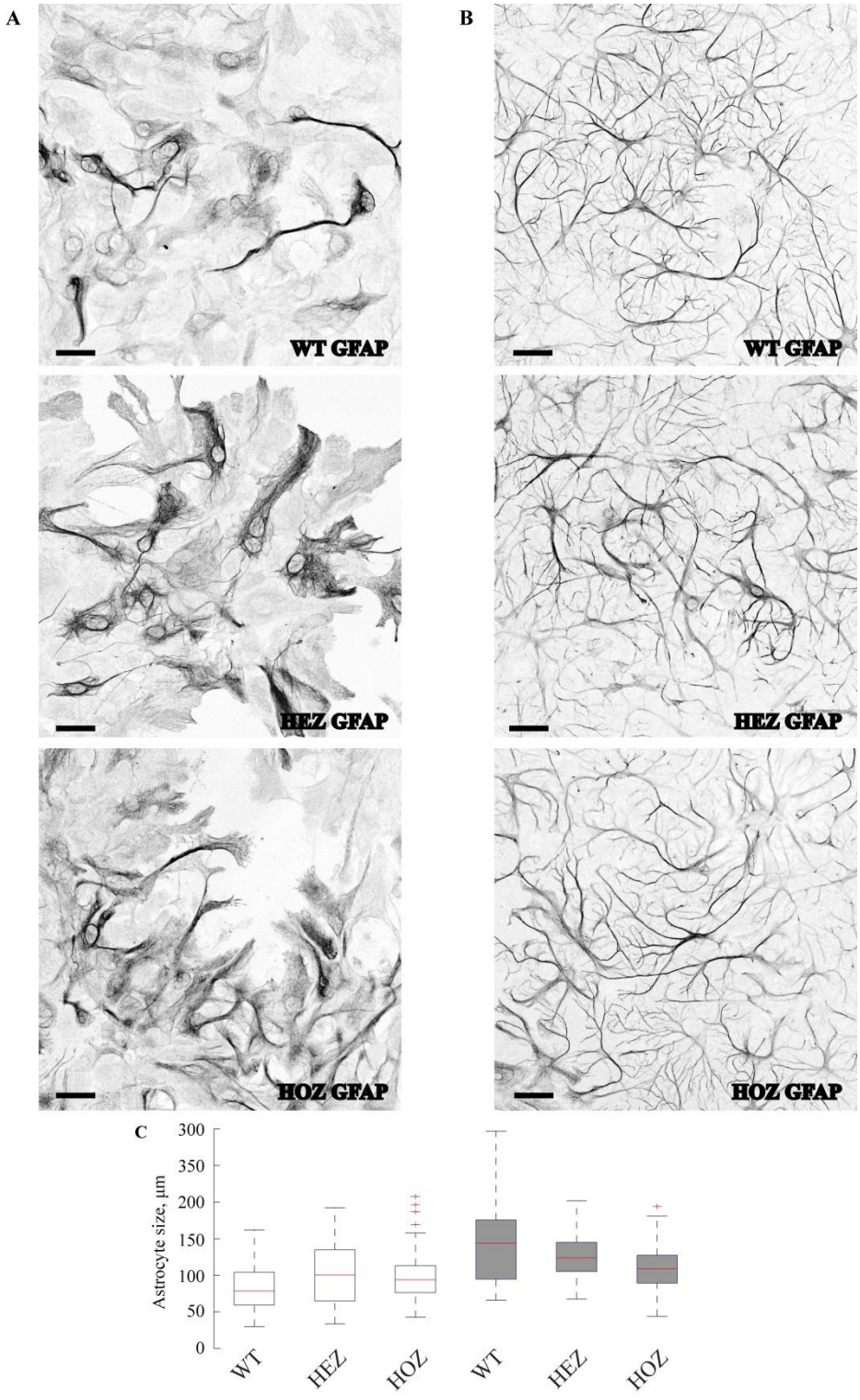

A
B
WT GFAP
WT GFAP
HEZ GFAP
HEZ GFAP
HOZ GFAP
HOZ GFAP
C
Astrocyte size, µm
300
350
200
150
100
50
0
WT
HEZ
HOZ
WT
HEZ
HOZ

Figure 4. Astrocyte GFAP immunostaining. A. Astrocyte enriched culture GFAP immunolabeling. Scale bar 40 μm. **B.** Astrocyte neuron mixed culture GFAP immunolabeling. **C.** Boxplot diagram of astrocyte diameter in μm (median value, interquartile range and minimum and maximum values) in astrocyte enriched cultures (white) and astrocyte and neuron mixed cultures (grey) from WT, HEZ and HOZ G301R α2 mutant mice. Data collected from 2 individual experiments. Number of astrocytes in each group N=40-60.

