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**STUDIES ON ASTROCYTE FUNCTION:
POTENTIAL ROLES IN BRAIN WATER
HOMEOSTASIS AND NEUROPROTECTION**

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Science has done all the easy tasks -- the clean simple signals. Now all it can face is the noise; it must stare the messiness of life in the eye.

«Out of Control» - Kevin Kelly

To my readers

ABSTRACT

Astrocytes are essential in brain homeostasis and function, including maintenance of water and ion balance. Astrocytes express the water channel aquaporin 4 (AQP4), implicated in both physiological functions and injury processes associated with brain edema, a common consequence of brain diseases. As part of the tripartite synapse astrocytes are tightly coupled to normal brain function via neuron-astrocyte interactions and by providing metabolic support to neurons as well as controlling extracellular potassium and glutamate. The overall aim was to explore the regulation of astrocyte water permeability and study aspects of astrocyte function of relevance for the interplay between astrocytes and neurons in physiology and ischemia.

The molecular mechanisms involved in short term regulation of astrocyte AQP4 were investigated by exploring the effects of glutamate and potassium on astrocyte water permeability. Glutamate was found to significantly increase astrocyte water permeability via activation of group I metabotropic glutamate receptors (mGluRs), an effect attributable to an effect on AQP4. An essential conclusion in this study is that AQP4 can be short-term regulated via gating. The evidence supports that this effect is dependent on phosphorylation, that the AQP4 serine 111 residue is a molecular target for the regulation and that this residue can be phosphorylated by particular protein kinases directly.

Next we showed that elevations in extracellular potassium increase astrocyte water permeability via a cAMP-dependent mechanism involving AQP4. The role of AQP4 serine 111 in the regulation of astrocyte water permeability was confirmed in this study. A prolonged upregulation of astrocyte water permeability was dependent on Kir-channel function. The effect could be modulated by calcium when such signaling was triggered by high extracellular potassium. The findings point to a functional coupling between water transport and potassium handling in astrocytes. Hence, as fundamental 'messengers' from neurons, glutamate and potassium were found to regulate astrocyte water permeability. The results indicate that astrocyte water permeability can be dynamically regulated in response to neuronal activity and that modulation of astrocyte signaling is dependent on both dose and duration of exposure to its regulators.

Due to its neuroprotective potential, the effects of EPO on astrocyte function were examined with regard to water permeability and aspects of astrocyte metabolism. EPO was found to counteract the glutamate-induced upregulation of astrocyte water permeability and significantly reduce neurological symptoms in an animal model of brain edema. It was shown that EPO modifies mGluR-mediated intracellular calcium signaling. In oxygen-glucose deprivation, a cellular model of ischemia, EPO was found to enhance astrocyte glutamate uptake. The effect was depended on the sodium pump Na,K-ATPase and coupled to intracellular pH regulation. The evidence also suggested that astrocyte metabolism is enhanced by EPO under oxygen-glucose-free conditions, a finding that indirectly supports a therapeutic potential of EPO or EPO analogs in ischemia.

Taken together, our data indicate a dynamic role for astrocytes in the regulation of brain water and ion homeostasis. Upregulation of AQP4 water permeability facilitates water transport across the plasma membrane. In conditions associated with perturbed brain water balance, this may accelerate or attenuate brain edema depending on the phase of edema formation or resolution. EPO protects against water overload by modulation of astrocyte water permeability. Moreover, by enhanced astrocyte metabolism and restored astrocyte function in ischemia, EPO should favor local homeostasis and promote neuroprotection via astrocytes.

LIST OF PUBLICATIONS

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

- I. Gunnarson E, Zelenina M, Axehult G, **Song Y**, Bondar A, Krieger P, Brismar H, Zelenin S, Aperia A.

Identification of a molecular target for glutamate regulation of astrocyte water permeability. *Glia*. 2008 Apr 15;56(6):587-96.

- II. **Song Y**, Gunnarson E.

Potassium dependent regulation of astrocyte water permeability is mediated by cAMP signaling. *PLoS One*. 2012;7(4):e34936. Epub 2012 Apr 6.

- III. Gunnarson E, **Song Y**, Kowalewski JM, Brismar H, Brines M, Cerami A, Andersson U, Zelenina M, Aperia A.

Erythropoietin modulation of astrocyte water permeability as a component of neuroprotection. *Proc Natl Acad Sci U S A*. 2009 Feb 3;106(5):1602-7.

- IV. **Song Y**, Gunnarson E.

Erythropoietin enhances astrocyte glutamate uptake in an *in vitro* model of ischemia. *Manuscript*

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LIST OF ABBREVIATIONS

AQP4	aquaporin 4
AQP4 S111A	mutated aquaporin 4 with Ser111 substituted to alanine
CaMKII	calcium(Ca^{2+})/calmodulin-dependent protein kinase II
CKII	casein kinase II
CNS	central nervous system
DHPG	(<i>S</i>)-3,5 dihydroxyphenylglycine
ECS	extracellular space
EPO	erythropoietin
ERK	extracellular signal regulated kinase
FBS	fetal bovine serum
GFP	green fluorescent protein
GFAP	glial fibrillary acidic protein
GPCRs	G-protein-coupled receptors
IP_3	inositol 1,4,5-trisphosphate
Kir	inwardly rectifying K-channel
L-NAME	N ω -nitro-L-arginine methyl ester
mGluR	metabotropic glutamate receptor
NHE	sodium hydrogen exchanger
Na,K-ATPase	sodium potassium ATPase
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
OGD	oxygen-glucose deprivation
PBS	phosphate buffered saline
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
tPA	tissue plasminogen activator
WT	wild type

1 INTRODUCTION

The breakthrough of studies on astrocytes has intensely improved our understanding of operational mechanisms of the central nervous system (CNS). The roles of astrocytes have been honored or disputed, but have never been highlighted as today. In this thesis, I focus on the short term regulation of astrocyte water permeability homeostasis and discuss potential functions of astrocytes in brain water homeostasis and neuroprotection. Due to my background in clinical medicine, our exciting results have inspired me to reflect about possible contributions to clinical therapeutic strategies. I don't consider this thesis as the final destination of my education, but rather a starting point of my scientific vision.

1.1 ROLES OF ASTROCYTES IN BRAIN WATER HOMEOSTASIS

Neurons are never alone. They have billions of astrocytes as housekeepers at home. It is now generally accepted that astrocytes are highly complex cells and respond to diverse external stimulations (Gordon et al. 2007; Kimelberg 1983; Simard and Nedergaard 2004). The essential function of astrocytes is to keep stability of brain internal environment, by means of multiple dynamic equilibrium adjustments, including water, ions, and neurotransmitters. That is, in other words, to maintain homeostasis in the brain.

As up to 87% of brain mass and more than 90% of the molecules in the body, water is inextricably bound to brain function (Amiry-Moghaddam and Ottersen 2003; Go 1997). There are at least two major aspects of astrocyte regulation of brain water homeostasis: water flux in or out of astrocytes, which can be observed as volume changes of astrocytes at the cellular level; and the speed of water transport through astrocyte plasma membrane, defined as astrocyte water permeability.

The extracellular space (ECS) in the brain constitutes typically 13-22% of the tissue volume and can be dramatically regulated by astrocytes (Hoffmann 1992; Risher et al. 2009). The concept of the tripartite synapse, which proposes that astrocytes can be considered as an integral component of the synapse (Araque et al. 1999), further highlights this spatial regulation. The fact that, surrounding synapses, extracellular space is very limited, implies that even small extracellular volume fluctuations will affect extracellular ion concentrations and neuronal activity. Glial cell swelling has

been studied and discussed widely (Bourke et al. 1980; Hoffmann 1992; MacAulay and Zeuthen 2010). Mediators such as neurotransmitters, pH value and extracellular potassium are involved in astrocyte swelling, even though the mechanisms are not fully clear so far. Swollen astrocytes are generally observed in most brain pathological processes, as seen with brain cellular edema, indicating an abnormal accumulation of water in the cell. However, current evidence suggests that neuronal activity-dependent astrocyte swelling is the result of homeostatic mechanisms as well (Kempinski and Volk 1994; Sykova 2005).

