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STUDIES ON AQUAPORIN 4, A MOLECULAR DETERMINANT OF BRAIN WATER HOMEOSTASIS

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Water is life's mater and matrix, mother and medium.
There is no life without water.
Albert Szent-Györgyi
1937 Nobel Price in Medicine

Til mine gutter

ABSTRACT

A well controlled brain water homeostasis is of utmost importance for an appropriate control of neuronal activity. Astrocytes express the water channel aqauporin 4 (AQP4) and play a key role for the maintenance of brain water homeostasis. Brain edema is a result of perturbed water homeostasis and involves astrocyte swelling. Emerging evidence supports that AQP4 is important in physiological potassium clearance, but also that AQP4 aggravates brain edema. The overall aim of this study has been to study the regulation of water permeability in astrocytes with particular emphasis on the role of AQP4.

In study I we presented a new; third form of mouse AQP4 mRNA not previously known. It was found to be predominantly expressed in brain and to be developmentally regulated. The finding may be relevant for the understanding of regulation of water homeostasis in the immature brain.

In study II and III we investigated the short term-regulation of astrocyte water permeability and the role of two substances associated with perturbed water homeostasis, the heavy metal lead and the neurotransmitter glutamate. Lead and glutamate were both found to induce a significant increase in astrocyte water permeability, an effect attributable to an effect on AQP4. The molecular target for the effects of lead and glutamate was identified as the AQP4 serine 111 residue, which is in a strategic position for control of water channel gating. The glutamate effect was mediated by activation of group I metabotropic glutamate receptors (mGluRs), intracellular calcium oscillations, activation of CaMKII and the NOS pathway. NOS can activate cGMP/PKG. We could show that PKG phosphorylates a peptide corresponding to I96-C123 of WT AQP4. It is suggested that the glutamate-effect on AQP4 play a physiological role, but also that the lead- and glutamate-triggered stimulation of water transport in AQP4-expressing astrocytes may contribute to adverse effects on brain water metabolism.

Erythropoietin (EPO) is neuroprotective in several models of brain injury that may induce cytotoxic brain edema as a secondary event. In study IV we found that EPO and an EPO-derivate significantly reduced the neurological symptoms in a rodent model of primary brain edema. We showed that EPO counteracts the effect of glutamate, mediated by mGluRs, on AQP4 water permeability in astrocytes. Our data indicate that EPO modifies the fast intracellular calcium oscillations caused by activation of mGluRs. Thus EPO may reduce the risk of astrocyte swelling in stroke and other brain insults.

In conclusion, this thesis demonstrates that astrocyte water permeability can be dynamically regulated via changes in the opening state of AQP4. The data support that the phosphorylation state of a serine residue in the first intracellular loop of AQP4 determines the opening state of AQP4 and that the regulation involves a calcium signaling pathway including CaMKII, NOS and PKG. We propose that up-regulation of astrocyte AQP4 water permeability is involved in the development of cytotoxic brain edema, and that inhibition of astrocyte AQP4 activity may offer a new therapeutic strategy in situations of perturbed brain water homeostasis.

LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers that will be referred to by their Roman numerals:

I Zelenin S, **Gunnarson E**, Alikina T, Bondar A, Aperia A.

Identification of a New Form of AQP4 mRNA That is Developmentally Expressed in Mouse Brain. *Pediatric Research* 48(3): 335-339, 2000.

II **Gunnarson E**, Axehult G, Baturina G, Zelenin S, Zelenina M, Aperia A.

Lead induces increased water permeability in astrocytes expressing AQP4. *Neuroscience* 136, 105-114, 2005.

III **Gunnarson E**, Axehult G, Song Y, Zelenina M, Bondar A, Brismar H, Zelenin S, Aperia A.

Identification of a molecular target for glutamate regulation of astrocyte water permeability. *Submitted for publication.*

IV **Gunnarson E**, Kowalewski JM, Aizman O, Axehult G, Zelenina M, Brismar H, Brines M, Cerami A, Andersson U, Aperia A.

Erythropoietin modulation of astrocyte water permeability as a potential component of neuroprotection. *Manuscript.*

LIST OF ABBREVIATIONS

AQP3	aquaporin 3
AQP4	aquaporin 4
AQP4 S111A	mutated aquaporin 4 with Ser111 substituted to alanine
Asialo-EPO	(desialylated) asialo- erythropoietin
BIM	bisindolylmaleimide
CaMKII	calcium(Ca^{2+})/calmodulin-dependent protein kinase II
CEPO	carbamylated erythropoietin
DDAVP	1-Deamino(8-D-arginine) vasopressin
DHPG	(*S*)-3,5 dihydroxyphenylglycine
DMEM	Dulbecco's Modified Eagle's Medium
DMSA	meso-2,3-dimercaptosuccinic acid
EPO	erythropoietin
FBS	fetal bovine serum
GFP	green fluorescent protein
GFAP	glial fibrillary acidic protein
L-NAME	Nω-nitro-L-arginine methyl ester
mGluR	metabotropic glutamate receptor
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
P_f	water permeability
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
WT	wild type

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INTRODUCTION

This thesis aims to elucidate some aspects of the regulation of water permeability in astrocytes with particular emphasis on the role of aquaporin 4 (AQP4) for brain water homeostasis. My personal goal as a clinician, working with children, is to be a good doctor for the patient. In this lies that I want to cure, soothe and help. This goal has also been my motivation for doing research. I believe that basic research may open up possibilities for new therapies or reveal new principles for therapeutic approaches. However, when starting this project I could not imagine that it would bring me "down to" the level of individual amino acids. This is so far from the clinic, it seems. Yet, I have realized along the way that in order to understand mechanisms in biology in general and, specifically, for the aim of this thesis it is necessary to study processes on the molecular level or beyond that.

Clinical background

The importance of normal brain water homeostasis has been acknowledged for many years. Several clinical conditions lead to perturbations in the fine-tuned balance of distribution of water within the different intracranial compartments. Disturbed water homeostasis leading to brain edema has crucial impact on both morbidity and mortality. Head trauma, stroke, hypoxia/ischemia, infections, intoxications, tumors and water overload can all lead to brain edema. Brain edema may be viewed as a final common pathway of several, in particular of acute, neurological conditions. The subsequent increase in intracranial pressure put the tissue at risk through impaired cerebral perfusion and is potential life threatening due to the risk of herniation.

Head trauma is the leading cause of mortality in childhood. Children are particular prone to develop diffuse cerebral swelling after head trauma. The reason for this susceptibility is not known (Bauer and Fritz, 2004, Noppens and Brambrink, 2004).

It is well documented that diffuse brain edema, observed on CT scans, often leads to substantial neurological sequela and is associated with a high mortality rate. In trauma care the concept of a primary brain injury followed by a secondary injury, caused by brain edema and increased intracranial pressure (ICP), is well established (Bayir et al., 2003). In the Nordic countries, the incidence of stroke exceeds 100,000 per year. Cerebral edema often follows severe cerebral hypoxia-ischemia in adults and is common in severe hypoxic-ischemic encephalopathy (HIE) in neonates (Whitelaw and Thoresen, in: The newborn brain. Cambridge University Press 2002). Severe HIE is a major cause of death and disability in neonates and children (Robertson and Finer, 1985). Other major health problems that involve perturbations of ion and water homeostasis in brain include hydrocephalus, epilepsy, and syndrome of inappropriate ADH secretion (SIADH). Disturbances in S-Na^+ levels are among the most common electrolyte disturbances and are frequently seen in postoperative or intensive care settings. Severe hyponatremia leads to hyponatremic encephalopathy, the clinical manifestation of cerebral edema, and is associated with a poor prognosis, particularly in women and children (Fraser and Arieff, 1997, Achinger et al., 2006).

In general, neurology as a discipline continues to lag behind other disciplines when it comes to the range and efficacy of medical treatment. Brain edema, followed by an increase in intracranial pressure, is commonly the direct cause of death after stroke and head trauma and contributes significantly to the morbidity of those patients who survive. Despite the magnitude of this health problem and its severe clinical consequences, the treatment of brain edema is mainly based on empirical grounds and has remained largely unchanged for the past decades. It is generally acknowledged that the lack of specific drugs for edema treatment reflects, at least partly, the incomplete understanding of the molecular mechanisms involved in the formation and resolution of brain edema (Papadopoulos et al., 2002). The current principles of treatment for brain edema include reduction of intracranial pressure, maintenance of cerebral blood flow and increased reabsorption of fluid (Grande et

al., 2002). In spite of elaborate intensive care and pharmacological therapies, surgical removal of brain tissue is sometimes required to avoid herniation and death (Albanese et al., 2003).

As a pediatrician with special interest in the neurological field, I have experienced the detrimental effects of brain edema and other neurological conditions in children. The need for new therapies is, in particular, urgent for brain edema, but even within other areas of neurology new principles for therapy should be welcomed.

Brain water and ion homeostasis

The concept of homeostasis—i.e., that all living things maintain a constant internal environment—was first suggested by Claude Bernard, a 19th-century French physiologist (Encyclopædia Britannica). Homeostasis is described as "the property of an open system, especially living organisms, to regulate its internal environment to maintain a stable, constant condition, by means of multiple dynamic equilibrium adjustments, controlled by interrelated regulation mechanisms". The term was coined in 1932 by Walter Cannon from the Greek homoios (same, like, resembling) and stasis (to stand, posture) (source: Wikipedia). The major functions important in the maintenance of homeostasis are fluid and electrolyte balance, acid-base regulation, thermoregulation, and metabolic control.

Water accounts for about 75% at birth and gradually declines to about 50-60 % of body mass in adults. From fetal life and during maturation also the distribution of water changes from a relative higher proportion of extracellular fluid compared to intracellular fluid in early life. These aspects require intricate and fine-tuned systems governing the exchange of water and ions in the body. The brain water and ion homeostasis is extremely complex and the description of all aspects of this is beyond the scope of this introduction. However, some important points should be made. Normal brain function is tightly coupled to water homeostasis. On the

macroscopic level, brain water homeostasis can be viewed as the balance between the production and drainage of cerebrospinal fluid. This is of great importance in the case of various types of hydrocephalus. There is presumably continuous transport of water in neurons, astrocytes and other cells accompanying glutamate and potassium transport, although *in situ* or *in vivo* such water movements are not readily measured. Magnetic resonance imaging (MRI) or diffusion weighted imaging, from which an apparent diffusion coefficient (ADC) can be obtained, are today available for average measurements of water contents in the brain *in vivo* (Fatouros and Marmarou, 1999). Indirect assessment of water movement may also be accomplished in animal models *in vivo* using confocal microcopy (Binder et al., 2004). However, *in vitro* studies of regulation of water transport in the individual cell types in the brain should offer valuable information of the mechanisms and targets for this regulation.

At the microscopic level, water transport is involved in cell volume regulation and in controlling the dimensions of the extracellular space (Sykova and Chvatal, 2000, Nagelhus et al., 2004). The extracellular space is narrow, as most of the volume between neurons is occupied by astrocytes. Modulation of water transport across the plasma membrane of neurons and glial cells will affect the concentration of ions and other solutes in the extracellular fluid and subsequently affect neuronal excitability. The ionic transmembrane shifts that are required to maintain ion homeostasis during neuronal activity must be accompanied by water, as neurons respond very sensitively to changes in the ionic composition in the extracelluar space. This is in particular true for K^+ and H^+ ions concentrations. Extracellular K^+-concentrations in the mammalian is normally around 3mM. It is crucial for a normal function of neurons that the increases in K^+-concentrations that occur during neuronal excitation, seizures and hypoxia or ischemia are rapidly and accurately restored to normal levels (Walz and Hertz, 1983). This potassium buffering is mainly taken care of by astrocytes and is mediated both through astrocyte cytoplasm and gap junctions between astrocytes (Kofuji and Newman,

2004, Leis et al., 2005). In the case of K^+-uptake into astrocytes, the excess K^+-ions are temporarily sequestered into the glial cells by transporters or K^+-channels. A net uptake of K^+ must be accompanied by water. Water can pass the plasma membrane by three distinct mechanisms; either by specialized water channels; aquaporins, by cotransport with ions (MacAulay et al., 2004) or by diffusion through the lipid bilayer. While the last process is slow and cannot be regulated, water transport via aquaporins is fast and may be regulated.

Water and ionic homeostasis is regulated by hormones in the body as well as in the brain. In the brain, centrally released neuropeptides such as vasopressin (AVP), ANP, and angiotensins appear to regulate fluid and ionic environment (Simard and Nedergaard, 2004). There is evidence that AVP plays a crucial role in the regulation of both brain water and of ion homeostasis, and these effects are mainly mediated via effects on astrocytes (Hertz et al., 2000).

Although the concept of normal brain and ion homeostasis since long has been acknowledged, several recent studies have drawn attention to the importance of a well-controlled brain water homeostasis for the stability of neuronal function (Li and Verkman, 2001, Amiry-Moghaddam et al., 2003b, Simard and Nedergaard, 2004, Binder et al., 2006, Seifert et al., 2006). Both physiological and pathological perspectives have raised questions concerning the mechanisms governing the regulation of brain water homeostasis. It is now generally agreed that astrocytes, which express the water channel aquaporin 4 (AQP4), play an important role in brain water homeostasis (Amiry-Moghaddam and Ottersen, 2003, Simard and Nedergaard, 2004, Kimelberg Harold, 2005) and, furthermore, that AQP4 is implicated in K^+-clearance from the extracellular space (Amiry-Moghaddam et al., 2003b, Leis et al., 2005, Binder et al., 2006) (see below).

Brain edema

Edema derives from the Greek word oidema (οίδημα) which means swelling, and the term brain edema therefore is applied to the pathological condition of brain

swelling. Occuring within the constraints of a closed skull, overall fluid accumulation in the brain leads to raised intracranial pressure, impairs vascular perfusion and can lead to brain ischemia, herniation and death. Klatzo classified brain edema into *vasogenic edema*, occurring when the blood-brain barrier (BBB) becomes leaky and plasma fluid enters the brain parenchyma, and *cytotoxic edema*, consisting of intracellular fluid accumulation/swelling without increased permeability of BBB (Klatzo, 1967). Kimelberg suggested that the term *cytotoxic* edema should be replaced by *cellular* edema, as this type of edema is seen in a wide variety of clinical conditions (such as trauma , hypoxic-ischemic injuries and metabolic disease), not only following a "noxious" or "toxic" factor, as classically suggested (Kimelberg, 1995). However, today the terms cytotoxic and cellular edema are still used interchangeably. Finally, the hydrostatic, or *interstitial*, edema is a result of cerebrospinal fluid (CSF) hydrostatic pressures. This occurs primarily in lesions associated with obstruction of CSF flow.