4.3.2 Na,K-ATPase a2 G301R mutant plasma membrane expression

In the G301R knock-in mouse we found significant downregulation of the Na,K-ATPase α2 G301R in embryonic (E17) and in adult (P50) brain (Paper III, Fig. 1). There are contradictory reports regarding the targeted expression of G301R in the PM. Santoro, Manganelli et al. reported biased expression of Na,K-ATPase α2 G301R mutant in the PM of transfected HeLa and HEK293 cells using imaging and biochemical techniques (Santoro, Manganelli et al. 2011). However, in *Xenopus* oocytes the Na,K-ATPase α2 G301R mutant was found to be normally targeted to the PM, but to have reduced activity (Tavraz, Durr et al. 2009). Possibly the expression system used may influence the expression of Na,K-ATPase on the PM. We used rat astrocyte derived cell line and primary astrocyte mouse cultures to determine the PM expression of Na,K-ATPase α2 (Fig. 5A and 5B). The expression of the Na,K-ATPase α2 G301R mutant was reduced in the PM in comparison to α2 WT, as shown by the fluorescence intensity distribution between the PM and cytosol using Venus-tagged α2 (Fig. 5D and 5E). If the mutant G301R is not targeted to the PM at all there will be a difficulty to accurately identify the edge of the cell where the PM should be (Fig. 5C), thus the reduction in the PM/cytosol ratio could be even greater. Reduced PM targeted expression of the mutant α2 could be due to incorrect folding of Na,K-ATPase G301R α2 and/or interaction with chaperone proteins, resulting in premature disruption of the protein.