In order to maintain water and ion homeostasis during neuronal activity, water transmembrane shifts must be rapidly modulated. Modulation of plasma membrane water permeability is one of the potential functions of astrocytes to optimize the synaptic transmission. Water can pass through the lipid layer of astrocytes by slow diffusion, but the speed of this passive movement is too slow for robust modulation of neuronal activity. Thus, fast regulation of astrocyte water permeability is mainly controlled by proteins, such as ion transporters and water channels (Gunnarson et al. 2004; MacAulay et al. 2004). There are basically two distinct manners for water transport via the astrocyte plasma membrane: osmotic gradient independent or osmotic gradient dependent. For example, the glutamate transporter EAAT1, one of the most efficient water co-transporters in astrocytes, admits an influx of 436 ± 55 water molecules per glutamate anion (MacAulay et al. 2001). Accordingly, there is only 165 water molecules needed per particle in order to achieve isotonicity (MacAulay and Zeuthen 2010), revealing that active water uptake takes place in the process (osmotic gradient independent water transport). Acting as ‘water pumps’, these co-transporters could initiate astrocyte swelling induced by neuronal activity. Theoretically, osmotic gradient independent water transport will require considerable energy to maintain the operation. As opposed to this, it is generally accepted that osmotic gradient dependent water transport by water channels is rather passive and ‘eco-friendly’. This characterization of water channels suggests that they are not as sensitive as co-transporters to brain injuries when energy failure is expected to occur. It may therefore be proposed that astrocyte water channels are the potential therapeutic targets in pathological situations with energy depletion, such as cytotoxic brain edema or brain ischemia. Furthermore, with regard to the capacity for water transport, astrocyte water channels are unchallenged for water traffic crossing the plasma membrane. The single-unit water permeability of aquaporin 4 (AQP4), the

predominant water channel in astrocytes, is 120 times that of the EAAT1 (Amiry-Moghaddam and Ottersen 2003). Also, due to multiple functions and couplings of the co-transporters, outcomes of modulation of co-transporters could be difficult to predict. As a pure water channel, AQP4 is a more practical therapeutic target to be explored. For clinical application, accordingly, one critical question is what mechanism is governing regulation of astrocyte water channels and, in particular, the dynamic regulation of astrocyte AQP4.

1.2 REGULATION OF AQP4

AQP4 is the predominant water channel in the brain. The expression of AQP4 was reported two years after identification of the first member of the aquaporin family (Chip28, later renamed AQP1) (Preston et al. 1992), characterized by its insensitivity to extracellular mercury (Hasegawa et al. 1994). Primary astrocytes from AQP4 deficient mice showed sevenfold reduction of osmotic water permeability (Solenov et al. 2004), suggesting its crucial role for water trafficking in the CNS. The potential physiological clinical roles of AQP4 were further explored in the AQP4-null animal model. An initial study showed that AQP4-null mice were partially protected from brain swelling in response to acute water intoxication and in ischemic stroke (Manley et al. 2000; Papadopoulos et al. 2002). However, this protection was later found to be confined to cytotoxic but not vasogenic brain edema (Papadopoulos and Verkman 2007), which makes a clinical application of drugs targeting AQP4 more complicated. Nevertheless, AQP4 and its regulators could be potential targets for treatment of brain disorders involving disturbances in water homeostasis. Inhibition of AQP4 may provide therapeutic options for preventing brain edema in cerebral diseases such as stroke and water intoxication.

Regulation of AQP4 has been reported in several studies (Han et al. 1998; Madrid et al. 2001; Zelenina et al. 2002). Astrocyte water permeability can be significantly modified by AQP4 protein expression, trafficking and protein interaction (Carmosino et al. 2007; Pan et al. 2010; Silberstein et al. 2004; Yukutake and Yasui 2010). Due to the outstanding single-unit water permeability, expression of AQP4 can dramatically change astrocyte water permeability (Solenov et al. 2004). Internalization of AQP4 has been reported in gastric cells and AQP4 expressing *Xenopus* oocytes (Carmosino et al. 2001; Moeller et al. 2009). Protein–protein interaction and changes of protein conformation are also suggested to be involved in the AQP4 regulation (Illarionova et

al. 2010; Nagelhus et al. 1999; Noell et al. 2007; Silberstein et al. 2004). Nevertheless, most studies regarding regulation of AQP4 in one way or another involve protein phosphorylation.

Phosphorylation of Ser 276 by casein kinase II (CKII) has been shown to regulate AQP4 cell surface expression via protein degradation in epithelial MDCK cells (Madrid et al. 2001). For protein trafficking, Ser 180 has been suggested to be the key regulation site (Moeller et al. 2009). Ser 180 is a potential phosphorylation site of protein kinase C (PKC). The idea was also supported by functional studies, such as in a glioma cell line migration test (Mccoy et al. 2010). That study showed that activation of PKC enhances AQP4 phosphorylation, reduces water permeability and significantly decreases glioma cell invasion. Phorbol esters, a PKC activator, did not change single channel permeability of AQP4 (Verkman and Mitra 2000) and AQP4 S180D, a mutant mimicking phosphorylated AQP4 Ser 180, showed no indications of obstruction near the cytoplasmic pore entrance (Mitsuma et al. 2010). Both reports suggest that Ser 180 phosphorylation regulation on AQP4 water permeability is not dependent on single channel gating.

It was demonstrated that the Ser 115 of spinach aquaporin SoPIP2;1, a serine residue in the first intracellular loop, is an important determinant of the diameter of the water channel (Tornroth-Horsefield et al. 2006). The channel gating was predicted to be regulated by phosphorylation of the serine. When aligning AQP4 to SoPIP2;1, Ser 111 of AQP4 corresponds to Ser 115 of spinach AQP. It is plausible that, depending on its state of phosphorylation, Ser 111 could restrict or facilitate AQP4 water permeability. An AQP4 mutant (M23-AQP4-S111E), mimicking phosphorylated AQP4 on Ser 111, produced approximately 1.5 fold greater single channel permeability in transfected LLC-PK cells, suggesting that Ser 111 is key site for AQP4 channel gating (Silberstein et al. 2004). Ser 111 has also been reported to be involved in long term regulation. Forskolin, a protein kinase A (PKA) activator, can stimulate AQP4 expression in LLC-PK cells, dependent on AQP4 Ser 111 (Van Hoek et al. 2009). However, surprisingly, short term regulation of AQP4 gating has rarely been explored, even though it may be the most efficient and convenient way to modify water permeability of astrocytes.

The expression of AQP4 is found on the astrocyte plasma membrane end-feet domain, indicating its important role for water transport crossing the blood–brain barrier (Nielsen et al. 1997). There is also a low but significant AQP4 expression in the

membrane domains surrounding glutamatergic synapses (Nagelhus et al. 2004). Thus, it is interesting to explore whether factors related to neuronal activity, like neurotransmitters and ions, can regulate AQP4. A rapid regulation of astrocyte AQP4 via gating could serve as a component of the integrated tripartite synaptic function.

1.3 GLUTAMATE AND ITS SIGNALING IN ASTROCYTES

Glutamate is the major mediator of excitatory signals in the CNS and is probably involved in all aspects of normal brain function including cognition, memory and learning. Besides activating postsynaptic neurons, glutamate released from presynaptic terminals also stimulates astrocytes (Bradley and Challiss 2012; Usowicz et al. 1989). The excitatory neurotransmitter considerably affects the physiology and pathology of astrocytes. On the other hand, astrocytes play an important role for the kinetics of extracellular glutamate. Astrocyte-neuron interaction can therefore be established by glutamatergic signaling.