In vasogenic brain edema increased osmoles and water enter the extracellular space of brain parenchyma due to a breakdown of BBB. The break down of the BBB is, as yet, relatively ill-defined (Kimelberg, 2004). It is generally accepted that even in vasogenic edema, astrocyte swelling (i.e. cytotoxic/cellular edema) also commonly occurs, and that these two types of edema often coexist. Cytotoxic edema is probably the major type of edema both in hypoxic-ischemic and in trauma situations (Unterberg et al., 2004). Intracellular swelling of astrocytes constitutes the major form of cytotoxic edema. In fact, neurons and oligodendrocytes basically never swell, even in severe conditions of experimentally induced hypoosmotic states (Kimelberg, 1995). The pathophysiological mechanisms leading to cytotoxic brain edema are not fully elucidated, and partly depend on the primary cause of injury. One mechanism of cellular swelling involves the uptake of KCl when extracellular K^+-concentrations increase following stroke or trauma. Failure of energy-demanding ion pumps, as the Na^+,K^+-ATPase, lead to accumulation of intracellular Na^+. Transport of HCO_3^- and H^+ out of the cell in exchange for Cl^- and

Na^+, respectively, and a breakdown of macromolecular glycogen to metabolic products contribute to the shift of water from the extra- to the intracellular space. Glutamate, released in excess amounts in several conditions leading to brain edema, can induce astrocyte swelling (Hansson et al., 1994, Simard and Nedergaard, 2004). This may be, at least in part, meditated by the increase of intracellular osmotic activity due to the concomitant uptake of Na^+ and glutamate via the glial glutamate transporters (EAATs), and partly because the EAAT1 is known to transport water (MacAulay et al., 2002). Also, hypoosmolality in itself (most often due to lowered plasma Na^+) is a well known and feared cause of cytotoxic brain edema, again, strikingly observed almost exclusively in astrocytes (Gullans and Verbalis, 1993).

Several lines of evidence suggest that the astrocyte water channel AQP4 is involved in the development of brain edema. In conditions associated with edema, including ischemia, meningitis and water intoxication, AQP4 have a deleterious effect on brain water homeostasis and contribute to the development of cytotoxic brain edema. AQP4 knockout mice develop less brain edema after acute water intoxication and have improved neurological outcome after ischemic stroke (Manley et al., 2000). Alpha-syntrophin knockout mice, which lack the perivascular pool of AQP4 in astrocytes, have less pronounced brain edema following transient cerebral ischemia than wild type mice (Amiry-Moghaddam et al., 2003a). These mice and MDX-mice, which also have reduced perivascular AQP4, have a delayed development of edema after water intoxication (Vajda et al., 2002, Amiry-Moghaddam et al., 2004). In an animal study of meningitis, it was shown that about 80% of the excess water in meningitis entered the brain parenchyma through AQP4 water channels, and that AQP4 null mice with meningitis had a much better outcome than wild type mice, with lower intracranial pressure and less brain water accumulation (Papadopoulos and Verkman, 2005). In summary, deletion of AQP4 improves outcome and reduces brain swelling following as disparate conditions as ischemia, meningitis and water intoxication.

These finding on one hand should support the concept of cytotoxic brain edema as a "common denominator" of these conditions, and on the other hand support the important role of astrocyte AQP4 in the development of brain edema.

Lead intoxication

Lead poisoning in children has been known as a serious environmental problem for decades and is associated with both long-term, cognitive, and acute manifestations (Perelman et al., 1993, Winneke et al., 1996, Gordon et al., 1998). Fortunately, the incidence of lead poisoning has declined during the last years (Meyer et al., 2003), although the problem still remains mainly in poor areas of the world. Acute lead intoxication is most common in young children (Aicardi J; in: Disease of the Nervous System in Childhood, 2nd Ed., Cambridge University Press) and is associated with brain edema and increased intracranial pressure (Pappas et al., 1986, Perelman et al., 1993, Rojas-Marcos et al., 2002). The acute form of lead encephalopathy is often presented as generalized convulsions, altered consciousness or coma and is associated with a high mortality rate if untreated. Experimental acute lead intoxication has been shown to be associated with astrocyte swelling (Lefauconnier et al., 1983, Selvin-Testa et al., 1997). Treatment of lead intoxication has involved different chelators, of which meso-2,3-dimercaptosuccinic acid (DMSA, also called succimer) is now commonly used (Angle, 1993, Bernal et al., 1997, Zhang et al., 2004). The mechanism of lead intoxication is not fully understood. It has been suggested that lead may activate intracellular signaling pathways that include either Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Westerink et al., 2002, Suszkiw, 2004) or protein kinase C (PKC) (Markovac and Goldstein, 1988, Bressler et al., 1999), but also that the long-term effects may be mediated via effects on glutamatergic synapses (Toscano and Guilarte, 2005).

Water intoxication

Water overload in clinical settings is observed in polydipsia (mainly in psychiatric disorders), in DDAVP treatment combined with inappropriate excess water intake and in endurance sports (Lebl et al., 2001, Whitfield, 2006). Impaired renal elimination of water can cause water overload. Massive oral intake of water in mothers during labour may cause life-threatening hyponatremia in newborns (Johansson et al., 2002). Water overload is always associated with hyponatremia. Hyponatremia is the most frequent electrolyte disorder in clinical medicine and causes hypoosmolality. Severe hyponatremic water retention is a much-feared complication after major surgery. The syndrome of inappropriate antidiuretic hormone secretion (SIADH) is one of the principal causes of hyponatremia in patients with CNS disorders such as meningitis, hemorrhages and traumatic brain injury. Regardless of the cause of acute, severe hyponatremia, the rapid development of hypoosmolality is a life-threatening medical emergency and frequently causes marked neurological symptoms due to hypoosmotic brain edema (Fraser and Arieff, 1997). The clinical manifestations include decreased sensorium, coma, seizures and death, with the mortality rates as high as 50% if untreated (Gullans and Verbalis, 1993). An animal model of hypoosmotic brain edema is represented by the water intoxication model. This is a well documented model of cellular/cytotoxic brain edema where the resulting hypoosmotic situation will create an osmotic gradient that favors water entry into the brain, without disruption of the blood-brain-barrier (Gullans and Verbalis, 1993, Manley et al., 2000). During the acute phase of this brain edema, experimental animals exhibit signs of neurological dysfunction secondary to brain swelling. The cellular response to the hyponatremic hypoosmolality is known to be astroglial swelling (Manley et al., 2000).

Astrocytes and their role for regulation of brain water homeostasis

The vertebrate central nervous system is comprised of two predominant cell types, neurons and glial cells. Astrocytes are subtypes of glial cells. Their star like shape includes processes which surround synapses and end feet which face capillaries. Astrocytes have long been considered as mere providers of structural and chemical support for the neurons. Studies in recent years have shown that astrocytes, constituting a prominent part of the number and volume of brain cells, play an active role in brain function by affecting or facilitating the activity of neurons ("the tri-partite synapse"). Several recent publications have highlighted the important role of astrocytes in neuron-astrocyte communication, their possible involvement in neurological disorders and their role in regulation of blood flow (Haydon, 2001, Mulligan and MacVicar, 2004, Volterra and Meldolesi, 2005, Seifert et al., 2006). Glutamate is considered to be the key mediator of the crosstalk between astrocytes and neurons (Volterra and Meldolesi, 2005). In an *in vivo* study in mice, it was recently demonstrated that sensory stimulation evokes increases in astrocytic cytosolic calcium and that this is mediated via glutamate acting on group I metabotropic glutamate receptors (Wang et al., 2006). As already referred to, astrocytes play a key role for the maintenance of brain water and ion homeostasis. With the aid of transporters, water channels and a variety of ion channels astrocytes are crucial in the clearance of neurotransmitters from the synaptic cleft and in controlling water and ion homeostasis (Simard and Nedergaard, 2004). Astrocytes are highly sensitive to changes in extracellular osmolarity and are capable of exhibiting prominent swelling and also of cell volume recovery through extrusion of ions and osmolytes (Pasantes-Morales et al., 2002). One of the main functions of the astrocyte is to optimize the extra-cellular space for synaptic transmission. To serve this function, astrocytes possess highly efficient systems for clearance of extracellular K^+ to avoid increases in concentration of more than 5 mM above resting level (which is about 3 mM) (Nagelhus et al., 1999, Walz, 2000, Butt and

Kalsi, 2006). When these systems are disrupted or overwhelmed, extracellular K^+ concentrations can reach values as high as 60 mM which leads to severely compromised CNS function (Kofuji and Newman, 2004). Potassium uptake into astrocytes are mediated through K^+-channels, Na^+K^+-ATPase and cotransporters, especially the Na^+, K^+, Cl^+ cotransporter. Potassium and water homeostasis are intimately linked. K^+ uptake results in water uptake and local astrocyte swelling. The extracellular osmolarity decreases following astrocyte uptake of K^+, and, since K^+-channels do not admit water transport, water flux must be mediated through distinct channels. Notably, AQP4 is expressed only in astrocytes and not in neurons; and is co-localized with the K^+-channel Kir4.1.

The extracellular space (ECS) and its volume is considered of great importance for extracellular ion concentrations and neuron-astrocyte communication (Sykova, 2004). Water flux through AQP4 not only affects astrocyte volume but inversely will contribute to ECS volume changes (Nagelhus et al., 2004). Several recent studies have provided evidence that water transport via AQP4 is important for efficient removal of the increase in extra-cellular K^+ that follows synaptic transmission (Nagelhus et al., 1999, Amiry-Moghaddam et al., 2003b, Nagelhus et al., 2004, Binder et al., 2006). In support of this, mice lacking AQP4 have more severe seizures when challenged with electrical stimulation, suggesting an impairment in K^+-clearance (Binder et al., 2006). In summary, there is considerable support of the importance of astrocytes in water and ion homeostasis in the brain and for the importance of this astrocyte "task" for neuronal function. Moreover, a role for AQP4-mediated water flux in brain volume and ion homeostasis is strongly indicated. However, so far less has been known about the mechanisms governing short-term regulation of astrocyte water permeability and, in particular, about the dynamic regulation of astrocyte AQP4.

Aquaporins

One of the major advances in biology in the last decade was the identification of a family of molecules - the aquaporin (AQP) water channels - that are responsible for regulated water transport across cell membranes (Preston et al., 1992). The discovery of water channels was recognized by the Nobel Prize in Chemistry 2003, awarded to Professor Peter Agre. It is now generally accepted that AQPs are critically involved in most if not all absorptive and secretory processes in our body and are vital for tight regulation of body water homeostasis (King et al., 2004). Cell membranes are composed of lipid bilayers and thus are nearly impermeable to water. AQPs constitute integral membrane channel proteins that enable passive yet remarkably efficient and rapid permeation of water across lipid bilayers. The AQPs exist as tetramers with each subunit having a separate pore. Structural studies have revealed that the hour-glass shaped pore constricts to about 3 Å at its narrowest point, thus limiting the size of molecules that can pass and constrains water molecules (D~2.8 Å) to flow in single file (Jung et al., 1994). Water flow via AQPs is bidirectional and dependent on osmotic gradients (Agre et al., 2002). They are found in a wide range of species and mammalian AQPs are expressed in multiple tissues, especially where rapid transmembrane water movement is important, including the kidney, lung, brain, eye lens, skin and red blood cells. To date, 13 mammalian aquaporins have been described, each with a specific tissue distribution and membrane localization pattern. They are also subdivided into three functional groups according to their permeability characteristics. In addition to water transport some members of the aquaporin family transport small molecules such as glycerol and urea (King et al., 2004).

Three water channels have been reported to be located in the brain: AQP1, AQP4, and AQP9. AQP1 is expressed in the choroid plexuses, AQP4 is expressed in astrocytes and ependymal cells, and AQP9 is expressed in astrocytes and in subpopulations of neurons (Amiry-Moghaddam and Ottersen, 2003, Badaut and Regli, 2004, Amiry-Moghaddam et al., 2005). AQP4 is the most extensively

studied brain water channel. The function of astrocyte AQP4 has different impacts in physiological and pathophysiological situations, as referred to above. Generally, aquaporins can be regulated both long-term (transcriptionally) and short-term (dynamically). For a review on regulation of brain aquaporins, please refer to the Appendix of this thesis.

Regulation of AQP4 (see also Appendix)

AQP4 exists in two isoforms that differ in length at the NH_2 terminus, the longer AQP4 M1 and the shorter AQP4 M23, both of which are expressed in the brain. The AQP4 water channel exists as a heterotetramer of these two isoforms. Immunohistochemical analyses have shown that AQP4 is primarily expressed in astrocytes and that the expression is polarized in plasma membrane domains of perivascular and subpial endfeet; i.e. at the blood-brain interface and at the processes facing CSF, respectively. There is also a low, but significant, expression in membrane areas facing glutamatergic synapses (Nielsen et al., 1997b, Nagelhus et al., 2004). The expression of AQP4 is regulated by interacting proteins. AQP4 is tethered at the perivascular membranes by association with the α-syntrophin and dystrophin anchoring complex. α-syntrophin knockout mice exhibit a loss of AQP4 expression in the endfeet but no decrease in other membrane domains (Amiry-Moghaddam et al., 2003a). In addition to this, there are data that the expression of AQP4 in the brain can be regulated developmentally, by steroids, by hypoxia and by hyperosmotic stress (see Appendix). Expressional changes of AQP4 protein have also been implicated in various experimental conditions such as trauma and ischemia in the brain (Kiening et al., 2002, Ribeiro Mde et al., 2006). With regard to short–term regulation, aquaporins can be dynamically regulated via gating or via trafficking (see Appendix). AQP2 (in the kidney) is typically regulated by trafficking, that is by recruitment of AQP2 molecules to the membrane upon stimulation. In “non-astrocyte”cell systems, AQP4 has been shown to be subject to

short term regulation via activation of PKC when expressed in oocytes or in cultured kidney cells, leading to a decrease in water permeability (Han et al., 1998, Zelenina et al., 2002). The target of this effect was found to be Ser180, a consensus site for PKC phosphorylation. Indirect evidence suggests that the activity of AQP4 in astrocytes is stimulated by vasopressin (AVP) (Sarfaraz and Fraser, 1999, Niermann et al., 2001). At present, though, there is no direct evidence that AVP-induced effects observed in astrocytes (increased swelling rate) or radial water flux in the brain are mediated via an effect on AQP4. Interestingly, vasopressin V1 receptors has been demonstrated to activate CaMKII in astrocytes (Zhao and Brinton, 2003), one of the kinases found to be of importance for up-regulation of AQP4 water permeability in this thesis. With respect to heavy metals, AQP4 is unique among the mammalian AQPs in that it is insensitive to mercury. Several divalent heavy metals have been shown to modulate water permeability of other aquaporins. Nickel and copper can inactivate AQP3 (Zelenina et al., 2003, Zelenina et al., 2004) and gold and silver has been reported to inhibit the water channel in soybean and human erythrocytes, presumably AQP1 (Niemietz and Tyerman, 2002). In summary, up till now relatively little has been known about whether and how any short-term regulation of AQP4 occurs in its native cell in the brain; the astrocyte.