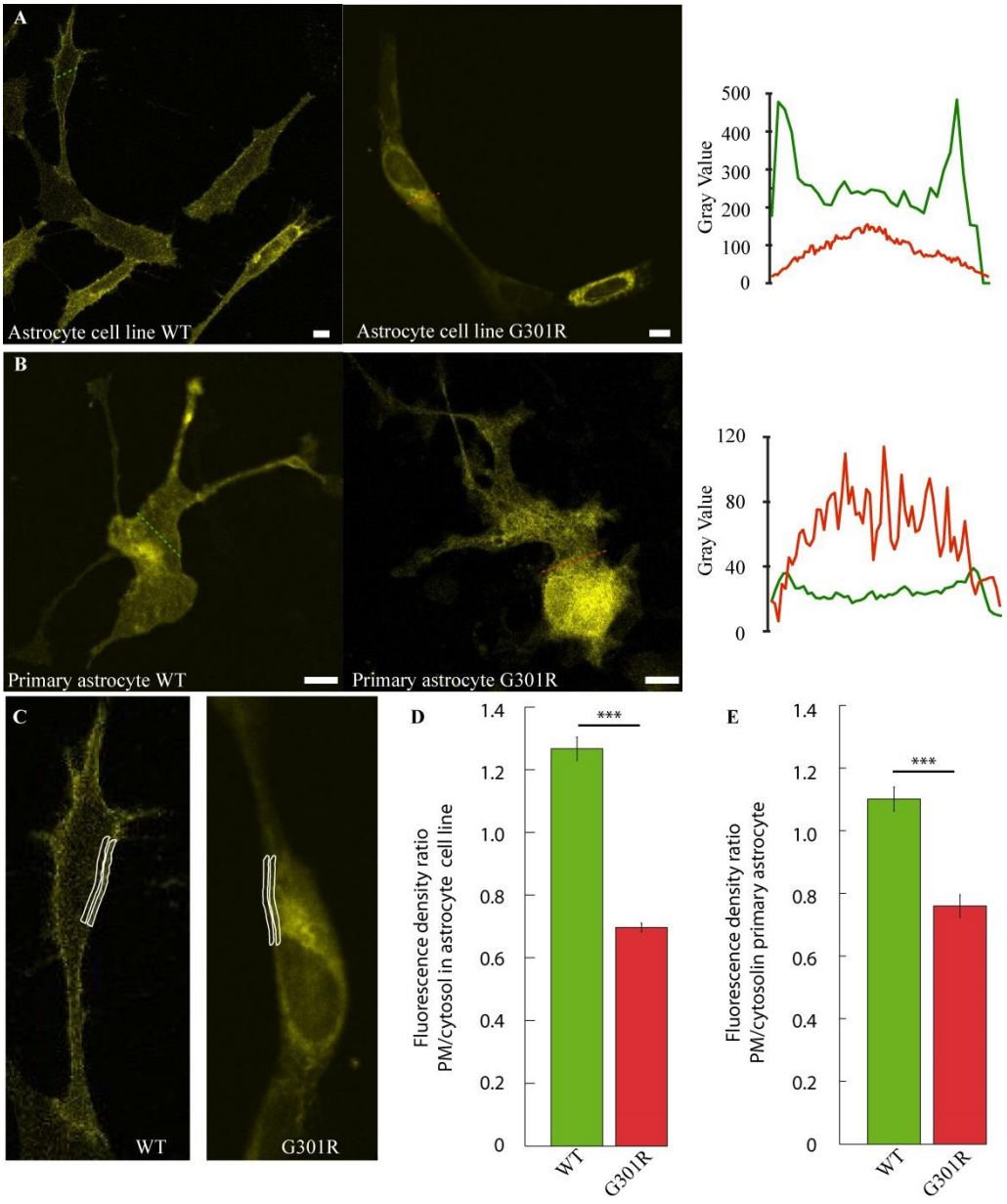


Figure 5. Hindered plasma membrane expression of Na,K-ATPase α2 G301R mutant. A. Confocal live cell image of Na,K-ATPase α2 WT and G301R expression in astrocyte cell line. Insert shows distribution of signal across the section of a cell, which is indicated with dashed lines: α2 WT (green) or α2 G301R mutant (red). Scale bar 10 μm. **B.** Confocal live cell image of single primary astrocyte cell expressing either Na,K-ATPase α2 WT or G301R. Insert shows distribution of signal across the section of a cell, which is indicated with dashed lines: α2 WT (green) or α2 G301R mutant (red). **C.** Confocal live cell image of single cell expressing either Na,K-ATPase α2 WT or G301R. Regions of interest for each transfected cell were selected on a

border of the cell and adjacent region in the cytosol. **D-E.** Ratio of these signals was taken for normalization and is compared between α2 WT and G301R.

Several FHM2 mutations were reported to have impaired PM expression: delK935-S940, S966 frame shift, P979L, W887R (Tavraz, Durr et al. 2009; Leo, Gherardini et al. 2011). The first knock-in mouse model generated for FHM2 mutation was the W887R. The homozygous mice died just after birth, and heterozygous mice had decreased threshold for cortical spreading depression (Leo, Gherardini et al. 2011). Already in the embryonic brain (E19.5) the Na,K-ATPase W887R mutant was downregulated to barely detectable level. In the HeLa expression system the W887R mutant was retained in the endoplasmic reticulum to a large extent. It is possible that the phenotype of the G301R knock-in mouse model studied in this thesis, due to its altered expression in the PM, will be similar to the heterozygous W887R knock-in model and α2 heterozygous knock-out model. In conclusion, it may be that mutant Na,K-ATPase α2 may not express fully in the PM and instead of dysfunctional α2 in the PM may create an effect of haploinsufficiency, i.e. the absence of protein activity of one of two alleles.

4.4 GENERAL DISCUSSION

In this thesis I explored astrocyte plasma membrane protein complexes with focus on the role of the Na,K-ATPase. Na,K-ATPase is a key enzyme for the cell viability and it is not surprising that its function is linked to the function of several other PM and intracellular proteins. Molecular interactions between functionally linked proteins are often beneficial for efficient and optimal activity of protein complexes. Through the work with this thesis it has become clear that Na,K-ATPase is participating in a variety of different protein complexes in the astrocyte PM also with regard to Na,K-ATPase catalytic subunit specificity.