Glutamate receptors have been found on astrocytes (Hertz et al. 1984). Most studies have focused on metabotropic glutamate receptors (mGluR). Different from ionotropic glutamate receptors, mGluRs are not functional as channels but as G-protein-coupled receptors (GPCRs). Evidence supports that there is a diversity of mGlu receptor subtypes expressed on astrocytes (Biber et al. 1999). The mGluR5 was reported to be predominant in astrocytes (Ciccarelli et al. 1997; Miller et al. 1995), especially in early postnatal brain (Romano et al. 1996). There are other reports concerning sub-groups of glutamate receptors in astrocytes, suggesting different patterns of expression (Conadorelli et al. 1997; Silva et al. 1999). It can be speculated that astrocytes from different parts or at special developmental stages of the brain exhibit diverse glutamatergic characterizations.

Group I mGluRs, including mGluR1 and mGluR5, cause phosphoinositide hydrolysis. Classic mGluR5 signaling is coupled to G protein signaling to stimulate phospholipase C activity, which releases calcium through inositol 1,4,5-trisphosphate (IP3) receptor from intracellular stores (Abe et al. 1992; Masu et al. 1991). Different from peak-like changes of intracellular calcium patterns induced by mGluR1, mGluR5 leads to oscillatory changes of intracellular calcium (Kawabata et al. 1996). These calcium oscillations have been reported to be related with PKC phosphorylation on different sites of mGluR5 (Kawabata et al. 1996; Kim et al. 2005).

Intracellular calcium oscillations induced by mGluR5 on astrocytes are accompanied by repetitive calcium elevations in adjacent neurons (Pasti et al. 1997). This elegant calcium coupling therefore set a cross talk between neurons and astrocytes. The neuron response could be induced by calcium dependent release of transmitters from astrocytes (Bradley and Challiss 2012), even though the detailed mechanisms are still under debate (Hamilton and Attwell 2010).

The calcium signaling cascades of astrocytes are diverse, but the effects are expected to serve as components of modulation of neuronal activity. For instance, stimulation of astrocyte mGluR5 enhances the function of glutamate transporters (Vermeiren et al. 2005), indicating that mGluR5 could act as a glutamate sensor to precisely regulate glutamate uptake. Neuronal activity is also related to water homeostasis. Therefore, it is logical to raise the question whether dynamic water transport is involved in glutamate signaling, which could be of importance for both physiological and pathological processes in the CNS.

1.4 AQP4 FUNCTION IN BRAIN POTASSIUM HOMEOSTASIS

Elevated extracellular potassium concentration ($[K^+]_o$) has been observed during neuronal activity and propagated seizures (Frankenhaeuser and Hodgkin 1956; Hotson et al. 1973). Compared with robust changes of glutamate concentration in the ECS, the potassium concentration in the brain is tightly controlled, although with vigorous kinetic changes. With intensive stimulation of neuronal pathways, $[K^+]_o$ could increase from a basal level 2.5-3mM to 8mM (Walz 2000), with a ceiling level of 12mM (Heinemann and Lux 1977). In a pathological situation as ischemia or hypoglycemia, the ceiling level of $[K^+]_o$ can be exceeded (Hansen 1985). In cortical spreading depression, $[K^+]_o$ can reach as high as 30-80mM in intact nervous tissue (Leis et al. 2005). As a fundamental biological factor in the brain, potassium kinetics can influence almost all aspects of neuronal activities. Therefore, potassium has been attracting attention from the very beginning of neuroscience.

One essential function of astrocytes is to keep potassium homeostasis in the brain. Astrocytes were suggested to control neuronal excitability by manipulation of $[K^+]_o$ around fifty years ago (Hertz 1965; Orkand et al. 1966), but the current evidence is still unclear regarding the specific mechanisms (Macaulay and Zeuthen 2012). It has been reported that astrocyte AQP4 is involved in the regulation of CNS potassium

kinetics, as knock down or dislocation of AQP4 will slow down extracellular potassium clearance (Amiry-Moghaddam et al. 2003; Binder et al. 2006). Interestingly, prolonged half time to the peak of $[K^+]_o$ after stimulation, as well as higher seizure threshold are also observed in AQP4 deficient animals, suggesting that the water channel, besides potassium clearance, may also be involved in potassium buildup (Binder et al. 2006; Strohschein et al. 2011). However, there is concern about whether the effect is due to functional interaction between AQP4 function and potassium kinetics or secondary effects of genetic elimination of AQP4 (Macaulay and Zeuthen 2012). Therefore, it should be of importance to explore the coupling between water and potassium homeostasis, particularly the functional interactions between water and potassium channels on astrocytes.

Astrocyte potassium transporters may be involved in rapid $[K^+]_o$ regulation during neuronal firing. The Na-K-Cl co-transporter (NKCC) has been suggested to actively take part in potassium clearance by astrocytes (Macaulay and Zeuthen 2012). However, ion transporter activity is normally coupled to ion gradients. The activity of NKCC is sensitive to intracellular chloride concentration (Payne and Forbush 1995), but elevation of $[K^+]_o$ from 5.8mM to 75mM only increased NKCC dependent potassium influx by 60% at most (Su et al. 2000). Conversely, changes in potassium gradients caused by neuronal activity are strictly restricted by dynamic regulation. For instance, the increase in $[K^+]_o$ is within 1mM during spontaneous activity in the reticular formation (Sykova et al. 1974), and with maximum stimulation intensity, changes in $[K^+]_o$ are less than 5mM (Strohschein et al. 2011). Taken together, this evidence suggests that only limited potassium transport is carried out by NKCC under physiological conditions.

As AQP4 is for water transport, the inwardly rectifying potassium channel 4.1 (Kir4.1) is the predominant potassium channel in astrocytes (Kucheryavykh et al. 2007). The co-enrichment of AQP4 and Kir4.1 has lead to the hypothesis of functional interaction between both channels (Fort et al. 2008; Nagelhus et al. 1999), although there has been arguments against the theory (Ruiz-Ederra et al. 2007). It should be noted that, regarding potassium clearance, conclusions of studies in Kir4.1 knock-out mice are not consistent with acute hippocampus slices treated with a specific blocker of Kir4.1 (Haj-Yasein et al. 2011a; Meeks and Mennerick 2007). These incompatible

results indicate that the roles of Kir4.1 in potassium clearance are more complex than expected.

Taken together, brain water and potassium are intimately related. For brain potassium homeostasis, involvement of astrocyte function has been proposed but the details have been under discussion over time. Astrocyte AQP4 is involved in the regulation of potassium kinetics, but the cross point of AQP4 function and regulation of potassium kinetics is still missing. One contribution that can be made here is to explore potassium regulation of AQP4 and the mechanisms of that, and especially, the possible roles of Kir channels involved.

1.5 ASTROCYTES AND NEUROPROTECTION

Under physiological condition, astrocytes generally attribute functions of neuronal support, such as glutamatergic signaling transduction and regulation of potassium kinetics, as mentioned. In other words, astrocytes are waiting for an initial ‘sparkle’ from neurons. However, current evidence have demonstrated that, under certain conditions, astrocytes control highly dynamic blood vascular response and have complex interactions with neurons, particularly in acute phase of brain damage in ischemia (Trendelenburg and Dirnagl 2005; Gordon et al. 2008).

As a common complication in ischemic stroke (focal cerebral ischemia) as well as head trauma and brain tumor, brain edema is considered to be a major cause of permanent brain damage. The blood-brain barrier function plays a crucial role in brain edema formation (Gotoh et al. 1985; Nico and Ribatti 2012). As one of the functional 'neurovascular units', astrocytes are strategically located between brain microvessels and neurons (Abbott et al. 2006). Microenvironments of neurons are isolated by blood–brain barrier, which can be functionally regulated by astrocytes (Wolburg et al. 2009). Hence, astrocytes are capable of securing brain function by modulation of water and ion trafficking across the blood–brain barrier.