Glutamate and group I metabotropic glutamate receptors

Glutamate is the major excitatory neurotransmitter in the brain and its role in neurotransmission is since long well established. It is, besides mediating neuron to neuron signaling, one of the key mediators of neuron-astrocyte interactions (Nedergaard et al., 2002, Volterra and Meldolesi, 2005, Wang et al., 2006). In excessive amounts glutamate is neurotoxic. It is therefore imperative that the astrocytes clear glutamate from the extracellular space to maintain normal neuronal signaling. Glutamate transporters, located on both astrocytes and neurons, prevent accumulation of glutamate and tonic activation of its receptors. An increased

outflow of glutamate, exceeding the capacity of proper clearance, is implicated in several pathological conditions and is generally considered to be the main cause of neurological damage in conditions such as trauma and ischemia/hypoxia. It is assumed that the neurotoxic effect of glutamate mainly is caused by activation of the ionotropic receptor NMDA and a subsequent massive influx of calcium (Leist and Nicotera, 1998). Glutamate can act upon several other receptors besides NMDA, both ionotropic (AMPA, kainate) and metabotropic glutamate receptors, signaling via either influx of ions or via second messengers, respectively. Metabotropic glutamate receptors (mGluRs) can be divided into three groups; group I, II and III, and are all G-protein coupled receptors. Group I mGluRs are coupled to Gq and activation leads to phophoinositide hydrolysis via phospholipase C, which in turn leads to calcium release from intracellular stores through activation of the InsP3 receptor. Group I mGluRs consist of mGluR1 and mGluR5, of which mGluR5 is considered unique in that it elicits Ca^{2+} oscillations (Kawabata et al., 1996, Kim et al., 2005). DHPG, which is used in this thesis, selectively activates the group I mGluR receptors. mGluRs are widespread in the nervous system, and mGluR5 has been shown to be developmentally regulated in rat brain, with higher levels found in early postnatal brain (Romano et al., 1996).

mGluRs and astrocytes

A variety of glutamate receptors have been characterized in different preparations of glia *in situ* and in cultured astrocytes, but mGluRs are considered to mediate most biological effects. Astroglial cells express group I mGluRs, and the presence of functional mGluR5 receptors has been best characterized (Balazs et al., 1997). It is known that glutamate can initiate calcium increases and calcium oscillations in cultured astrocytes, and that these responses are mediated primarily by mGluRs (Nedergaard et al., 2002). In a recent publication, glutamate and group I mGluR agonists was found to evoke Ca^{2+} -oscillations in rat hippocampal astrocytes in situ (Zur Nieden and Deitmer, 2006). Furthermore, Wang et al. showed that sensory-

evoked Ca^{2+}-responses in astrocytes *in vivo* were mediated by group I mGluRs (Wang et al., 2006). Calcium-signaling in astrocytes have been implicated with several downstream effects, such as release of glutamate, prostaglandins and ATP from the astrocytes (Pasti et al., 2001, Simard and Nedergaard, 2004). A breakthrough in our knowledge about astrocyte Ca^{2+}-signaling was made when it was shown neuron-induced astrocyte Ca^{2+} elevations can modulate local cerebral blood flow (Zonta et al., 2003, Mulligan and MacVicar, 2004).

Erythropoietin

Erythropoietin, a major haemopoietic growth factor, has in recent years emerged as an efficient neuro-protective agent (Genc et al., 2004, Brines and Cerami, 2005, Brines and Cerami, 2006). It is well documented from both experimental and clinical studies that erythropoietin (EPO) attenuates the degree of brain damage following stroke (Brines et al., 2000, Ehrenreich et al., 2002, Liu et al., 2006). Stroke is associated with a massive release of glutamate and the results of experimental studies indicate that EPO also protects from glutamate-triggered neurotoxicity (Yamasaki et al., 2005). EPO acts by attenuating the destruction of viable tissue surrounding the site of an injury, i.e. the penumbra that develops during the first 24-48 hours following a brain insult (Brines and Cerami, 2005).

The neuro-protective action of EPO is generally considered to be mediated through a specific non-erythroid EPO receptor in the nervous system (Brines et al., 2004). The non-erythroid EPO ("tissue-specific") receptor is a heteromer consisting of EPO receptor and the beta-common receptor involved in cytokine signaling, including IL-3 and IL-5 signaling (Brines and Cerami, 2005). It is abundantly expressed in astrocytes that surround capillaries in white matter and is up-regulated following hypoxia/ischemia (Eid et al., 2004, Genc et al., 2004). Immuno-histochemical studies have suggested that EPO receptors and AQP4 are co-localized in astrocyte endfeet (Nielsen et al., 1997b, Eid et al., 2004). In Paper IV

in this thesis we used both EPO, that activates erythroid and non-erythroid EPO receptors, and two EPO analogues, asialo-EPO and CEPO, of which the latter only activates the non-erythroid EPO receptor (Leist et al., 2004).

The mechanisms of the neuro-protective effect of EPO has been shown to include anti-apoptotic and anti-inflammatory effects (Siren et al., 2001, Villa et al., 2006). However, the mechanisms have not been fully elucidated. It is intriguing that a single dose of EPO results in a very early onset of protection against injury, as observed in an animal model of spinal cord injury (Gorio et al., 2002).

AIMS OF THE STUDY

The overall aim of this study has been to elucidate the regulation of water permeability in astrocytes with particular emphasis on the role of aquaporin 4 (AQP4) for brain water homeostasis. The presented studies include animal studies and cellular studies using both astrocytes in cell line and in primary culture.

The main goals have been:

- To explore whether there is a short-term regulation of astrocyte water permeability and, if so, to determine if it would be attributed to an effect on AQP4

  Specifically I wanted:

  1) To identify the role of glutamate in the regulation of astrocyte water permeability. Glutamate is known to have a neurotoxic effect in many conditions associated with brain edema.
  2) To investigate the effect of the heavy metal lead on AQP4 water permeability in astrocytes. Lead intoxication is known to be associated with brain edema.
  3) To elucidate the signaling pathway involved in regulation of astrocyte AQP4 water permeability.

- To study the regulation of AQP4 mRNA expression in the brain

  Specifically I wanted:

  1) To identify the different species of AQP4 mRNAs in the brain and compare it with peripheral tissues in young and adult mice.
  2) To verify the expression of AQP4 mRNA in different regions of the rat brain and to examine whether it is age-dependent or could be modified by a toxic agent known to be associated with brain edema.

- To study the effect of the neuroprotective agent erythropoietin on aspects of brain water homeostasis

  Specifically I wanted:

  1) To study the effect of erythropoietin on astrocyte AQP4 water permeability
  2) To investigate the effect of erythropoietin in an animal model of brain edema

EXPERIMENTAL PROCEDURES

For detailed description of materials and methods used in this study, please refer to the original papers enclosed. Here, I present a brief summary and a general discussion of the methods.

Materials

Cell cultures

One of the main goals of this study was to study regulation of astrocyte water permeability and the dynamic regulation of AQP4. To approach this we used two different cell cultures:

- Astroglial cells in primary co-culture from rat striatum or hippocampus.
- An astrocyte cell line derived from rat transiently transfected with GFP-tagged WT or mutated aquaporin.

To study the regulation of water permeability in single cells, we needed to use cell cultures, as single cell measurements are difficult in tissue slices or *in vivo*. In order to have a relevant model system, we therefore prepared astroglial cells in primary co-culture. The presence of neurons in primary cultures of astroglial cells allows for a cross-talk between neurons and astroglia and provides a more physiological environment. To obtain this, rat hippocampi or striatum were dissected from pups at embryonic age of 18 days. The astroglial cells were subconfluent to confluent at the time of experiment. The cells had a typical appearance with polygonal shape and small extensions from a relatively large cell body, characteristic of astroglial cells in primary culture. These cells were grown without suppression of astrocyte proliferation, which resulted in a relatively small number of neurons. Immunostaining confirmed that the majority of the cells were positive for glial fibrillary acidic protein (GFAP), an astrocyte specific protein. In the co-cultures the

neuronal cells was commonly growing on top of the astroglial cells, which made it possible, in addition to the difference in size and shape of the cells, to visually distinguish the astroglial cells from neurons during the experiments. Analysis with RT-PCR showed that the astroglial cells in the primary co-cultures express endogenous AQP4 mRNA. The endogenous expression of AQP4 allowed us to use these cells as a physiological relevant system for studying astrocyte function.

The astrocyte cell line (CTX-TNA2) was used for water permeability studies mainly. This is an immortalized cell line that is derived from rat cortical astrocytes. We performed several preliminary experiments that revealed that the subpassages 3-7 of this cell line provide the most optimal study conditions. We showed with RT-PCR that this cell line does not express endogenous AQP4. It is known that the expression of aquaporins is lost when cells are immortalized. Still, basic properties and signaling pathways of astrocytes are expected to be conserved and thus these cells are well suited to study astrocyte function. The absence of AQP4 expression in these astrocytes was an advantage, since we wanted to transiently transfect the cells with AQP4. Following transient transfection approximately 10% of the cells expressed the GFP-tagged AQP4. We were therefore able to compare effects on water permeability in cells that did and did not express AQP4, lying side-by-side on the same plate and exposed to exactly the same conditions. In this way we could distinguish between effects attributable to an effect on AQP4 permeability and general effects on the cells.

Animals

In Paper I we investigated expression of AQP4 mRNAs in adult and infant (2 day old) mice of the C57/Black strain. In Paper II studies were performed on ten and forty day old male Sprague Dawley rats. The animal experiments were approved by the local Committee on the Ethics of Animal Experimentation, Stockholm, Sweden. In Paper IV studies were performed on female C3H/HEN mice. This study was approved by the local Institutional Animal Use and Care Committee and

followed the Guide for the Care and Use of Laboratory Animals, U.S. National Research Council. All animals were kept under standard housing conditions with free access to food and water. Infant mice were kept with their dams.

Methodological approaches

Water permeability measurements

The method of studying the regulation of water permeability has been fundamental for the studies included in this thesis and will therefore be discussed a little more in detail. This method was originally developed by Zelenina and Brismar at our lab (Zelenina and Brismar, 2000) and is one of the first methods that allow the study of regulation of water channels in differentiated mammalian cells. I applied this method to explore the regulation of water permeability in astrocytes in both cell line and in primary culture.

Aquaporins account for an osmotic water permeability, which is significantly higher than the diffusional water permeability accounted for by water conductance of the membrane lipid bilayer. One of the main parameters in the characterization of osmotic water permeability is the osmotic water permeability coefficient, P_f (Farinas and Verkman, 1996, Zelenina and Brismar, 2000). All known methods for P_f measurements in living cells are based on the principle of exposure to hypo- or hyperosmotic shock and the time course of changes in cell volume. Changes in cell volume can be monitored in different ways. We have used recordings of changes in fluorescence intensity emitted by cells loaded with a fluorescent dye to study water permeability. By use of this method P_f in individual cells within cell monolayers can be determined and compared between cells that do and do not express GFP-labeled proteins, in the case of transiently transfected cells, or directly in primary cultured cells. Coverslips with the transfected astrocytes or primary cultured astroglial cells were mounted in a closed perfusion chamber (Focht Live Cell Chamber System) on the stage of a Zeiss 410 inverted laser-scanning microscope.

The chamber was filled with isoosmotic phosphate buffered saline (PBS) and scanned every 2 seconds with excitation at 488 nm and emission collected at 515–525 nm. At the beginning of each study, an image showing the distribution of GFP-tagged proteins in the transfected cell line was recorded. As the emission spectra for GFP and calcein are similar and non-separable in this system, calcein loaded cells were identified as GFP-positive (i.e. expressing GFP-AQP4) or GFP-AQP4 negative (i.e. not expressing GFP-AQP4) by superimposing the two images (Fig. 1). In our studies we chose to use GFP-AQP4 positive cells with a relatively low expression of AQP4, i.e. cells displaying a relatively weaker fluorescence, in order to avoid an excessive over-expression of AQP4 in the cells. Cells were loaded with the fluorescent dye calcein AM (Molecular Probes), which is inert to changes in intracellular pH and calcium. The loading solution was changed to 300 mOsm PBS containing drugs or vehicle to be studied and the cells were incubated for 2-15 minutes, depending on the protocol. To measure P_f, cells were initially perfused with isoosmotic PBS. The perfusate was then rapidly switched to a hypoosmotic PBS (150 or 200 mOsm). The temperature was kept low throughout the measurements (10 or 20°C, depending on the protocol) in order to minimize the diffusional water movement through the lipid bilayer of the plasma membrane. The obtained series of images were analyzed off-line by measuring the time course of the calcein fluorescence in individual cells. The part of the obtained curves recorded immediately after the solution switch was used for P_f calculation. During this period (~10 seconds), the cell swelling is assumed to be proportional to the permeability of the cellular membranes to water. To calculate the water permeability separately for cells that did and did not express AQP4, the GFP and calcein images were superimposed. The GFP-AQP4 negative cells thus served as "internal" controls underscoring that any observed effect was attributable to an effect on AQP4 and not a general effect on all cells. Using ImageJ software, a curve was obtained for each individual cell, showing changes in fluorescence intensity inside the cell during osmotic swelling. The P_f was calculated by a mathematical formula as previously described (Zelenina and Brismar, 2000,

Zelenina et al., 2002). The initial region of the fluorescence curve was fitted with a single exponential function and its time constant was used as a measure of the rate of cell swelling. This value is relative by definition and does not depend on the absolute values of fluorescence intensity at the beginning of the cell swelling. In order to calculate P_f for the astrocyte cell line, the surface to volume ratio and the osmotically active portion of cell volume in the astrocyte cell line were acquired by making image stacks of the entire cell before and after osmotic changes (not included in this thesis) (Zelenina et al., 2002). In this method, the fluorescence intensity of the intracellular fluorescent dye is measured in a thin (less than 0.5 μm) layer inside the cell. Due to the inherent instability of fluorescent molecules, the molecules are subject to photobleaching, i.e. intensity of fluorescence can decrease as an effect of excitation. This effect was accounted for by correcting the time course of fluorescent intensity change with the linear slope of the first part of the curve before establishing the osmotic concentration gradient. For the astroglial cells in primary culture the time constant of the fitting of the initial part of the curve by a single exponential function was used directly as a measure of water permeability.