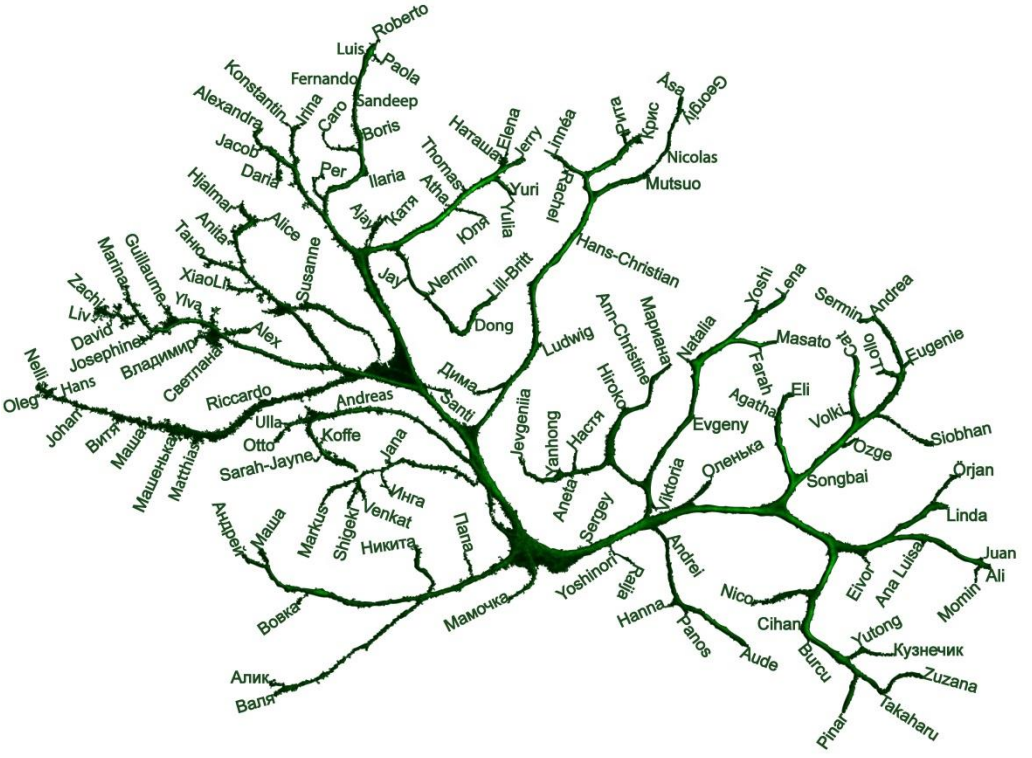
We have found a molecular link between the Na,K-ATPase and AQP4 in the astrocytes PM. We propose that this complex may be important for the water and K^+ homeostasis. Moreover the complex was found to be short-term regulated by the group I mGluR via regulation of AQP4 increase in water permeability, which shows a potential to respond to neuronal activity. It may be beneficial for the astrocyte ion homeostasis during excessive K^+ uptake, for which Na,K-ATPase is a key enzyme, to maintain osmotic balance by increased permeability of AQP4.

Astrocytes, as few other cell types in the body express not only the ubiquitous Na,K-ATPase α1 subunit, but also a glia specific α2. Unlike cells with only the ubiquitous α1 subunit, astrocytes experience pulsatile changes in $[Na^+]_i$ during excitatory activity of nearby neurons. The released neurotransmitter is effectively taken up by astrocytes via Na^+-coupled transporters. We suggest that the uneven inward Na^+ currents require for astrocytes to express an additional second Na,K-ATPase α subunit with relatively low apparent Na^+ affinity to ensure efficient maintenance of $[Na^+]_i$ during excessive $[Na^+]_i$ load. Spatial

proximity of Na,K-ATPase to glutamate transporters may also be required to maintain basal $[Na^+]_i$ and retain functional glutamate uptake.

Rare genetic diseases, such as FHM2, give us insight into the molecular mechanisms of pathology and disease development. With the identification of several genetic mutations expressed in neurons and astrocytes it becomes more clear that the migraine types have a neurological component and are complex disorders involving several systems. Novel mutations discovered in Na,K-ATPase α2 and the coupling to glutamate transporters and efficient glutamate uptake identifies new potential molecular drug targets for migraine treatment and gives better understanding of molecular mechanisms in disease processes.

5 ACKNOWLEDGEMENTS



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