Kir4.1 and AQP4 are both abundant on the perivascular endfeet of astrocytes, which are geographically close to microvessels (Nagelhus et al. 2004). Kir4.1 has been suggested to be responsible for potassium siphoning, a special form of astrocyte potassium spatial buffering. Notably, in normal brain activity, neurotransmitters and potassium are released but generally not lost from the brain (Abbott et al. 2006),

indicating that potassium siphoning is more likely to be observed under pathological situations when potassium needs to be expelled from the brain. On the other hand, glucose metabolism generates water at the rate of $\sim 28 \text{ nl g}^{-1} \text{ min}^{-1}$ (Rapoport 1976), leaving 'net' water that must be removed from the brain. Hence, theoretically, the brain water efflux should be exceeding the influx. If brain water flux is shut down while brain metabolism persists, in principle, this will result in brain edema. Not surprisingly, AQP4 play an important role for brain water flux via blood–brain barrier (Nicchia et al. 2004). Indeed, AQP4 knockout mice showed protection against ischemic brain edema (Verkman 2002). But it has to be kept in mind that deletion of AQP4 can also give the result that more water is retained in the brain (Haj-Yasein et al. 2011b). To access astrocyte AQP4 as a therapeutic target for neuroprotection, diverse strategies must be taken into consideration according to specific phases of brain edema formation.

Besides water management, glutamate uptake and potassium clearance also emphasize the roles of astrocytes in protecting neurons from toxicity of glutamate and potassium overload. Both processes can enhance metabolisms in the CNS and highly increase the demands of ATP (Bittner et al. 2011; Longuemare et al. 1999). Therefore, the protective function of astrocytes was further highlighted by characterization of astrocyte metabolism (Rossi et al. 2007). In line with the ischemic tolerance of astrocytes (Vega et al. 2006), astrocyte glycolysis may benefit neuronal function via anaerobic metabolism during ischemia (Genc et al. 2011). The fact that the coupling between astrocyte metabolism and neuron activity has been revealed (Magistretti 2006; Pellerin and Magistretti 1994) encourages studies to explore roles of astrocyte on CNS metabolism. As an outstanding theory for the past 18 years, the astrocyte–neuron lactate shuttle has been attracting increasing attention from neuroscientists (Pellerin and Magistretti 1994; Pellerin and Magistretti 2011). Although the model is still under debate (DiNuzzo et al. 2010; Mangia et al. 2009), evidence showed that lactate could be a valuable energy substrate to sustain neuronal activity (Schurr et al. 1988; Wyss et al. 2011), and contribute to the neuroprotection in cerebral ischemia (Berthet et al. 2009).

In conclusion, the special location and unique metabolism of astrocytes render prospects for neuroprotection, particularly in brain edema and injuries caused by acute conditions such as water intoxication or stroke. Considering their vital role for

energy supply to neurons, the various functions of astrocytes have to be assessed in the case of brain ischemia. Unfortunately, until now, investigations of the role of astrocytes in ischemia neuroprotection have been remarkably few.

1.6 CEREBRAL ISCHEMIA AND NEUROPROTECTION

As one of leading causes of death and long-term disability, ischemic stroke can be described as focal cerebral ischemia induced by insufficient blood flow for the metabolic demand (Nemoto et al. 2000). Basically, it can be defined as a local metabolic stress induced by energy depletion. Within the affected site, it should be noted that metabolic conditions change significantly from the core of the lesion to its outermost boundary, caused by the gradation of ischemia (Lipton 1999). Hence, the very nature of ischemic stroke provides insights at different regions and developmental stages of focal ischemia.

Focal ischemia induce contiguous damaged brain tissue, defined as infarct (Osborne et al. 1987). Quantified by blood flow reduction at stroke onset, some area of the infarct could be further classified to ischemic core, where blood flow is below 15% of normal tissue, and penumbra where blood flow is 15~40% (Back et al. 1995; Ginsberg and Pulsinelli 1994). Generally, in the ischemic core, a massive cell death is due to necrosis, which is characterized by rapid ATP deficiency and anoxic depolarization. Cell death in the penumbra is less drastic, normally combined with apoptosis, i.e. programmed cell death (Ueda and Fujita 2004). Even though there is no clear line between these two types of cell death for an individual cell (Lipton 1999), studies on neuroprotection traditionally have focused on treatments against apoptosis in the penumbra.

In the penumbra, ischemic cell death is usually delayed. Depending on the severity of insults and different regions, neuronal death can continue as long as days or even weeks (Du et al. 1996; Kirino et al. 1984), which provide a valuable treatment window for neuroprotection. It has been proven extremely difficult to prevent neuronal death in the ischemic core (Stankowski and Gupta 2011). Indeed, 10-20min of ischemia can induce DNA damage and neuronal necrosis in the core (Li et al. 1995; Memezawa et al. 1992), in line with severe brain damage found in a short time window after ischemic stroke (Rossi et al. 2007). Therefore, the main 'battlefield' of neuroprotection is currently in the penumbra.

Remarkably, normal homeostatic mechanisms (e.g. via reperfusion) are unable to prevent delayed cell death in the penumbra after a certain time-point. Compared to permanent ischemia, reperfusion after 2 hours ischemia does not significantly change the size of the brain lesion caused by delayed damage within 72 hours (Zhang et al. 1994). There is no solid evidence showing that increasing blood flow itself is protective during the post-ischemic period (Lipton 1999). It is still debated whether patients with intracranial arterial stenosis can benefit from treatment with stenting (Abou-Chebl and Steinmetz 2012; Chimowitz et al. 2011). Many clinical trials of acute stroke intervention showed negative results, raising significant concerns regarding neuroprotection alone as a strategy for therapeutic intervention in the treatment of stroke (Barone 2009). Considering metabolic level, glucose metabolism is actually enhanced in the cortical penumbral zone (Yao et al. 1995), even though levels of ATP are reduced about 30~50% (Folbergrova et al. 1992). Thus, neuroprotection in ischemia is not as simple as replenishing neurons with oxygen and glucose but rather reestablishing the homeostasis in the injured brain. Furthermore, in such complex circumstances as in ischemia, it is complicated to distinguish protective from destructive mechanisms. For example, it has been suggested that, in the event of brain ischemia, apoptosis can be considered as an outcome of self-protective action (Ueda and Fujita 2004). At the onset of stroke, anti-apoptosis may further increase the demand of oxygen supply to keep local homeostasis, impairing viability of the brain tissue if limited oxygen supply consists.

As mentioned, astrocytes are actively involved in the modulation of this pathological process. The definition of 'neuroprotection' should not be confined to neurons. That is, with the intimate metabolic coupling to neurons, these neighboring cells must be taken into consideration for intervention in the treatment of ischemic stroke.

1.7 ASTROCYTE GLUTAMATE UPTAKE

Neurons and astrocytes are capable to perform glutamate uptake by glutamate transporters. The function of these transporters is not only important for the modulation of neurotransmission during synaptic events but also essential for keeping the extracellular concentration of glutamate below the toxic dose. In resting condition, the glutamate concentration is in the range of 0.02~20 μ M in the different compartments of extracellular space (Moussawi et al. 2011). During neuronal firing, glutamate can reach concentrations as high as 1mM in the synaptic cleft (Dzubay and

Jahr 1999). It is generally accepted that the extracellular concentration of glutamate has to be dynamically restricted. It has been reported that cultured cortical neurons will undergo programmed cell death after 30min incubation of 2-4 μ M glutamate (Rosenberg et al. 1992). There is no clear border between concentrations of transmission and cytotoxic doses of extracellular glutamate (Hara and Snyder 2007). The physiological or pathological effects are relative, depending on multiple factors such as dosage, timing, cofactors, etc. Nevertheless, to achieve glutamate clearance, glutamate transporters are considered to be the only path for removal of glutamate from the ECS.