Astrocytes were introduced to our laboratory for the first time when the work included in this thesis was started. Our results from the study revealed that the passage of the astrocyte cell line was important for the phenotype of the cells. We therefore restricted the use of these cells to subpassages 3-7 and avoided high passages. Furthermore, the use of GFP fusion proteins as the mode of visualizing the expression of aquaporins in the cells raised the general concern that GFP-tagging of AQPs could have an influence on water permeability. This was addressed in control experiments by transfecting GFP and AQP4 separately into LLC-PK cells and then measure P_f. GFP-tagging of the AQP was shown not to affect water permeability of the AQP (Zelenina et al., 2003) and thus the use of GFP-tagged AQP4 for transfection in these studies was considered justified.

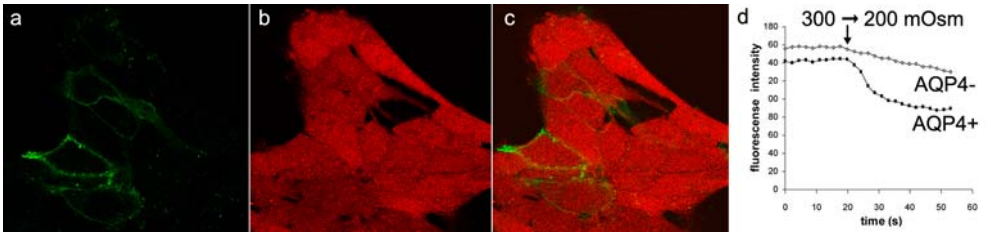

Figure 1. Measurements of water permeability in astrocyte cell line transfected with GFP-AQP4. a. Image showing the distribution of GFP-tagged AQP4. b. The same cells after being loaded with calcein. c. The GFP and calcein images are superimposed to allow identification of transfected cells (GFP-AQP4+ cells). d. Representative recordings from individual cells showing changes in calcein fluorescence emitted after switch from isoosmotic to hypoosmotic solution in GFP-AQP4 negative (AQP4-) and GFP-AQP4 positive (AQP4+)cells.

DNA constructs and mutations

The use of molecular technology has been of great importance in order to address the questions in the studies included in this thesis. In the water permeability studies we mainly used constructs encoding the short form of mouse AQP4, AQP4-M23. cDNA encoding the whole AQP4-M23 was amplified by RT-PCR using total mouse brain RNA as a template. Wild type AQP4.M23 was tagged with green fluorescent protein (GFP) at the NH_2-terminus and transiently transfected into the astrocyte cell line. This wild-type AQP4 fusion protein was used in studies II, III and IV and was fundamental for the understanding of the contribution and regulation of AQP4 to astrocyte water permeability. In order to explore individual amino acid residues as possible targets for water permeability regulation we performed analysis of AQP4 protein structure to predict putative phosphorylation sites and to estimate their phosphorylation potential, using NetPhos 1.0 and 2.0 software. This allowed us to predict possible protein kinases involved in AQP4 regulation and to introduce targets for point mutations. The point mutation of Ser111 (Paper II and III) to alanine (S111A) in NH_2-terminal GFP-tagged AQP4 fusion protein was generated using a U.S.E. Mutagenesis kit (Amersham Pharmacia). The mutations and absence of other modifications were confirmed by sequence analysis of the whole insert. The AQP4M23 S111 (WT) and AQP4M23

A111 (Ser111 to alanine point mutation) enabled us to create short peptides corresponding to AQP4 I96-C123. These peptides were fused to GST at the N-terminal, purified and used for the *in vitro* phosphorylation studies (Paper III). We also constructed sense and anti-sense riboprobes for the *in situ* hybridization study (Paper II). Structure of all constructs was analyzed using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Warrington, UK). As a control of specificity in Paper II, we transfected human AQP3 tagged with GFP at the COOH-terminus into the astrocyte cell line. The AQP3 construct was previously described (Zelenina et al., 2003).

Transfection of cell line

Transfection is the process of introducing nucleic acids into eukaryotic cells by nonviral methods. Essentially, transfection is a method to introduce DNA into cells. In general, transfection reagents designed as cationic liposomes are considered as more versatile than other traditional transfection methods. These are composed of cationic lipids in combination with a neutral lipid and will form multilamellar vesicles upon hydration. The neutralization of the negatively charged molecules, such as DNA and RNA, makes it easier for the DNA:transfection reagent complex to cross the membrane. Following cellular internalization, the complexes appear in the endosomes, and, in the case of DNA, later in the nucleus. The best transfection reagent and conditions for a particular cell type must be empirically and systematically determined because inherent properties of the cell influence the success of any specific transfection method. For the present studies, we wanted to perform a transient transfection of AQP4 in the astrocyte cell line. In preliminary studies, we tested several transfection reagents. Most of the methods tested, all based on the use of cationic lipids for the delivery of nucleic acids to the cells, resulted in a very low transfection efficacy. TransFast™ from Promega was finally our method of choice, since this reagent gave the best transfection efficacy, resulting in transfection of about 10% of the cells. The GFP-tagged AQP4 cDNA

construct was transfected into the cells via the liposomes, transcribed and translated. We performed control experiments where the size and shape of GFP-AQP4 transfected astrocytes were compared with non-transfected, wild type, astrocytes. There were no differences in the size; measured as the circumference, diameter or area of the cells, or in the shape between astrocytes transfected with GFP-AQP4 and non-transfected astrocytes.

Calcium measurements

In the calcium studies included in this thesis (Paper III and IV) we used the calcium-sensitive fluorophore Fura-2/AM for single cell ratiometric imaging. This fluorophore is highly selective and sensitive to Ca^{2+}-ions, can be easily loaded in a non-destructive manner into the cytosol of cells and have excellent spatial and temporal resolution. It can be applied to measure changes in intracellular Ca^{2+} concentration over long experimental time, e.g. several hours, if necessary. Fura-2/AM is a dual-excitation (ratiometric) fluorophore. This property makes it suitable for measurements over time because it avoids the problem associated with bleaching and leakage of fluorophore. Different fluorophores have different affinities to Ca^{2+}. Fura-2/AM has the capacity to detect wide ranges of intracellular Ca^{2+}-changes. Calcium measurements using Fura-2/AM, performed in other cell types, is a well established method in our laboratory (Uhlen et al., 2000, Aizman et al., 2001). The method was, however, not previously applied on astroglial cells in primary culture and astrocyte cell lines. Since fluorophores, being artificial substances, can disturb normal cellular function or even be toxic for the cells, it was of critical importance to experimentally determine how to explore intracellular changes of Ca^{2+} in the astrocytes. We therefore performed preliminary studies to determine the appropriate concentration, loading conditions and time for the astroglial cells used in this study. Different cell types are known to have considerable differences in their ability to load fluorophores. We found that incubation of the primary cultured astroglial cells with 3 μM Fura-2/AM for 20

minutes in PBS at 37°C was optimal for intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurements. The astrocyte cell line required substantially longer loading time, about 1-1.5 hours.

The primary cultures of astroglial cells are co-cultures that contain some neurons. The neurons could usually be distinguished from the astroglial cells by visual examination. A subset of the cells identified as neurons showed spontaneous Ca^{2+}-oscillations. This was not the case in the astroglial cells. Furthermore, NMDA induced a sustained calcium increase in neurons but had no effect in the astroglial cells (Fig. 2). In all the calcium-experiments, this differential response to NMDA was therefore used as a way to confirm neuronal cells. All neuronal cells were excluded from all the calcium analyses in Papers III and IV.

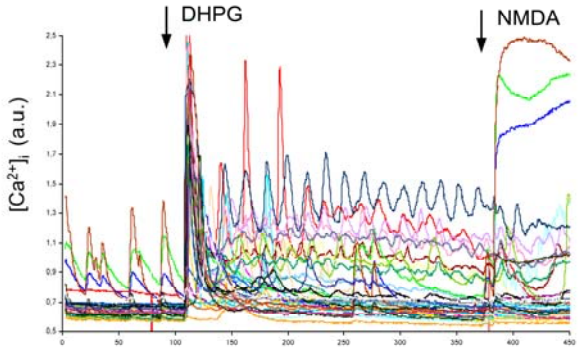

Figure 2. DHPG-induced Ca^{2+}-oscillations in astroglial cells in primary co-culture. DHPG induced a transient peak in $[Ca^{2+}]i$ followed by periodic oscillations in astroglial cells. A subset of the neurons showed spontaneous Ca^{2+}-oscillations. NMDA induced a sustained calcium increase in these cells. Observe that the same cells (neurons) that show spontaneous oscillations (brown, light green and blue) prior to application of DHPG respond with a sustained Ca^{2+}-increase to NMDA. There is no response to NMDA in the astroglial cells.

Frequency analysis

Calcium is an almost universal intracellular messenger that controls a diverse range of cellular processes (Berridge et al., 1998). The concerted actions of Ca^{2+} transporters located in the plasma membrane and in the membranes of the

intracellular stores generate Ca^{2+} oscillations. Different physiological stimuli can give rise to Ca^{2+} oscillations within a wide range of frequencies; from periods of fractions of a second to tens of minutes. Experimental recordings of intracellular Ca^{2+}- signals can be difficult to interpret and quantify with the naked eye. As a tool to detect and quantify Ca^{2+} oscillations recorded in our studies (Paper III and IV), we performed a frequency analysis of the data. This was modified from a method described by Uhlén (Uhlen, 2004). Spectral analysis is a powerful analytical method in which periodic signals can be converted from the time domain (e.g. in seconds) to the frequency domain (e.g. mHz). The relative contribution of different frequency components in a calcium signal was calculated using FFT (fast fourier transform) in MATLAB® (The Mathworks, Natick, MA). The frequency analysis allowed us to interpret the DHPG-induced Ca^{2+} oscillations and to describe the alterations in Ca^{2+} signaling caused by pre-treatment of the cells with CEPO or EPO.

In vitro phosphorylation

Protein phosphorylation and dephosphorylation are key processes that regulate cellular functions. They play a fundamental role in mediating signal transduction initiated by a wide range of signaling molecules such as neurotransmittors and hormones. The functional state of many proteins is modified by phosphorylation/dephosphorylation, which constitutes the most ubiquitous posttranslational modification in eukaryotes. Most protein kinases, responsible for the transfer of a phosphate to the target protein, are either Ser/Thr kinases or Tyr kinases. Ser/Thr protein kinases have broad substrate specificity and therefore regulate a wide variety of target proteins in the cells. The activity of Ser/Thr protein kinases is typically regulated by second messengers such as cAMP, cGMP or Ca^{2+}. Protein phosphorylation is typically detected either by using radioactive (^{32}P) labeling of the proteins or by phospho-specific antibodies. After having identified the serine 111 residue as a target for regulation of AQP4 water

permeability (Paper II and III), we wanted to explore which protein kinase that could phosphorylate this residue. In Paper III we therefore performed an *in vitro* phosphorylation of a GST-fusion protein corresponding to wild-type AQP4 I96-C123 and a corresponding peptide where the serine residue had been substituted to alanine. *In vitro* phosphorylation was performed with activated protein kinases; PKG and CaMKII. We are now in the process of producing a phospho-specific antibody against the serine 111 residue in AQP4, which will be a useful tool to detect in vivo phosphorylation of AQP4.

Whole animal studies

Water intoxication. In our studies of astrocyte AQP4 water permeability, we use the exposure of cells to hypoosmolar solutions to induce movement of water into the cells. For *in vivo* studies, the water intoxication model is that which mimics this situation best. In this model, cellular (cytotoxic) brain edema is produced as a result of the rapid intraperitoneal water infusion along with 1-Deamino 8-D-arginine vasopressin (DDAVP). The resulting hypoosmotic situation will create an osmotic gradient that favors water entry into the brain, without disruption of the blood-brain-barrier (Manley et al., 2000). In our study of the interaction of EPO and AQP4 (Paper IV), we therefore applied this model to C3H/HEN mice. During the acute phase of this brain edema, experimental animals exhibit signs of neurological dysfunction secondary to brain swelling. Neurological symptoms were evaluated by a scale modified from Manley et al. (Manley et al., 2000). The assessment variables included the following parameters: exploring of cage, visually tracking of objects, whisker movement, leg-tail movements, pain withdrawal, coordination of movement and stop at edge of table. Although C3H/HEN mice appeared to be relatively resistant to the symptoms of water intoxication, we found a protective effect of EPO and asialo-EPO on the symptoms as assessed by the neurological scale (see Results and comments).

Lead intoxication. The *in vivo* model for lead intoxication was performed in order to investigate whether detectable levels of lead induced redistribution or major changes in the levels of AQP4 mRNA in the brain of 10 and 40 day old male Sprague Dawley rats. Changes in mRNA or protein expression of a protein (e.g. AQP4) are results of long-term effects of drug or other impacts on the animal, as opposed to the short-term, dynamic effects on the protein that we can study with the water permeability measurements. Up- or down-regulation of AQP4 expression in the brain has been demonstrated in various models of brain insults (Ribeiro Mde et al., 2006). To investigate the effect of lead on AQP4 expression in the brain, adult animals (40 d old) were given lead acetate in a dose of 100 mg/kg by gavage twice daily and 10 day old rats received lead acetate in the same dose intraperitoneally once daily for three consecutive days. Lead content in brains was analyzed by the Inductive Coupled Plasma (ICP)-atomic emission spectrometry technique after wet digestion to confirm lead uptake and exposure levels. The analysis was done at the National Veterinary Institute of Sweden, where it is an accredited method for measuring lead in biological samples. The detection level of the method was 0.0015 mg/kg wet weight. The effect of lead exposure on the distribution of AQP4 mRNA in the brain was examined with *in situ* hybridization.

Structure analysis of AQP4 gene and AQP4 mRNAs

There are two isoforms of the AQP4 protein – M1 and M23. The M1 isoform contains an additional 22 more amino acids in the N-terminal compared to the M23 isoform. However, at the time of study I (Paper I), there were conflicting data concerning the mRNA sequence of AQP4 and the number of distinct AQP4 mRNA isoforms. We therefore reexamined the expression of AQP4 mRNA in mouse tissue. We used a fragment prepared by amplification of mouse genomic DNA by use of primers corresponding to exons of the AQP4 gene. This fragment was used for screening of the mouse genomic library. Individual clones of the AQP4 gene were isolated, analyzed for presence of 5′ end of the AQP4 gene and sequenced.

Total RNA was extracted from adult mouse brain and following RT-PCR, PCR fragments were extracted and sequenced. Comparison of genomic DNA sequence and AQP4 cDNA sequence data showed the presence of an exon that was interspaced between exon 0 and exon 1. By the use of a set of primers, all analyzed against Gene Bank sequence data for absent homology of some alien nucleotide structure, three AQP4 mRNAs were identified.

In situ *hybridization*

In situ hybridization is an efficient method to detect and determine the distribution of mRNA expression in tissue. We used riboprobes (RNA antisense and sense) derived from cDNA clones corresponding to AQP4. *In situ* hybridization with antisense riboprobes gives a strong signal with good sensitivity. The structure of all constructs was confirmed by sequencing. Sections from lead-treated rats and control rats (Paper II) were placed on the same slides in order to keep them in similar conditions during both hybridization and exposure to X-ray film. Also, control sections were incubated with the sense riboprobe under identical conditions to confirm specificity.

RT-PCR

The polymerase chain reaction (PCR) is a well established method to amplify DNA from a selected region of a genome, once the genome sequence is known, a billionfold and by this "purify" the DNA away from the remainder of the genome. We performed *reverse transcriptase* (RT)-PCR in order to amplify AQP4 cDNA from purified RNA from tissue or cells. Primers for detection of the different AQP4 mRNAs were designed from using the AQP4 gene structure. We applied RT-PCR in Paper I in a semiquantitative fashion to compare the expression of different AQP4 mRNAs in tissues from mouse brain, lung and kidney in young and adult mice. In Papers II and III we used RT-PCR to identify the presence of

endogenous AQP4 mRNA in the astrocyte cell line and astroglial cells in primary culture. RT-PCR is an extremely sensitive method. Therefore, strict mixing procedures and appropriate positive/negative controls should be applied to rule out unspecific amplifications or failing reactions. In addition, when using RNA as the source of amplification, as we did in our studies, control reactions without reverse transcriptase prior to PCR should be performed. Finally, amplification of β-actin mRNA should be added to the reaction or run in separate reactions as an internal control.

RESULTS AND COMMENTS

Expression of AQP4 (Papers I and II)

In Papers I and II we investigated the expression of AQP4 mRNA in tissues from young and adult animals using semiquantitative RT-PCR and *in situ* hybridization.

Identification of a new form of AQP4 mRNA

AQP4 is mainly expressed in astrocytes in the brain (Nielsen et al., 1997b). Two isoforms of AQP4 protein have been identified, M1 and M23. In Paper I we presented a new; third form of mouse AQP4 mRNA. First, screening of a mouse genomic library revealed an exon not previously described, interspaced between the previously described exons 0 and 1. We called this "exon X". By the use of a set of primers we identified three AQP4 mRNAs; AQP4.M1 mRNA, AQP4.M23X mRNA, a new form, and AQP4.M23 mRNA. Comparison of genomic and cDNA structures demonstrated that the new mRNA form, AQP4.M23X, included a sequence corresponding to the new exon X. Our analysis also indicated that alternative splicing of exon 1 had occurred. Nucleotide sequence analysis suggested that the AQP4.M23X mRNA encodes for the M23 isoform of the AQP4 protein. We then wanted to investigate the tissue distribution of the new AQP4.M23X mRNA and the previously described AQP4.M23 mRNA. We found, by use of semiquantitative RT-PCR, that the two mRNAs encoding AQP4.M23 had a tissue- and age-specific expression. The new AQP4 mRNA, AQP4.M23X, was predominantly expressed in brain, compared to lung and kidney. The expression was twofold higher in the adult than in the infant, 2 day old, brain and was very low in both infant and adult lung and kidney. In comparison, the relative expression of AQP4.M23 mRNA was very low in both infant and adult brain compared to lung and kidney. In conclusion, we identified a new form of "brain-specific" AQP4 mRNA in mice that is developmentally regulated.

In situ *hybridization of AQP4 in young and adult rat brain*

The expression of AQP4 in the brain was also studied in young, 10 day old, and adult, 40 day old, rats. We performed *in situ* hybridization studies on the brain using riboprobes against AQP4 (Paper II). AQP4 mRNA hybridization signal was detected throughout the rostrocaudal axis of the brain. The distribution of AQP4 mRNA was similar in young and adult brains. Strong labelling was found in cerebellum, around the lateral ventricles, around the third ventricle and in the area of the paraventricular nucleus. The pial surface was defined by a strong hybridization signal. Signals were also detected in the hippocampal formation and the striatum had a moderate labelling. Notably, it is from these two regions that we in the present thesis have obtained primary cultured astroglial cells. Hybridization with the sense probe showed no detectable labelling. Fig. 3 shows the distribution of hybridization signal in brain from 10 day old and 40 day old rats.

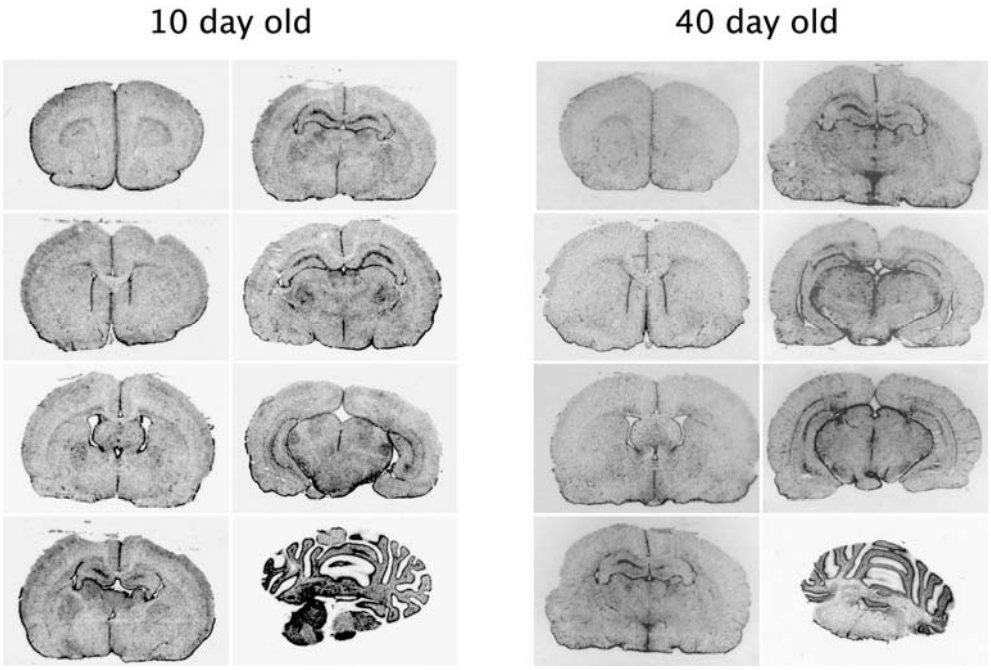

Figure 3. In situ *hybridization using AQP4 riboprobes in 10 d old and 40 d old rats.*

Effect of lead on AQP4 mRNA expression in brain

We then performed semiquantitative RT-PCR and *in situ* hybridization on rat brains from control animals and animals that had received three days of lead intake. There were several reasons to why we wanted to study the effect of the heavy metal lead on AQP4 regulation. Firstly, acute lead intoxication is associated with brain edema (Pappas et al., 1986, Perelman et al., 1993, Rojas-Marcos et al., 2002). Secondly, experimental acute lead intoxication has been shown to be associated with astrocyte swelling (Lefauconnier et al., 1983, Selvin-Testa et al., 1997). Finally, several divalent heavy metals are known to modulate aquaporin water permeability (Preston et al., 1993, Niemietz and Tyerman, 2002, Zelenina et al., 2003). Any effect of lead on AQP4 activity had, however, not previously been reported. The lead treatment in this protocol, as described in Methodological Approaches, resulted in significantly elevated brain lead levels, 0.26 + 0.01 mg/kg wet weight, compared to the control brains, where lead was nondetectable. A screening, using semiquantitative RT-PCR on rat cerebellum and cerebrum separately, showed no detectable differences in AQP4 mRNA abundance between lead-treated and control rats. When investigated with *in situ* hybridization, treatment with lead acetate did not appear to have any effect on the distribution of AQP4 mRNA in the brain in either 10 day old or adult rats. The intensity of the signal was also similar. In conclusion, the impact of lead intoxication in rats did not appear to have any long-term effects on AQP4 expression in the brain.

Potential impacts of the findings

In Paper I we found that the new AQP4.M23X mRNA was more abundantly expressed in brain than in peripheral tissue, indicating that this might represent a brain-specific mRNA of the M23 protein. Differences in mRNAs, although encoding the same protein, may indicate the need for differential regulation of the protein in brain and in peripheral tissues. Regulation may vary with regard to transcription of the gene, e.g. with different promoters, RNA stability and/or

translation of the protein. Furthermore, we found a pronounced postnatal increase in the expression of the AQP4.M23X mRNA, which may be relevant for the notion that water homeostasis is different in immature and adult brain. Lead exposure, on the other hand, did not appear to have any long-term effects on AQP4. Taken together, our studies on expression of AQP4 suggested that AQP4 can be both short- and long-term regulated. In this thesis we went on to study the short-term regulation of AQP4. In future studies we plan to further explore the long-term regulation of this water channel.

Lead increase astrocyte AQP4 water permeability (Paper II)

The finding that lead did not change AQP4 on the expressional level in brain encouraged us to proceed with studies on of the short-term regulation of AQP4-expressing astrocytes. We investigated the effect of lead on astrocyte AQP4 water permeability. Lead exposure induced a significant, 40%, increase in P_f in AQP4-expressing cells. The effect was specific for AQP4, since lead had no effect on astrocytes transfected with another aquaporin, AQP3. The lead-induced increase in P_f was dose-dependent. We found it highly unlikely that the lead effect was due to a general toxic effect on the cells. Cells not expressing AQP4 actually had a slightly lower water permeability following lead exposure. Also, the volume and appearance of the astrocytes did not change during the experimental period and no leakage of calcein was observed, indicating an intact plasma membrane. The increase in astrocyte AQP4 water permeability persisted after lead washout, suggesting that the lead effect on AQP4 was taken up by the cells and acted within the cells. This was supported by the finding that a commonly used lead chelator, DMSA, totally abolished the lead-induced increase in P_f. We also tested the effect of lead exposure on water permeability in astroglial cells in primary culture, expressing endogenous AQP4. Lead increased water permeability significantly, by more than 50%, in the astroglial cells in primary culture. It has been suggested that lead may activate calcium-dependent intracellular signaling pathways that include

either Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Westerink et al., 2002, Suszkiw, 2004) or protein kinase C (PKC) (Markovac and Goldstein, 1988, Bressler et al., 1999). When we preincubated the cells with an inhibitor of CaMKII, KN-62, the lead-induced increase in water permeability in GFP-AQP4 positive cells was abolished. The CaMKII inhibitor did not have any effect on water permeability of lead-exposed GFP-AQP4 negative cells or on water permeability of vehicle-treated GFP-AQP4 positive or -negative cells. Two different inhibitors of the classical PKC isoforms, BIM and calphostin, were tested separate protocols. Neither of the PKC inhibitors had any effect on the lead-induced increase in water permeability of GFP-AQP4 positive cells. In summary, we found that lead increases astrocyte water permeability via an effect on AQP4 and a calcium-signaling pathway involving CaMKII.

Glutamate increase astrocyte AQP4 water permeability (Paper III)

The finding that the lead-induced increase in astrocyte AQP4 water permeability was mediated via a calcium-signaling pathway raised the question whether other agents/substances activating calcium-signaling could regulate astrocyte water permeability. We reasoned that glutamate, a major neurotransmitter in the brain, would be an obvious candidate for exploring this question. Glutamate can mediate its effects via increases in intracellular calcium through activation of ionotropic and/or metabotropic glutamate receptors (mGluRs). Moreover, excessive outflow of glutamate is implicated in several acute brain disorders associated with cytotocix/cellular brain edema. In Paper III we therefore investigated the effect of glutamate on astrocyte and AQP4 water permeability. Glutamate caused a significant increase in water permeability in astroglial cells in primary culture and caused a 50% increase in P_f in astrocytes transfected with WT AQP4 (GFP-AQP4 positive cells). Glutamate had no effect on P_f in GFP-AQP4 negative cells. When

we explored the role of metabotropic glutamate receptors, mGluRs, for the glutamate-triggered increase in astrocyte AQP4 water permeability, we found that the group I mGluR agonist DHPG mimicked the glutamate effect. DHPG caused a significant, 34%, increase in water permeability in the astroglial cells in primary culture. In the astrocyte cell line DHPG also caused a significant, 40%, increase in P_f in GFP-AQP4 positive cells but had no significant effect on GFP-AQP4 negative cells. The ionotropic receptor agonist NMDA had no effect on water permeability neither in astroglial cells in primary culture nor in GFP-AQP4 positive astrocytes. When exploring interactions between effectors and a target in biological system, in this case DHPG and AQP4, respectively, it is important to consider the dose-response relationship. We found that the DHPG-effect on water permeability in GFP-AQP4 positive cells was dose-dependent; underlining the physiological role of group I mGluRs for astrocyte AQP4 water permeability.

In the GFP-AQP4 positive cells we found that the GFP signal was evenly distributed in the plasma membrane. The signal from the cytoplasm was generally very low. When we applied glutamate or DHPG to the cells, there was no apparent redistribution of the signal. This was to us an important observation, suggesting that the observed increase in water permeability was attributable to a change in the opening state of the channel rather than to exocytosis of AQP4.