The high-affinity sodium dependent transporters are dominant in astrocytes, although there are other types of glutamate transporters in the cells. Astrocyte glutamate uptake is highly dependent on the sodium gradient across the plasma membrane, coupled to the energy consuming sodium pump, sodium potassium ATPase (Na,K-ATPase) (Rose et al. 2009). Also, the robust conversion of glutamate to glutamine in astrocytes, commonly referred to as a component of glutamine–glutamate cycle, is ATP-dependent (Martinez-Hernandez et al. 1977; Ottersen et al. 1996). Therefore, the glutamate uptake is highly sensitive to changes in energy supply (Danbolt 2001). Disturbance of energy supply severely impedes the function of astrocyte glutamate transporters. Indeed, at the onset of stroke, astrocytes are sharply depolarized in the ischemic core. This has been suggested to cause glutamate transporters to operate in the reverse way, causing rapid neuronal damage due to glutamate excitotoxicity (Rossi et al. 2007).

Together with three sodium ions, the glutamate transporter also transports one proton into the cell for every cycle of the transport (Levy et al. 1998), inducing acidification of the astrocyte (Amato et al. 1994). Interestingly, the pH of mitochondria also decreases as a result of astrocyte glutamate uptake (Azarias et al. 2011), indicating that the metabolism of mitochondria actually is repressed in astrocytes in response to neuronal activity. In physiological conditions, the acidification of astrocytes and their mitochondria can increase local oxygen availability and supply more lactate to neurons as an energy substrate. However, in ischemia, the severe acidosis induced by a massive glutamate load may inhibit glycolysis in astrocytes and consequently impede their functions (Peak et al. 1992; Swanson and Benington 1996). Thus, modulation of astrocyte intracellular pH can be a feasible therapeutic strategy against ischemia.

Astrocyte functions can be retained by restoration of intracellular pH and accordingly preserve neuronal viability.

1.8 ERYTHROPOIETIN AND NEUROPROTECTION

Given that the treatment of cerebral ischemia remains a great challenge, clinicians have long been searching for effective candidates to minimize consequences of ischemic stroke. Erythropoietin (EPO), originally identified for its role in erythropoiesis, shows robust tissue protective effects in diverse models (Brines and Cerami 2005). Particularly, in line with the topic of this thesis, it has been shown that EPO have neuroprotective functions via non-hematopoietic mechanisms (Genc et al. 2004).

Purified several decades ago (Miyake et al. 1977; Winkert et al. 1961), EPO has also been found to be expressed in the brain. Upregulation of the protein can be induced by ischemia (Masuda et al. 1994; Tan et al. 1992). EPO has been reported to have anti-apoptotic effects involving signaling of JAK2, MAPK and PI3K, as well as inhibition of caspase (Fisher 2003; Xiong et al. 2011). In view of the EPO receptor, there are two alternative concepts regarding assembly of the receptor. The original model is that the EPO receptor is formed as homodimer for protective signals, as the formation of the erythroid-specific receptor (Livnah et al. 1999). The other hypothesis suggests that the tissue-protective EPO receptor comprises a heteroreceptor incorporating one EPO receptor and two common beta receptors (Brines et al. 2004). Although non-hematopoietic analogues will relieve the concern of hematopoietic effects of EPO (Brines and Cerami 2005), in principal, EPO assembled in both combinations have been suggested to have tissue protective functions. In fact, short-time treatments with EPO do not elevate hemoglobin, hematocrit, and erythrocyte levels above normal range in stroke patients (Ehrenreich et al. 2009), which indicates that hematopoietic effects are not the major ‘side effect’ for the stroke clinical trial. Both hematopoietic and nonhematopoietic EPO analogues have shown efficacy in experimental stroke (Minnerup et al. 2009), but the basic mechanisms besides anti-apoptosis are not fully explored. Nevertheless, the surprising tissue protective effects of EPO have inspired neuroscientists to explore EPO for neuroprotection in ischemia. According to recent results from clinical trials (Ehrenreich et al. 2009), however the current evidence is still not enough to provide comprehensive guidelines for application of EPO in the treatment in stroke (Xiong et al. 2011).

EPO is widely used for anemia and extensively evaluated for clinical safety, but several issues need to be underlined and explored for the neuroprotection in ischemia. First, EPO has been shown to have neuroprotective effects in ischemic animal models (Bernaudin et al. 1999; Wang et al. 2004), but protocol designs included pre-treatment. It has been shown that EPO was more effective with a treatment in the first 6 hours of stroke compared to a later treatment initiation (Minnerup et al. 2009), even though EPO did not show significant effect for post-injury treatment in a cellular model of ischemia (Ruscher et al. 2002). All the evidence indicates that the therapeutic window of EPO need to be further investigated. Secondly, there are reports regarding 'overdose' effects of EPO in ischemic models (Li et al. 2004; Sakanaka et al. 1998). A double dose of EPO can totally reverse its effect on the production of nitric oxide (NO) (Beleslin-Cokic et al. 2004). With still unclear mechanisms of EPO crossing the blood-brain barrier, appropriate doses of EPO require more investigation for clinical applications. Finally, for short-time regulation, Studies of the effects of EPO mainly focused on neurons in ischemic models. There are remarkable few studies concerning astrocytes, even though the metabolic coupling between astrocytes and neurons should be crucial for ischemic neuroprotection.

Markedly, clinical translation of promising results from preclinical models has failed almost universally in treatments of stroke, which has been widely discussed (Fisher et al. 2009; Hsu 1993; O'Collins et al. 2006; Wiebers et al. 1990; Zivin and Grotta 1990). One successful proof-of-concept clinical study using EPO has been performed (Ehrenreich et al. 2002), dramatically raising the hope of EPO in the treatment of acute ischemic stroke. However, the following clinical trial showed unexpected disappointing result (Ehrenreich et al. 2009), demonstrating that more studies should be conducted to comprehend the translation from viability of a single neuron to neuroprotection in the CNS.

2 AIMS OF THE STUDY

The overall aim of this study has been to study the dynamic regulation of astrocyte water permeability with the goal to elucidate roles in brain water homeostasis. Further, the aim has been to explore aspects of astrocyte function and metabolism of potential relevance for neuron-astrocyte interaction and neuroprotection.

The main goals have been:

- **To explore molecular mechanisms involved in short term modulation of astrocyte water permeability and to determine the regulation of the water channel AQP4**

Specifically, the aims were:

- To identify the role of glutamate in the regulation of astrocyte water permeability and study molecular mechanisms involved
- To investigate the effects of extracellular potassium on astrocyte water permeability, and to elucidate the coupling between water and potassium homeostasis

- **To explore potential neuroprotective effects of EPO via astrocyte function**

Specifically, the aims were:

- To study the effect of EPO on regulation of astrocyte water permeability and the signaling involved
- To investigate the protective role of EPO in brain water intoxication
- To shed light on the effect of EPO on astrocyte metabolism and glutamate uptake in a cellular model of ischemia

3 DISCUSSION OF EXPERIMENTAL PROCEDURES

Please refer to original papers or the manuscript for thorough information of materials, chemicals, reagents and methods used in this study. A summary of materials and methods is rendered in this part. Motivations, advantages and limitations will be further discussed.

3.1 MATERIALS

3.1.1 Cell cultures

1. To study the dynamic regulation of AQP4, astrocyte cell lines (CTX TNA2 and DI TNC1) derived from rat, transiently transfected with GFP tagged WT or mutated AQP4, were employed for water permeability measurements.