Mechanisms of increased astrocyte AQP4 water permeability (Papers II and III)

The glutamate-mediated increase in astrocyte water permeability was due to an effect on AQP4 and was mediated by group I mGluR activation. Group I mGluRs are shown to play a pivotal role in the neuron-astrocyte crosstalk. Group I mGluRs, which include mGluR1 and 5, are known to activate a Gq-phospholipase C pathway and to generate InsP3, leading to release of calcium from intracellular stores. We found that DHPG triggered an oscillatory calcium response in the

astroglial cells in primary culture and in the astrocyte cell line. This response is considered to be a typical effect of group I mGluRs, and of mGluR5 in particular (Kim et al., 2005), and confirmed that the cells used in this study express functional group I mGluRs. NMDA did not trigger an increase in intracellular calcium in astrocytes. As mentioned, glutamate and DHPG did not induce any redistribution of the GFP signal in the GFP-AQP4+ cells. This suggested that the observed increase in water permeability was due to a change in the opening state of the water channel rather than to recruitment of new AQP4 molecules. It was recently reported that in the spinach aquaporin SoPIP2;1, the state of phosphorylation of the Ser115 residue in the first intracellular loop is an important determinant of the opening diameter of the water channel (Tornroth-Horsefield et al., 2006). When we aligned the mammalian AQP4 to spinach AQP, we found that the serine 111 residue of AQP4, which is located in the first intracellular loop, corresponds to the serine 115 residue in spinach AQP. We had, prior to this, identified the AQP4 serine 111 residue as a putative consensus site for phosphorylation (see Appendix). Accordingly, the next step was to study AQP4 where Ser111 was substituted to alanine. GFP-tagged AQP4 S111A was expressed in the astrocyte cell line. Lead failed to increase water permeability in GFP-AQP4 S111A expressing cells. Moreover, neither glutamate nor DHPG had any significant effect on P_f in cells expressing GFP-AQP4 S111A. These results strongly indicate that the AQP4 serine 111 residue is the molecular target for the up-regulation of astrocyte AQP4 water permeability and, specifically, that it is the target for both the lead- and the group I mGluR mediated increase in water permeability.

The Ser111 residue is conserved in human, mouse and rat AQP4 (see Appendix). Analysis by NetPhos 1.0 and 2.0 revealed that this residue is a potential phosphorylation target for several Ser/Thr protein kinases such as Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein kinase G (PKG). We next tested the effect of two protein kinase inhibitors on the DHPG response on AQP4 water permeability, the highly specific CaMKII inhibitor KN-93 and the fairly unspecific

serine kinase inhibitor H-89. The astrocyte cell line transfected with AQP4 was preincubated with either KN-93 or H-89 before applying glutamate or DHPG. Both inhibitors significantly inhibited the DHPG-induced increase in water permeability in GFP-AQP4+ cells. KN-93 was also found to significantly inhibit the glutamate-induced P_f increase in GFP-AQP4+ cells. Here it is appropriate to point out that the lead-triggered increase in water permeability in AQP4-expressing astrocytes also was abolished in the presence of a CaMKII inhibitor, KN-62, although this inhibitor is considered to be less (brain)-specific than KN-93.

To test which protein kinase that might phosphorylate the Ser111 residue, we generated two recombinant peptides corresponding to the residues I96-C123 of WT AQP4 or to AQP4 S111A. The peptides were fused to GST for purification purpose. *In vitro* phosphorylation was performed with activated CaMKII and PKG, respectively. Surprisingly, CaMKII was not found to phosphorylate the wild type AQP4 peptide. Since CaMKII has been reported to phosphorylate nitric oxide synthase (NOS) (Schneider et al., 2003) and thereby activate the NO-cGMP-PKG signaling pathway, and since Ser111 was predicted to be a consensus site even for PKG, we proceeded to test whether PKG could phosphorylate this site. We found that the wild type AQP4 peptide, corresponding to the residues I96-C123, was phosphorylated by PKG. The use of the mutant S111A peptide confirmed that Ser111 was the target for PKG phosphorylation, as PKG did not phosphorylate this peptide. It should be noted that the protein kinase inhibitor H-89, which is a strong inhibitor of PKG at the concentration used in this study (Wang, 2000), also abolished the effect of DHPG on AQP4 water permeability.

Protein kinase G is known to be activated by cGMP, which in turn can be activated by a NOS-NO signaling pathway. As mentioned, it has been reported that NOS can be regulated by CaMKII. We therefore wanted to explore whether NOS could be involved in the glutamate-induced regulation of AQP4. Preincubation with the NOS inhibitor L-NAME completely abolished the DHPG effect on GFP-AQP4 expressing astrocytes. L-NAME had no effect on DHPG-exposed cells not

expressing GFP-AQP4. In summary, our results support the group I mGluR-CaMKII-NO-cGMP-PKG signaling pathway as a modulator of astrocyte water permeability. Fig. 4 depicts the proposed signaling pathway for the glutamate-induced increase of water permeability in AQP4-expressing astrocytes.

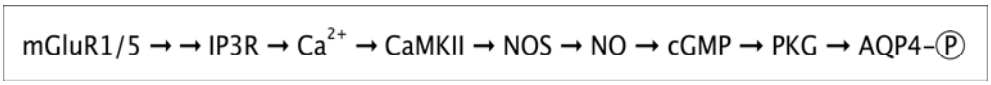

Figure 4. Proposed signaling pathway for the glutamate-induced increase of water permeability in AQP4-expressing astrocytes.

Erythropoietin and astrocyte AQP4 water permeability (Paper IV)

During recent years erythropoietin (EPO), a major haemopoietic growth factor, has emerged as a promising neuro-protective agents (Brines and Cerami, 2005). EPO appears to act by preventing the destruction of viable tissue surrounding the site of an injury. We hypothesized that EPO might modulate astrocyte water permeability and asked the question whether EPO might have a tissue protective role in brain edema. To address these questions, we tested the neuroprotective effect of EPO in an experimental model of primary brain edema and investigated the effect of EPO on astrocyte water permeability.

Effect of EPO in an animal model of brain edema

EPO has been shown to be neuroprotective in several experimental models of brain injury and is both neuroprotective and safe in human stroke. Evidence indicates that it prevents destruction of viable tissue in the penumbra (please see Introduction for references). Common for many of the experimental conditions tested, such as hypoxic-ischemic, traumatic and excitotoxic injuries, is that they may induce cytotoxic edema as a secondary detrimental event. This led us to hypothesize that

EPO may attenuate brain edema. We tested this in a rodent model of primary brain edema. Female C3H/HEN mice were exposed to water intoxication by a combination of intraperitoneal water injection and DDAVP. Animals were pre-treated with vehicle (saline), recombinant human erythropoietin (EPO) or asialo-erythropoietin (asialo-EPO) before the water load. Asialo-EPO is a desialylated EPO derivate (i.e. removal of sialic acid) that has no erytropoietic effects due to its extremely short half-life (Leist et al., 2004). Pre-treatment with EPO and asialo-EPO significantly reduced neurological symptoms caused by this brain edema. The neurological symptoms started to appear after 45 minutes in both the saline- and the EPO- treated groups, but were delayed and started to appear after 60 minutes in the asialo-EPO treated group. After 75 minutes the symptoms gradually decreased in all groups but were consistently lower in the EPO- and the asialo-EPO treated groups. At the end of the assessment, 180 minutes after water load, the neurological scores remained pathological in all three groups, but the EPO-treated and asialo-EPO treated groups had less neurological symptoms than the saline-treated group. Statistical analysis of the area under the curve showed that the neurological symptoms were significantly less in the EPO- and the asialo-EPO treated groups compared to the saline-treated group. There was no statistical difference between the EPO and asialo-EPO groups. In summary, we found that EPO and its derivate asialo-EPO have a neuroprotective effect in an acute model of primary brain edema.

Cell studies of EPO effect on astrocyte water permeability

We hypothesized that EPO might modulate astrocyte water permeability, and that such an effect could be implicated in the neuroprotective effects of EPO. The following studies were consequently performed on the astroglial cells in primary culture and on the astrocyte cell line transfected with AQP4. We first tested if EPO had any effect on astrocyte water permeability. We found that EPO alone had no effect on water permeability in astroglial cells in primary culture. Nor did EPO

have any effect on water permeability in GFP-AQP4 positive cells or GFP-AQP4 negative cells. We applied EPO to the cells for 5, 10, or 15 minutes, respectively, and all gave equivalent results, i.e. no significant effect on water permeability. Two different concentrations of EPO were tested, 10^{-7} g/ml and 10^{-8} g/ml, and there was no effect of any of these concentrations on water permeability.

Glutamate modulates astrocyte AQP4 water permeability via activation of group I mGluRs, as shown in this thesis. Having found that EPO itself did not affect water permeability in astrocytes, we proceeded to test whether EPO might affect the mGluR-triggered increase in astrocyte water permeability. The group I mGluR agonist DHPG, again, significantly increased water permeability in the astroglial cells in primary culture. The cells were then preincubated with EPO for ten minutes before exposure to DHPG. Pre-treatment with EPO significantly attenuated the DHPG-induced increase in water permeability in the astroglial cells in primary culture. To examine whether the inhibitory effect of EPO on water permeability was mediated via an effect on AQP4 permeability, we used the astrocyte cell line transiently transfected with GFP-tagged AQP4. DHPG caused a significant increase in water permeability in the GFP-AQP4 positive cells. Pre-treatment of the cells with EPO completely abolished the DHPG-induced increase in water permeability in GFP-AQP4 positive cells. There was no significant difference in P_f between GFP-AQP4 positive cells exposed to vehicle and GFP-AQP4 positive cells exposed to EPO followed by DHPG. In cells that did not express AQP4, GFP-AQP4 negative cells, neither DHPG nor pre-treatment with EPO before DHPG had any effect on water permeability, underscoring that the observed effect was attributable to an effect on AQP4 and not a general effect on all cells. We found that neither DHPG nor EPO induced any redistribution of the GFP-signal in the cells transfected with the GFP-AQP4 fusion protein. This finding suggested that the observed up-regulation and the inhibited up-regulation, respectively, of AQP4 water permeability was due to gating of the water channel rather than to exo- or endocytosis of AQP4.

The mechanism by which EPO affects the water permeability was explored. Activation of group I mGluRs typically triggers increases in intracellular calcium. The group I mGluR agonist DHPG caused an immediate and consistent $[Ca^{2+}]_i$ increase followed by periodic calcium oscillations. This response was observed in 20-30% of all cells. Frequency analysis revealed a highly regular appearance of the oscillatory changes in intracellular calcium with an approximate periodicity of 15 sec, corresponding to a frequency of 65 mHz. This type of response is considered to be typical for the group I mGluR5. The oscillatory response was also observed when a calcium-free buffer was used, indicating that the rise in $[Ca^{2+}]_i$ was a result of release of Ca^{2+} from intracellular stores. Next we examined the calcium response to DHPG in cells pre-treated with EPO or carbamylated EPO (CEPO). CEPO is a non-erythropoietic EPO-analogue where all lysines have been modified to homocitrulline (Leist et al., 2004, Coleman et al., 2006). Pre-treatment of the cells with CEPO for 10 minutes altered the appearance of the DHPG-triggered intracellular calcium oscillations. Approximately 20% of the cells still responded with periodic changes in $[Ca^{2+}]_i$, but the oscillations had a less regular pattern and the synchronicity of the response was disappeared. The effect of CEPO was confirmed when analyzed by a frequency analysis. Following pre-treatment with CEPO, the DHPG response no longer showed any peak at 65 mHz. Instead, the analysis of the DHPG response following pre-treatment with CEPO resulted in a frequency spectrum that lacked any peak frequency. The difference between the response to DHPG with and without pre-treatment with CEPO was significant at 65 mHz. Pre-treatment of the cells with EPO for 10 minutes before applying DHPG resulted in an equivalent change in the calcium oscillatory response as CEPO. CEPO or EPO alone did not induce any calcium response in the astroglial cells.

In conclusion, we found that EPO counter-acts the effect of glutamate, mediated by mGluRs, on AQP4 water permeability in astrocytes. Our data indicate that EPO and carbamylated EPO interferes with the fast calcium oscillations triggered by the mGluRs. Calcium oscillations is maybe the most specific and most versatile of all

intracellular signals since the cell can decode the frequency of intracellular Ca^{2+} oscillations (Berridge et al., 1998). Thus we find it likely that the different calcium responses to DHPG recorded in cells incubated with DHPG alone and in cells preincubated with EPO/CEPO, may explain why EPO abolishes the DHPG effect on astrocyte AQP4 water permeability. The distal mechanism for this interaction remains to be clarified. It is possible that one of the targets is CaMKII, since different $[Ca^{2+}]_i$ oscillation frequencies have been shown to affect the level of phosphorylation and activity of this kinase (Hudmon and Schulman, 2002), and, as shown here, activation of CaMKII is one of the steps in the signaling pathway by which glutamate and DHPG increases the water permeability of AQP4 expressing astrocytes. We will proceed with studies to further investigate how EPO interferes with the signaling pathway of AQP4 regulation. These will include studies to elucidate the role of nitric oxide and explore further downstream effects of EPO or EPO-analogues on the NOS-NO-cGMP-PKG signaling pathway involved in the regulation of AQP4. In addition we will study whether EPO/EPO-analogues affect phosphorylation of the identified target for AQP4 regulation, the serine 111 residue. We are in the process of developing a site-specific phospho-antibody for AQP4 S111 that will be useful for this purpose.

GENERAL DISCUSSION

In the present thesis I have focused on exploring whether astrocyte water permeability can be short-term regulated and investigated the role of AQP4 and signaling pathways involved in this regulation. I have also studied some aspects of regulation of AQP4 in the brain on the expressional level. The data presented show that astrocyte water permeability can be dynamically regulated via changes in the opening state of AQP4. The findings may have both physiological and pathophysiological implications.

AQP4 permeability is regulated by gating

To study whether short-term regulation of astrocyte AQP4 water permeability occurred we chose to study the effect of one exogen (lead) and one endogen (glutamate) substance. Both lead and glutamate are neurotoxic. Lead neurotoxicity is associated with brain edema. Glutamate neurotoxicity occurs when glutamate is released in excess, generally secondarily to hypoxia and ischemia, i.e. conditions that are associated with brain edema. We found that astrocyte water permeability was increased by both lead and glutamate and that this increase was attributable to an effect on AQP4 water permeability. The glutamate-mediated increase in astrocyte water permeability was mediated by group I mGluR activation. Both the lead-triggered and the DHPG-triggered increase in AQP4 permeability were dose-dependent, which should underline the physiological relevance of the findings. Moreover, the neuroprotective agent EPO could counter-act the increase in AQP4 water permeability caused by activation of the mGluRs. Our results provide strong evidence that AQP4 water permeability in astrocytes is indeed short-term regulated. Short term regulation of AQPs can occur in two principally different ways, namely by subcellular trafficking (exo- and endocytosis) or by gating of the channel inserted in the plasma membrane (see Appendix). Our results strongly

indicate that the observed regulation of AQP4 is taking place through gating of the water channel rather than to recruitment of new AQP4 molecules. We did not at any time find that exo- or endocytosis of AQP4 occurred. This is in line with the fact that AQP4 never has been reported to be found in subcellular compartments in the brain or in other locations. We used a whole cell system to study AQP4 regulation in its native environment; the astrocyte. The observed increase in astrocyte AQP4 water permeability may take place in two different ways. The opening diameter of all AQP4 molecules in a cell can increase, i.e. allow for passage of more water molecules per time unit, resulting in an increase in water permeability. On the other hand, assuming that AQP4 can be closed, one can envision a situation where equilibrium between the number of open and closed AQP4 water channels exists in a cell. By stimulation, e.g. by group I mGluR activation, this equilibrium may be shifted towards a situation where more channels will be open, thereby increasing overall water permeability. These two situations are graphically illustrated in Fig. 5.

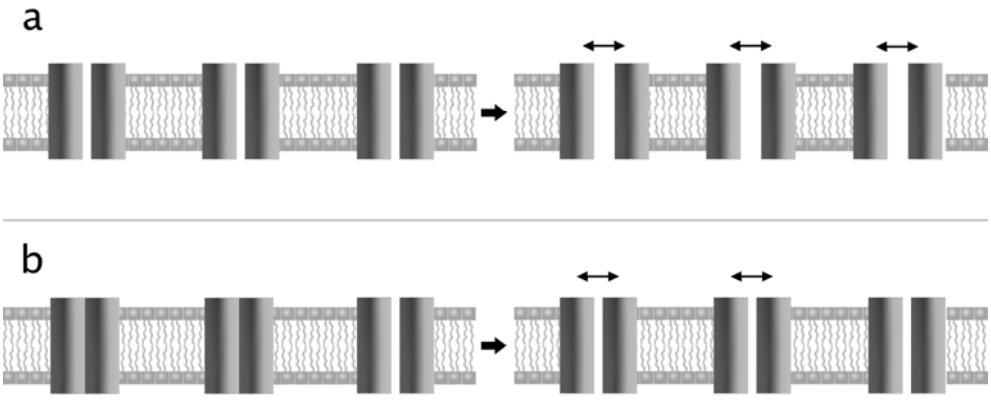

Figure 5. Graphical illustration of two potential modes of increased whole-cell AQP4 water permeability. a. The opening diameter of all AQP4 molecules in a cell increases. b. Shift of equilibrium between the number of open and closed AQP4 water channels.

It should be mentioned that when transfecting AQP4 into astrocytes, the cells that express the water channel always increase their water permeability, even under control situations. We have never found the basal water permeability of GFP-AQP4 expressing cells to be equal to non-transfected cells. This can be interpreted that AQP4 water channels are never all closed. However, we can not determine which of the above mentioned scenarios; the more-or-less open state of AQP4 or the all-or-none open state of each AQP4 molecule is in fact responsible for the increased water permeability.

It should be mentioned that glutamate transporters, known to be expressed in astroglial cells, have been shown to transport water together with glutamate (MacAulay et al., 2002). As DHPG, acting on mGluRs, mimicked the effect of glutamate, we excluded the possibility that the increase in water uptake into the cells were due to glutamate transporters. Furthermore, control experiments were performed when we exposed the cells to an isoosmotic solution containing glutamate. No decrease in calcein fluorescence was detected, i.e. there was no indication that water was entering the cells via glutamate transporters.

We have found that a highly conserved serine, the serine 111 residue, is a putative phosphorylation site for several protein kinases. In the present thesis we show that the Ser111 residue, located in the first intracellular loop, was the target for the observed increase in water permeability caused by both lead and glutamate. It was recently shown that the state of phosphorylation of the Ser115 residue in the first intracellular loop of the spinach AQP SoPIP2;1 is, depending on whether it carries negative charges or not, an important determinant of the diameter of the water channel (Tornroth-Horsefield et al., 2006). The channel was predicted to be open when the serine residue was phosphorylated and closed following dephosphorylation. When aligning AQP4 to spinach AQP, the serine 111 residue of AQP4 was found to correspond to the serine 115 residue in spinach AQP. The identification of the Ser111 residue as the target for the both the lead and the glutamate/DHPG effects adds strong support to the concept that AQP4 is regulated

via gating. In control experiments, not included in the thesis, we could exclude the Ser180 residue (shown to be implicated in regulation of AQP4 in cultured kidney cells) as a target for the glutamate-triggered increase in astrocyte AQP4 permeability.

We found that both the lead- and the glutamate-triggered increase in AQP4 water permeability were mediated by a calcium signaling pathway that involved CaMKII. In Paper II we present evidence that support the role of the group I mGluR-CaMKII-NO-cGMP-PKG signaling pathway as a modulator of astrocyte AQP4 water permeability. Calcium signaling has been recognized as an important way of response in glial cells (Deitmer et al., 1998) and the role of group I metabotropic glutamate receptors as key mediators in neuron-astrocyte communication *in vivo* has been underlined (Wang et al., 2006). Our findings that the group I mGluR agonist DHPG resulted in regular calcium oscillations with a periodicity of 15 seconds, supports that the effect probably was mediated by mGluR5. CaMKII is known to be present in astrocytes (Vallano et al., 2000, Song et al., 2006) and different $[Ca^{2+}]_i$ oscillation frequencies have been shown to affect the level of phosphorylation and activity of this kinase (Hudmon and Schulman, 2002). Protein kinase C (PKC) is a calcium-dependent protein kinase and has been shown to regulate AQP4 in cultured kidney cells (Zelenina et al., 2002). PKC was not found to affect the lead-induced increase in astrocyte AQP4 water permeability and thus the regulation of AQP4 may differ between kidney and brain.

It is well established that AQP4 water permeability can be regulated by reversible protein phosphorylation (Han et al., 1998, Zelenina et al., 2002). The NetPhos analysis indicated that the identified target for lead- and mGluR up-regulation of AQP4 activity, Ser111, was a potential phosphorylation site for several Ser/Thr protein kinases, among these CaMKII. We were therefore surprised to find that CaMKII did not phosphorylate a short peptide of wild type AQP4 that included Ser111. Instead, we found that the WT peptide was phosphorylated by PKG. We reason that the activation of PKG is downstream of CaMKII. The effects of

CaMKII on the NO-NOS-PKG pathway may be cell specific. Thus it has been shown in endothelial cells that CaMKII phosphorylates NOS (Schneider et al., 2003) and thereby activates the NO-cGMP-protein kinase G signaling pathway. The activation of NOS and/or NO production by CaMKII in our cells remains to be proven. As NOS, like CaMKII, is known to be activated by Ca^{2+} and calmodulin (Boucher et al., 1999), we cannot rule out the possibility that the mGluR-triggered calcium signal acts by activating NOS directly. Nonetheless, the involvement of PKG was supported by the finding that the protein kinase inhibitor H-89, which is a strong inhibitor of PKG at the concentration used in our study, also abolished the effect of DHPG on AQP4 water permeability. The finding that the NOS inhibitor L-NAME completely abolished the DHPG-triggered increase in AQP4 water permeability added further support to the role of the group I mGluR-CaMKII-NO-cGMP-PKG signaling pathway for regulation of astrocyte AQP4 water permeability. The NOS-NO pathway in astrocytes has, to the best of our knowledge, never been implicated in water permeability or volume regulation of astrocytes. Interestingly, it was reported that glutamate induced a rapid and reactive NO production in astrocytes in neocortical slices (Buskila et al., 2005). The receptors responsible for this effect were, however, not identified. Activation of constitutive NOS has been shown to be coupled to increased intracellular cGMP in cultured astrocytes (Oka et al., 2004). It will be an important topic for future studies to further reveal the role of NO for regulation of brain water homeostasis. In conclusion, the findings strongly support that the opening state of AQP4 is dynamically regulated by gating of the water channel, that the signaling pathway for this regulation involves calcium oscillations and activation of CaMKII-NOS-PKG and, that the target for this regulation is the Ser111 residue.

Role of AQP4 in perturbed brain water homeostasis

Normal brain function is tightly coupled to water homeostasis. Several recent studies have drawn attention to the importance of a well-controlled brain water

homeostasis for the stability of neuronal function (Li and Verkman, 2001, Amiry-Moghaddam et al., 2003b, Simard and Nedergaard, 2004, Binder et al., 2006, Seifert et al., 2006). Perturbations in water homeostasis are common in acute brain diseases including stroke (Hacke et al., 1996, Ayata and Ropper, 2002), trauma (Unterberg et al., 2004) and meningitis (Saez-Llorens and McCracken, 2003). A common denominator of these conditions is brain edema, which represents the prime example of a neurological condition involving loss of a proper control of brain water homeostasis. It is illustrative for the role of appropriate brain water homeostasis that in the model of primary brain edema (water intoxication), as included in this thesis (Paper IV), the brain is the first and seemingly only organ to be affected, as symptoms from the CNS evolve rapidly following the water load. Other major health problems that involve perturbations of ion and water homeostasis in brain include brain tumors, hydrocephalus, epilepsy, intoxications and severe hyponatremia. Hyponatremia in combination with excess water is the clinical counterpart of the water intoxication model, and represents a serious clinical problem following surgery and intensive care (Fraser and Arieff, 1997). Astrocytes, which express AQP4, play a key role in the regulation of brain water homeostasis (Amiry-Moghaddam and Ottersen, 2003, Simard and Nedergaard, 2004, Kimelberg Harold, 2005). In several conditions associated with perturbed brain water homeostasis, AQP4 is hown to have a deleterious effect on brain water homeostasis and contribute to the development of cytotoxic brain edema. Cytotoxic brain edema is, as mentioned, a phenomenon mainly consisting of astrocyte swelling (Kimelberg, 1995). Verkman's group calculated that 80% of the excess water in meningitis entered the brain parenchyma through AQP4 water channels (Papadopoulos and Verkman, 2005). Deletion of AQP4 has, accordingly, been shown to improve outcome and reduces brain swelling following ischemia, meningitis and water intoxication (Manley et al., 2000, Vajda et al., 2002, Amiry-Moghaddam et al., 2003a, Amiry-Moghaddam et al., 2004, Papadopoulos and Verkman, 2005).

The findings presented here; that glutamate and lead increase water permeability in astrocytes expressing AQP4 may support the concept of a deleterious role of AQP4 in conditions associated with perturbed water homeostasis. Glutamate has been observed to induce astrocyte swelling *in vitro* when employing high extracellular concentrations, mimicking pathological situations (Hansson et al., 1994, Kimelberg, 2005). Here, we have identified AQP4 as a target for the glutamate-triggered increase in astrocyte water permeability. The effect was dose-dependent. Although we believe that the effect of glutamate on AQP4 in astrocytes may have a physiological role under normal conditions, it is quite possible that during pathologically increased glutamate-concentrations the effect on AQP4 may contribute to the development of cytotoxic brain edema. We found that the glutamate effect was mediated via activation of group I mGluRs. It is interesting to note that in a study from China, microinjection of a mGluR agonist (that activates group I mGluRs) into the brain resulted in increase in brain water content and a remarkable swelling of astrocyte endfeet processes (Yuan et al., 2000). We are in the process to investigate whether antagonists of group I mGluRs will be protective against brain edema and neurological damage. The findings that EPO antagonizes upregulation of AQP4 water permeability *and* is protective in the water intoxication model, previously shown to be attenuated by removal of AQP4, gives further support to the role of AQP4 in brain edema formation. Acute lead intoxication is also associated with brain edema and astrocyte swelling. We found that lead specifically increased water permeability in astrocytes expressing AQP4. Taking the adverse effects of AQP4 in brain edema into account, our results may offer an explanation to some of the pathology of acute lead intoxication. Assuming that the lead-induced activation of AQP4 water permeability is potentially hazardous, the lack of downregulation of AQP4 mRNA as a response to lead may prove to be disadvantageous in the situation of acute lead intoxication.

Water flow via AQPs is bidirectional and dependent on the osmotic gradient across the plasma membrane (Agre et al., 2002). So, osmotic gradients are needed in order

for water movement to occur. The *direction* of transmembranous water movement in each physiological or pathophysiological situation is not obvious. In fact, to understand the temporal and spatial resolution of the driving forces for water is an extremely complex, but necessary, task in order to predict the direction of water movement. Often times this prediction is limited by our static view on a dynamic event. Nevertheless, in many of the above mentioned conditions associated with perturbed brain water homeostasis, including ischemia and meningitis, astroglial cells can become unable to regulate ionic gradients across the plasma membrane. In ischemia/hypoxia, energy-demanding processes like the Na^+,K^+-ATPase will be affected, resulting in a progressive loss of membrane ionic gradients (Dirnagl et al., 1999). Ion homestasis will be further perturbed through a number of mechanisms, including effects on cotransporters (e.g. NKCC) and ion exchangers (e.g. NHE) (Kimelberg, 2005). The resultant accumulation in intracellular Na^+ and other ions will create an osmotic driving force for entry of water into the cells. Thus, given a situation of equal osmotic forces, the amount of water entering into the cells should to a certain extent be determined by the open state of water channels. By this it follows that, in a situation where dissipation of ionic gradients across the plasma membrane favors water leakage into the cells, stimuli that up-regulates the water permeability of AQP4 may have a deleterious effect on brain water homeostasis and contribute to the development of cytotoxic brain edema.

AQP4 – the double-edged sword

Astrocytes are direct communication partners of neurons and dynamically interact with synapses through the uptake and release of neurotransmitters and receptor-mediated intracellular Ca^{2+}-signaling (Seifert et al., 2006, Wang et al., 2006). Glutamate is suggested to be the key mediator of the neuron-astrocyte signaling, and mGluRs appear to mediate most of the biological effects in astrocytes. Astrocyte must optimize the extra-cellular space for synaptic transmission and in order to avoid excessive stimulation of neurons they possess highly efficient

systems for clearance of K^+ and glutamate (Nagelhus et al., 1999, Schousboe, 2003, Butt and Kalsi, 2006). The extracellular level of K^+ has to be tightly controlled and uptake of K^+ into astrocytes is followed by water, leading to activity dependent swelling. The results from several studies indicate that water transport via AQP4 is indeed important for efficient removal of the increase in extra-cellular K^+ that follows synaptic transmission. Specifically, studies on animals that lack AQP4 have provided evidence that a build-up of K^+ might be secondary to a deficient water transport (Amiry-Moghaddam et al., 2003b, Binder et al., 2006). The delayed clearance of K^+ was not attributable to any change in the expression of Kir4.1. Logically one can therefore assume that perturbed expression or altered conformation of AQP4, if present, might impede water clearance from active neuropil and indirectly cause an activity-dependent overflow of K^+. In support of this, mice lacking AQP4 have more severe seizures when challenged with electrical stimulation (Binder et al., 2006) and patients with temporal lobe epilepsy have been found to have very low levels of AQP4 immunoreactivity in hippocampus (Eid et al., 2005). The results from the present thesis would suggest that the concomitant neuronal release of K^+ and glutamate serves to facilitate K^+-uptake into astrocytes in a physiological setting. The transient increase in extra-cellular glutamate concentration during neuronal activity should activate astrocyte mGluRs, increase water permeability of AQP4 and thus promote K^+ clearance.