Several initial and pilot experiments were performed to optimize the method. The 3rd-7th passages of the cell lines showed to provide the most optimal study conditions. There is no endogenous AQP4 expressed in immortalized cell lines, which provides a practical way to isolate AQP4 water permeability after transfection. Due to a more efficient transfection of DI TNC1, this cell line was chosen for FRET based cAMP measurements.

2. Pure primary cultured astrocytes or astrocyte-neuron co-cultures were used for individual projects in the study.

To obtain primary cultures, rat hippocampus, cerebellum or cortex was dissected from pups at the embryonic age of 18 days.

To study the glutamate-signaling regulation of water permeability, astrocyte-neuron co-cultures from hippocampus, where mGlu5 receptors are reported to be highly expressed (Romano et al. 1995), were used to measure astrocyte water permeability at the single cell level. Astrocytes had a typical polygonal or star-shaped appearance in the culture and immunostaining confirmed that glial fibrillary acidic protein (GFAP), a specific astrocyte protein, was expressed in the primary cultures. The cultures were used after two weeks when cells were 60~70% confluent. AQP4 mRNA was also analyzed by RT-PCR. This type of primary culture is expected to maintain interaction between neurons and astrocytes. The endogenous expression of mGluR5 and AQP4 allowed us to explore mechanisms involved in glutamatergic regulation of water permeability. For

data analysis, the selection of neuronal cells could be excluded by their distinct morphology and extraordinarily low water permeability.

To investigate potassium regulation of astrocytes, pure astrocyte cultures obtained from cerebellum were used to measure the production of cAMP, membrane potential, calcium signaling and water permeability. AQP4 and Kir channels are abundantly expressed in astrocytes in the cerebellum (Amiry-Moghaddam et al. 2003; Kang et al. 2008). The expression of AQP4 and Kir4.1 in these cells were confirmed by immunostaining in a preparatory pilot study (data not shown). The advantage of using pure astrocyte cultures is that the effect of potassium on astrocytes can be analyzed without interfering effects from neurotransmitters released by neighboring neurons in response to changes of potassium kinetic.

To explore effects of EPO on astrocytes, astrocyte-neuron co-cultures from hippocampus and cortex were used to evaluate glutamatergic signaling and changes of metabolic products, respectively. Cortical astrocytes, cultured in neuron-conditioned medium, were used to study effects of EPO on astrocytes in oxygen-glucose deprivation (OGD). Interactions between neurons and astrocytes were to a large degree preserved in astrocyte-neuron co-cultures. For intrinsic fluorescence measurements, Sulforhodamine 101 (SR101) was used as a specific marker to identify astrocytes (Nimmerjahn et al. 2004). SR101 negative cells were used as internal control for NADH measurements. Pure astrocytes cultured by neuron-conditioned medium were advantageous for functional assays, such as lactate measurements, glutamate uptake and rubidium uptake assay.

3.1.2 Animals

Water intoxication was 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. This model was designed to assess primary brain edema, known to depend upon AQP4 water transport (Manley et al. 2000).

3.2 METHODS

3.2.1 Water permeability measurements

The method of water permeability measurements has been developed in our lab (Zelenina and Brismar 2000; Zelenina et al. 2002). The principle is to evaluate plasma membrane water permeability by the initial rate of cell swelling induced by hypoosmotic solutions ($\Delta=50\text{-}100\text{mOsm}$). The method is based on the fact that, during the initial phase of cell swelling, the rate of fluorescence decrease is proportional to the permeability of the cellular membrane to water. Within the initial 10 seconds of cell swelling (paper II, Figure 1A), the obtained fluorescence curves were fitted with an exponential function to estimate plasma membrane water permeability. The unit of water permeability is m/s, but can be also expressed as arbitrary units (a.u.), representing relative value of time constants of water permeability. This method is one of the first techniques that allowed studies of the regulation of water channels in differentiated mammalian cells. There are two major aspects I would like to discuss:

First, for calculations of cell swelling rate, water permeability measurements are dependent on swelling of the cell induced by hypoosmotic solutions. Therefore, the estimations may be affected by other factors related to cell volume regulation. Swelling of astrocytes is a complex process, and several contributing mechanisms have been ascribed to the phenomenon (Aschner 2011). Considering the studies included in this thesis, astrocyte swelling induced by glutamate uptake and high extracellular potassium will be discussed.

Glutamate-driven swelling of astrocytes can be caused by an osmotic gradient independent way, by which 'net' water can be transported into the cell against the osmotic force (MacAulay et al. 2001; MacAulay and Zeuthen 2010) (also see introduction 1.1). To isolated mGlu receptor's signaling in the study, the group I mGluR agonist (S)-3,5 dihydroxyphenylglycine (DHPG) was used to assess the effect of glutamatergic signaling. When using glutamate, the incubation time was limited to 2min and glutamate was removed before the recording. Thus, glutamate uptake and long term metabolic effects of glutamate uptake that may complicate the process of cell swelling *in vivo* was avoided.

Increased extracellular potassium concentrations, applied in study II, can cause astrocyte swelling (Aschner 2011). In control experiments, we quantified recordings

of changes in calcein fluorescence intensity in cells exposed to isosmotic high potassium (paper II, Figure 1B). Analyses showed that there is the decrease in calcein fluorescence intensity within the time frame relevant for actual permeability recordings. However, this fluorescence intensity decrease (representing cell swelling) was minimal ($<0.6\%$) and found to be linear for the whole experimental time window. The slope of the fluorescence intensity decrease could therefore be adjusted in the off-line water permeability estimations (Zelenina and Brismar, 2000). Hence, we could conclude that our method is not hampered by cell volume changes induced by isosmotic high potassium.

As a common effect, cell volume regulation can be influenced by regulatory volume decrease (RVD). However, as signaling effects of RVD usually will be observed at a later point, up to minutes after a hypoosmotic challenge (Benfenati et al. 2011). Data collected from the first few seconds were used to assess water permeability in our method, thus measurements should not be influenced by mechanisms involved in RVD.

Secondly, to study the dynamic regulation of AQP4 gating, an astrocyte cell line transfected with GFP-tagged AQP4 was used. The feasibility of this approach was addressed in control experiments by transfecting GFP and AQP4 separately into LLC-PK cells. The GFP-tag on the water channel was shown not to affect water permeability of the channel (Zelenina et al. 2003) and thus the use of GFP-tagged AQP4 in these studies was considered to be justified. It should be noted that AQP4-M23 was used in these measurements. No protein internalization was observed during the experiments, indicating that the changes in AQP4 water permeability were caused by a gating effect of the water channel rather than trafficking.

3.2.2 Brain slice swelling measurements

Experiments performed on acute hippocampus slices showed that the hypotonic challenge increased water uptake, primarily in astrocytes, probably due to their unique AQP4 expression. However, it should be noted that group I mGluR is also expressed in neurons, why the glutamatergic signaling may not be confined to astrocytes at the tissue level. Stimulated by the group I mGluR agonist, a significant increase in water permeability was finally shown to exclusively occur in astrocytes (paper I, Figure 7). The evidence provided by measurements in acute slices supported the physiological

relevance of our findings in cell cultures. The results from brain slices were in line with the *in vitro* cellular responses, suggesting a physiological and pathological relevance of dynamic regulation of water transport *in vivo*.

3.2.3 *In Vitro* Phosphorylation

Protein phosphorylation appears to be a plausible way to regulate the function of the water channel AQP4. In our study, the serine 111 residue was identified as a target for regulation of AQP4 water permeability. We tested the candidate kinases that may phosphorylate this residue. There were no phospho-specific antibodies available for this site. Therefore, GST-fusion proteins corresponding to wild-type AQP4 I96-C123 and mutant peptides with serine 111 substituted to alanine were designed and purified for *in vitro* phosphorylation. To exclude artifacts due to non-physiological conditions, control experiments were performed by applying several different kinases. The data showed that AQP4 S111 was not phosphorylated by CaMKII or casein kinase II. In addition, mutated AQP4 with A111 (serine 111 substituted to alanine) was employed for water permeability measurements. These studies supported that phosphorylation is involved in the regulation of AQP4. However, we cannot exclude that phosphorylation of other residues in AQP4 and/or other proteins also are involved in the regulation of astrocyte water permeability.

3.2.4 Calcium measurements

The calcium-sensitive fluorophore Fura-2/AM was used for single cell ratiometric imaging. Acetoxymethyl AM esters of fura-2 can passively diffuse across cell membranes, avoiding the use of invasive loading techniques. Once inside the cell, these esters are cleaved by intracellular esterases to yield fluorescent indicators. The fluorophore is highly selective and sensitive to calcium changes. Because of the property of ratiometric signals, the measurements can relieve problems associated with focus shifts and fluorophore bleaching. For experiments using astrocyte-neuron co-cultures, the ratiometric signals of neurons could be excluded by their differential response to NMDA.

Due to the high affinity of Fura-2, calcium concentrations above 1 μ M produce almost complete binding saturation of the fluorophore. Hence, for measuring intracellular calcium concentrations beyond 1 μ M, other indicators should be applied, such as Fura-

4F and Fura-6F. In our studies, calcium responses induced by glutamatergic signaling were all/none effects rather than the dose dependent. Furthermore, calcium influx caused by extracellular potassium was normalized to the response to 50 μ M ATP, which revealed that the elevation in calcium was below the saturation level. We conclude that Fura-2 was well suited to explore changes in intracellular calcium in our studies.

3.2.5 Cyclic AMP Measurements

Two different methods were used to explore cAMP production induced by potassium. The production of cAMP can increase remarkably in some models and, specifically, cAMP production induced by potassium has very recently been quantified in astrocytes (Choi et al. 2012). We found that potassium induced a statistically significant increase in fluorescence resonance energy transfer (FRET) ratio in astrocytes. The FRET sensor, kindly provided by NKI, was recently developed and is based on the conformational change of EPAC to indicate cAMP production in living cells. The FRET ratio has however not been shown to be linear to cAMP production in transfected cells. For this reason, we could not determine the concentration changes of cAMP based on FRET signaling. Therefore, we also used enzyme immunoassay to measure cAMP production in primary astrocytes and found an increase in cAMP production up to 46% (paper II, Figure 2C).

3.2.6 Membrane potential measurements

The plasma membrane potential was measured by using a DiO/DPA membrane potential detection kit (Biotium, Inc). The method is based on the principle of FRET, where DiO is the FRET donor while DPA acts as a mobile acceptor (Bradley et al. 2009). Primary cultured astrocytes grown on glass coverslips were labeled by 5 μ M DiO-C16(3) for 10 min in serum-free medium containing 1 μ M DPA at 37°C. Imaging was performed using a heated chamber (FCS2, Biopetechs, Butler, PA, USA) mounted on a Zeiss Axioskop 2 microscope with a 40X/1.3 NA epifluorescent oil-immersion objective. DiO was excited at wavelength 485nm and fluorescence emission was filtered using a 540/80nm bandpass filter. All experiments were performed at 37°C with perfusion of aCSFs containing 1 μ M DPA with continuous oxygenation. Data were analyzed using Meta-Fluor software (Molecular Devices, Downingtown, PA).

DiO/DPA can report voltage-dependent fluorescence changes within milliseconds and

the method can provide data from a group of cells. To keep the photostability of DiO, the images were obtained every 5 seconds. As a donor, DiO is a bright, nontoxic membrane label that permits repeated imaging of viable cellular structures and does not perturb electrical activity. The FRET signal has a sensitivity of up to 60% $\Delta F/F$ per 100mV, which is more competent than traditional membrane potential ANEP dyes (10% fluorescence changes per 100mV) for our study.

3.2.7 Water intoxication

The *in vivo* water intoxication model mimics the cellular edema induced by hypoosmotic solutions *in vitro*. In this model, cellular brain edema developed as a result of a rapid intraperitoneal water infusion along with 1-Deamino 8-D-arginine vasopressin. The hypotonic situation creates an osmotic gradient that favors water entry into the brain, without disruption of the blood brain barrier (Manley et al. 2000). During the acute phase of this brain edema (within 3 hours), experimental animals exhibit signs of neurological dysfunction secondary to brain swelling. C3H/HEN mice appear to be relatively resistant to the symptoms of water overload. It is therefore reliable to employ these mice for water intoxication.

3.2.8 Oxygen-glucose deprivation

Oxygen–glucose deprivation (OGD) is a widely used *in vitro* model of ischemia. OGD was achieved by bubbling D-glucose-deficient aCSF with 95% N₂ and 5% CO₂ for 30mins, while maintaining the pH at ~7.3-7.4 as in the aCSF solution. Thus, oxygen was depleted in the OGD buffer, which led to the anaerobic metabolism predominating in astrocytes.

The OGD model has been reported to produce apoptotic and necrotic cell death (Kalda et al. 1998). Given the unique glycogen stores in astrocytes, it should be noted that astrocytes are resistant to the oxygen-glucose-free situation. Indeed, we found no significant cell death in the astrocyte cultures during the recordings. A pilot study also showed that the fluorescence produced by cleaved AM esters was stable for at least 60 min in OGD, suggesting that the astrocyte glycogen stores and anaerobic metabolism ensured viable cells during the experimental processes.

3.2.9 pH measurements

BCECF (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein)-AM is the most commonly used fluorescent indicator for intracellular pH. BCECF-AM itself is nonfluorescent. The conversion to fluorescent BCECF via the action of intracellular esterases can therefore be used as an indicator of cell viability.

The fluorescence excitation profile allows implementation of ratiometric measurements, avoiding problems caused by focus shifts or photobleaching. However, the fluorescence excitation isosbestic point of BCECF AM is quite far from the excitation maximum, giving poor signal-to-noise characteristics in ratio imaging microscopy. Due to the negative charges of BCECF at pH 7-8, this pH indicator can be easily loaded into the cell and will keep the intracellular retention. To minimize phototoxicity, the loading concentration of BCECF AM was as low as 1 μ M and images were recorded every 6 seconds.

3.2.10 Intrinsic NADH fluorescence measurements

As an electron donor, reduced nicotinamide adenine dinucleotide (NADH) can be utilized as a sensitive indicator of glycolytic energy metabolism. The ultraviolet absorption spectra is different between the oxidized and reduced forms of this coenzyme (NAD^+/NADH), which makes it feasible to evaluate glycolysis by measuring auto-fluorescence of NADH. It has been shown that both the resting fluorescence and the fluorescence fluctuations are dominated by NADH with two-photon fluorescence imaging *in vivo* (Kasischke et al. 2004). Astrocyte-neuron co-cultures were used in our study to monitor NADH changes in response to EPO. With sulforhodamine 101 (SR101) staining, astrocytes were indentified after experiments. The SR101-negative cells were used as internal controls for focus. Given that the metabolism of astrocytes is anaerobic in OGD, NADH will be rapidly turned to NAD^+ by lactate dehydrogenase at the end of glycolysis. Therefore, the increase in NADH fluorescence will be transient and thus the maximal fluorescence change was used as a measure of enhanced glycolysis. The method can assess real time changes of metabolism in living cells. However, in order to obtain auto-fluorescence, a 355-375nm stimulation was used. This type of short-time stimulation may however cause phototoxicity. In addition, the intensity of auto-fluorescence was low in the

measurements. Additional method, such as the lactate release assay, can be performed to evaluate metabolic changes in astrocytes in a comprehensive way.