The first studies exploring the function of AQP4 in brain revealed its role in aggravating brain edema (Manley et al., 2000, Vajda et al., 2002) and thereby indicated that the presence of AQP4 in the brain represents a risk. Accordingly, the goal to find an inhibititor or a blocker of AQP4 was mentioned by several investigators. Now, including more recent studies, the concept emerges that AQP4 indeed has an important, physiological role in providing a proper extra-cellular milieu for neuronal function and avoiding epileptic seizures. This function must be taken into consideration when modulation of AQP4 activity is planned. It has been speculated that efflux of water via AQP4 may be facilitating in the resolution phase

of an established brain edema, but so far there are no evidence to support this. Taken together, AQP4 has emerged as a double-edged sword with both beneficial and detrimental effects. It is tempting to speculate that modulation of AQP4 could prove to be therapeutically useful not only in curtailing water uptake, but also in controlling neuronal excitability. It will be an important topic for future studies to explore whether AQP4 is implicated in the pathogenesis of epilepsy. The evidence presented in this thesis, disclosing receptors and molecules in the signaling pathway of AQP4 regulation, may provide new targets for modulation of the function of AQP4 in astrocytes.

The potential neuroprotective effect of inhibition of AQP4

Brain edema, followed by an increase in intracranial pressure, is commonly the direct cause of death after stroke and head trauma and contributes significantly to the morbidity of those patients who survive (Ayata and Ropper, 2002, Unterberg et al., 2004). Diffuse brain swelling after TBI occurs more commonly in children than adults and the presence of diffuse swelling is associated with threefold higher mortality rate (Bauer and Fritz, 2004). Secondary injuries to the brain are presumably caused by brain edema and increased intracranial pressure. Despite the magnitude of this health problem the treatment for brain edema is largely the same for decades. Childhood brain tumors; the second most common pediatric malignancy, hydrocephalus and epilepsy represent diagnoses which are expected or shown to involve perturbations of brain ion and water homeostasis.

The term “neuroprotection” is widely used in the search for agents that may reduce neurological injury. The goal of neuroprotection is to rescue neuronal cells from cell death and includes the prevention of inflammatory cascades and apoptotsis. Neuroprotection may however, in my opinion, turn out to include the maintenance of normal astrocyte function. Brain edema will not only cause increased intracranial pressure (and impaired cerebral blood flow) but will also compromise

neuronal function on a cellular level. The prime goal of neuroprotection in stroke has been suggested to be the salvation of the ischemic penumbra. Despite a large number of therapeutic interventions that decrease neurological damage in ischemic stroke in experimental animals, a number of clinical trials have failed to prove protection of the same agents in humans (Dirnagl et al., 1999). Brain edema is common in severe neonatal hypoxia-ischemia. Several therapeutic interventions for this condition have shown very limited effects, including hyperosmotic agents, calcium channel blockers and steroids (A.D. Edwards et al., in: The newborn brain, Cambridge University Press 2002). In traumatic brain injury, prevention of edema formation is acknowledged as one of the most crucial points in the efforts to avoid an additional, secondary brain insult (Noppens and Brambrink, 2004). Regardless of which the primary mechanism of injury to the brain is (ischemia, hypoxia, trauma, infection), the pathophysiological mechanisms involved are complex and certainly involve factors such as excitotoxicity, oxygen free radicals and immunoinflammatory processes, which may eventually cause cell death. However, when reviewing some of the vast literature around these conditions, there is agreement among several investigators that cytotoxic brain edema is present at some point in most, if not all, acute brain injuries.

The discovery of water channels have evoked a hope of the development of novel therapies based on a molecular understanding of the disease processes. AQP4 has been identified as a passageway for water from blood to brain and between extracellular and intracellular compartments. This makes AQP4 a prime target for an entirely new approach to the treatment of brain edema and dysregulated water homeostasis. As already pointed to, there is now ample evidence that in conditions like ischemia, meningitis and water intoxication, AQP4 aggravates brain water homeostasis and contribute to the development of cytotoxic brain edema. Thus AQP4 could be the long sought-after therapeutic target in cytotoxic brain edema of various etiologies. However, since AQP4 is expressed in several organs (Terris et al., 1995, Nielsen et al., 1997a, Hamann et al., 1998), it has been suggested that

inhibitors of AQP4 up-regulation may be better drugs than AQP4 channel blockers for a more specific treatment of brain edema (Amiry-Moghaddam and Ottersen, 2003, Papadopoulos and Verkman, 2005). To date, no specific AQP4 blocker has been reported, but it may be predicted that AQP4 channel blockers may impair hearing and vision and have peripheral side effects, e.g. in kidney and lung. Verkman's group suggested that inhibitors of AQP4 *expressional* up-regulation would be beneficial (Papadopoulos and Verkman, 2005). It could be argued, though, that up-regulation of AQP4 expression has not been established as causative for brain edema development. We propose that inhibitors of AQP4 *activity* up-regulation might be preferred. Our finding that glutamate, released in excess in many conditions associated with brain edema, up-regulates the activity of astrocyte AQP4 via activation of mGluRs make these receptors and the molecules in the signaling cascade attractive targets for new therapeutic strategies in brain edema. Antagonists of mGluRs will probably be more brain-specific and give considerable less peripheral side effects than AQP4 blockers. We have recently initiated a study of the protective effects of group I mGluRs in a rat model of traumatic brain injury. Group I mGluRs are, however, also found outside the CNS (Gill et al., 1999), so peripheral side effects of specific antagonists can not be excluded.

Erythropoietin (and its non-erythroid analogues, here referred to as "EPO") has in recent years emerged as an efficient neuro-protective agent (Genc et al., 2004, Brines and Cerami, 2005, Brines and Cerami, 2006). It is well documented from both experimental and clinical studies that EPO attenuates the degree of brain damage following stroke (Brines et al., 2000, Ehrenreich et al., 2002, Liu et al., 2006). Experimental studies have indicated that EPO also protects from glutamate triggered neurotoxicity (Yamasaki et al., 2005) and neonatal hypoxia-ischemia (Kumral et al., 2003, Wang et al., 2004). The mechanisms of action are not fully revealed, but evidence indicate that EPO acts by preventing the destruction of viable tissue surrounding the site of an injury, i.e. the penumbra (Brines and

Cerami, 2005). Cell swelling, increased amounts of glutamate and transient elevations of K^+ are characteristic features of the penumbra (Di et al., 2003, Weinstein et al., 2004). A pathophysiologic role of AQP4 in some, if not all, of these features seems obvious. The results from this thesis suggest that EPO may attenuate the development of cytotoxic edema. Glutamate increases the permeability of AQP4 in astrocytes and here we showed that this effect was counteracted by EPO. Thus EPO might, at least partly, exert its neuro-protective effect by reducing the risk for astrocyte swelling and brain edema formation. In the search of "brain-specific" inhibitors of AQP4 up-regulation, EPO; appearing to work downstream of the mGluRs, may turn out to be ideal for such a purpose. We found that EPO has a neuro-protective effect in an acute model of water intoxication. This finding supports the concept that EPO may, in addition to other tissue protective effects (Villa et al., 2003, Ghezzi and Brines, 2004, Brines and Cerami, 2005), attenuate the pathophysiological consequences of brain edema. Our finding, that EPO is protective in primary brain edema via an effect on AQP4, is supported by the study by Manley et al. that AQP4 knock-out mice were protected against brain edema caused by the very same model that we used in our study (Manley et al., 2000).

We found that the NOS-NO pathway is involved in the mGluR-triggered up-regulation of astrocyte AQP4 water permeability. From this it may be predicted that EPO, interfering with calcium-signaling from these receptors, inhibits the downstream activation of NOS. This is in conflict with reports that have found that EPO enhance NO signaling. This NO-signaling, however, has been implicated in vascular relaxation and maintainance of local blood flow (Beleslin-Cokic et al., 2004). On the other hand, EPO treatment significantly reduced the increased brain nitrite levels in hemispheres of rat pups subjected to hypoxic-ischemic insults, suggesting that EPO may have an inhibitory effect on NO overproduction (Kumral et al., 2004). We found that EPO altered calcium oscillations induced by mGluRs. The distal mechanisms for this interaction remain to be clarified. EPO has been

reported to modulate calcium-channels in erytrhoid cells (Tong et al., 2004). We did not find that EPO itself induced a calcium response in our cells, suggesting that EPO-calcium interactions may be cell-specific. The mechanisms of EPO neuroprotection has been shown to include anti-apoptotic and anti-inflammatory effets (Siren et al., 2001, Villa et al., 2006). It is tempting to speculate that an early inhibition of astrocyte cell swelling might also have a positive effect on neuronal survival by maintaining astrocyte function and the extracellular environment. These last comments are speculative, and we will address the distal mechanisms of the interaction between EPO and AQP4 further in future studies. We will also assess the effect of EPO and its non-erythroid derivates on brain edema and neuroprotection in an experimental trauma model.

The reasons for the increased susceptibility of children to develop brain edema following TBI are not known. In mammals, brain water content is age-dependent and decreases with age. Also, the extracellular space volume is believed to be greater in immature brain tissue. This may challenge the systems involved in regulation of brain water homeostasis under pathological conditions. One can hypothesize that developmental aspects of AQP4 expression or function might affect increased tendency for brain edema in children. In the developing rat brain, AQP4 protein expression has been found to increase during the second postnatal week (Wen et al., 1999). We found no major differences in AQP4 mRNA in the brain between 10 and 40 day old rats using riboprobes against AQP4. However, in mice, we found a maturational change in the AQP4.M23X form, which suggests that the ontogeny of AQP4 expression might depend on which mRNA species and which protein isoform that is studied. In chicken, expression of AQP4 occurs in ependymal and astroglial cells during early embryonic development (Nico et al., 2001). We have unpublished data that AQP4mRNA is expressed in premature lamb brains. Interestingly, it was recently reported that AQP4 protein occurs in the human brain as early as gestational week 17, and that the level of AQP4 expression in astrocyte end-feet at term (40 weeks) was reached in the fetuses already at 23

weeks gestational age (El-Khoury et al., 2006). Hence, the development of AQP4 expression may be species-dependent. Differences in susceptibility for brain edema might also depend on differences in the dynamic regulation of AQP4, the receptors and/or the intracellular signalling pathways regulating this. Here it is interesting to note that both mGluR5 expression and phosphoinositide metabolism in response to glutamate have been shown to be much higher in immature brain compared to adult brain in rats (Romano et al., 1996, Simeone et al., 2004).

In conclusion, based on ours and others findings, it is suggested that astrocyte AQP4 represents a target for development of new therapeutic strategies in brain edema and that inhibition of astrocyte AQP4 activity may have a neuroprotective potential. Further studies are required and should also be aimed at revealing whether AQP4 may be a target for treatment in other conditions, such as epilepsy. Neurology as a discipline continues to lag behind other disciplines when it comes to the range and efficacy of medical treatment. I believe that studies of the regulation of water transport in brain cells is essential to our understanding of mechanisms involved in neurological diseases in which loss of water homeostasis is a primary or secondary event.

FUTURE PERSPECTIVES

The results included in this thesis have provided some answers but also raised new issues for future studies. The main focus of future research on brain aquaporins should be on their potential role in disease processes and on their role as promising targets for new therapeutic strategies.

We will further investigate how EPO interferes with the signaling pathway of AQP4 regulation, including studies of downstream elements in the pathway and the role of NOS-NO signaling in astrocytes. The results can be expected to shed additional light on mechanisms involved in brain edema formation and point to

potential targets for development of specific therapeutic agents for cytotoxic brain edema. We will also investigate the neuroprotective effects of both group I mGluR antagonists, EPO and tissue-protective EPO-analogues in a rat brain injury model. These studies will include assessment of edema formation and the expression of AQP4 in the brain.

Vasopressin is implicated in brain water homeostasis and in astrocyte swelling. We will study whether vasopressin can regulate astrocyte AQP4 water permeability. If so, vasopressin receptors or molecules in the signaling pathway can be explored as targets for treatment of neurological conditions associated with perturbed brain water homeostasis.

The role of AQP4 in astrocyte potassium homeostasis will be explored further in our group. This will include studies of interaction between AQP4 and other proteins involved in potassium uptake in astrocytes, such as Na^+,K^+-ATPase. An improved understanding of the role of AQP4 and interacting proteins involved in astrocyte potassium homeostasis are of utmost clinical importance and can be expected to reveal whether AQP4 may be a target for treatment in epilepsy. It will be an important topic for future studies to explore whether changes in AQP4 gene, protein structure or function are implicated in some forms of human epilepsy.

CONCLUSIONS

This thesis presents evidence that astrocyte water permeability can be dynamically regulated via changes in the opening state of AQP4. Our data support the concept that the phosphorylation state of a serine residue in the first intracellular loop of AQP4 determines the opening state of AQP4. We show that the intracellular pathway of astrocyte AQP4 regulation involves a calcium signaling pathway including CaMKII, NOS and PKG. These findings may have both physiological and pathophysiological implications, since both an exogen, toxic substance (lead)

and endogenous substance (glutamate) were found to affect the short-term regulation of astrocyte AQP4 water permeability. We show that the glutamate-mediated increase in astrocyte water permeability is mediated by mGluR group I activation, a finding that supports the role of mGluRs as key mediators of biological effects in astrocytes.

We have identified a new form of "brain-specific" AQP4 mRNA in mice and show that this AQP4 mRNA is developmentally regulated in the brain. We show that the toxic agent lead only affects the short-term and not the long-term regulation of AQP4. We show how the neuroprotective agent EPO can protect against primary brain edema. We present evidence that EPO counteracts the effect of glutamate, mediated by mGluRs, on AQP4 water permeability in astrocytes. Our data indicate that EPO modifies the fast intracellular calcium oscillations caused by activation of mGluRs.

These results, describing a dynamic regulation of astrocyte AQP4 water permeability, have an impact on the understanding of physiological astrocyte water and ion homeostasis and of the role of AQP4 in the development of cytotoxic brain edema. Modulation of astrocyte AQP4 activity may become a new therapeutic strategy in situations of perturbed brain water homeostasis. Receptors and downstream elements in the signaling pathway involved in AQP4 regulation may provide molecular targets for such new therapies.

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APPENDIX