3.2.11 Lactate assay

Lactate can specifically react with a commercial enzyme mix to generate a product, which interacts with a lactate probe to generate color ($\lambda = 570 \text{ nm}$) and fluorescence (Ex/Em = 535/587nm). Theoretically, this method provides a convenient way to detect L-Lactate in biological samples such as blood, cells and culture mediums. However, lactate degradation by lactate dehydrogenase (LDH) will complicate intracellular lactate measurements. Thus, measurement of lactate release was used for evaluation of astrocyte metabolism. The fluorometric assay was applied due to its high sensitivity.

To enhance stability of the assay, the experiments were performed at room temperature. It should be noted that a more robust reaction may be observed as temperature increases up to 37°C. Glutamate (200 μM) was employed as a positive control for every experiment to evaluate the cellular responses (data not shown). Due to the limited energy supply in OGD, the effects of a metabolic boost may be observed only within a certain time window. After pilot experiments, 5min was chosen as the time point for measuring lactate release, even though 15min of EPO incubation still yielded significant results.

3.2.12 Glutamate uptake assay

Due to the robust metabolism of glutamate, nonmetabolizable [^3H]D-aspartate was used to assess the function of excitatory amino-acid transporters. The experiments were performed at room temperature and the incubation time was set to 5min. The total D-aspartate uptake was actually found to be attenuated when glutamate was added, probably due to glutamate/aspartate competition for the transporter. The evidence showed that there is no essential difference between the uptake characteristics of L-glutamate and D-aspartate (Drejer et al. 1983). Therefore, D-aspartate uptake can be used as a functional indicator of excitatory amino-acid transporters in the presence of L-glutamate.

3.2.13 Rubidium-86 uptake assay

Ouabain dependent rubidium uptake can be used as a measurement to estimate the activity of sodium potassium ATPase (Na,K-ATPase). In OGD, 2mM ouabain was found to inhibit 30~50% of rubidium uptake by astrocytes, supporting the feasibility of this method to evaluate of sodium pumps. Consistent with a previous report (Pellerin and Magistretti 1997), glutamate was found to increase Na,K-ATPase activity by itself (~3 folds compared to control group, data not shown). Despite the increase caused by glutamate, co-incubation with EPO could further increase pump activity in the initial 5min, demonstrating the capacity of the method to assess effects of EPO with glutamate stimulation.

4 RESULTS AND DISCUSSION

4.1 GLUTAMATE REGULATION OF AQP4

4.1.1 Glutamate modulates AQP4 water permeability

We showed that glutamate can upregulate AQP4 water permeability by activation of metabotropic glutamate receptors (paper I). With a 2min pre-incubation, glutamate significantly increased water permeability in primary cultured astrocytes and in the astrocyte cell line transiently transfected with GFP-tagged AQP4 (GFP-AQP4 (+) cells), but had no effect on GFP-AQP4 (-) cells. The results indicate that AQP4 is the target for glutamate-regulation of astrocyte water permeability. To isolate the signaling of mGluRs, DHPG was employed (see method 3.2.1) and the results showed that the upregulation of AQP4 water permeability was induced by activation of group I mGluRs. Further, the effect was confirmed by the effect of DHPG on astrocytes in acute hippocampal slices.

As one of the key mediators of neuron-astrocyte interactions, glutamate has distinct physiological and pathological roles under certain circumstances (Stipursky et al. 2011). Regarding our findings, by elevating AQP4 permeability, glutamate can facilitate water transport and accelerate ion kinetics as a response to neuronal activity. On the other hand, in line with glutamate excitotoxicity, upregulation of AQP4 water permeability may deteriorate brain edema and augment neuronal death in the ischemic penumbra (Swanson et al. 2004). Clinical trials using antagonists of glutamate receptors have been unsuccessful at preventing brain damage (Rossi et al. 2007), which suggests the requirement of more specific therapeutic targets rather than universal glutamatergic blocking. Therefore, by revealing the mechanisms involved in glutamate regulation of AQP4 water permeability, therapeutic strategies may apply particularly to glutamate-induced cytotoxic brain edema.

In physiological situations *in vivo*, the extracellular glutamate concentration is tightly controlled by glutamate transporters to prevent excitotoxicity (Danbolt 2001; Herman and Jahr 2007; O'Kane et al. 1999). Robust glutamate uptake causes active water transport into astrocytes against the osmotic gradient, which requires energy preserved by the sodium gradient (MacAulay et al. 2001; MacAulay et al. 2004). Thus, due to the osmotic pressure, passive water flux via the water channel is potentially outward following glutamate uptake. It is well established that astrocytes

swell in response to neuronal activity and the molecular mechanisms of this physiological swelling has been discussed (Ostby et al. 2009; Schneider et al. 1992; Walz 1987). The upregulation of AQP4 water permeability can facilitate water turnover by water efflux, consequently assisting astrocyte volume recovery after swelling. Indeed, deletion of AQP4 causes more shrinkage of the ECS during a stimulation train (Haj-Yasein et al. 2012), indicating that AQP4 play an important role in the restoration of ECS (i.e. astrocyte volume recovery). The rapid restoration of ECS can reinstate water homeostasis and water that effluxes from astrocytes further decreases the concentrations of extracellular glutamate and/or potassium in an 'eco-friendly' way. In other words, it can be speculated that the 'clearance' of glutamate or potassium may then take place without transport of them across the cellular plasma membrane. The mGluR signaling was emphasized in our study. Glutamate was removed during the water permeability measurements. Considering the other consequences that can be induced by glutamate, such as intracellular acidification and membrane depolarization (Amato et al. 1994; Levy et al. 1998), it is too early to conclude that AQP4 water permeability is immediately upregulated by glutamate to blunt astrocyte swelling by water efflux. That is, it can be speculated that opening of AQP4 induced by mGluR signaling is restrained during glutamate uptake because of intracellular acidification. This is in line with the structural mechanism of aquaporin gating suggested from studies on plant plasma membranes (Tornroth-Horsefield et al. 2006). Upregulation of AQP4 water permeability via mGluR signaling will however eventually occur after glutamate uptake. Therefore, the dynamic regulation of AQP4 should be considered as a part of the response to neuronal activity.

In some pathological circumstances an osmotic overload can occur in astrocytes and in turn promote cytotoxic (cellular) edema (Liang et al. 2007). It has been suggested that blocking AQP4 may dramatically improve clinical outcome after a number of brain disorders (Manley et al. 2000; Papadopoulos and Verkman 2005; Vajda et al. 2002). Relevant to this thesis, accumulation of extracellular glutamate has been reported to occur in pathological situations in the brain, such as ischemic stroke and hyponatremia (Davalos et al. 2000; Haskew-Layton et al. 2008; Hyzinski-Garcia et al. 2011). Thus, glutamate regulation of AQP4 can be crucial in the development of cytotoxic edema related to ischemia and water intoxication. We discussed that potential therapeutic strategies may be raised by our findings (see Paper I,

Discussion). Meanwhile, it should be kept in mind that water flow via AQP4 is bidirectional. The roles of AQP4 in cerebral water related disorders have been studied and opposite roles of AQP4 in cytotoxic and vasogenic brain edema have been suggested (Papadopoulos and Verkman 2007). Different strategies of therapies are therefore required for particular brain water disorders. Besides, effects of glutamate on AQP4 should be further explored in mGluRs independent ways to provide a more comprehensive view on this regulation.

4.1.2 AQP4 is regulated by calcium/CaMKII

A transient increase in calcium increase was observed by the activation of group I mGluRs in the astrocyte cell line. In primary astrocytes, mGluR activation could also induce calcium oscillations. When intracellular calcium stores were depleted, the DHPG-induced upregulation of AQP4 water permeability was abolished, indicating that intracellular calcium signaling is required for the regulation. A calcium-free buffer did not abolish the observed calcium response, which supports that the intracellular stores of calcium are the major source of the calcium events. Further data showed that CaMKII is a key mediator for glutamate regulation of AQP4 water permeability.

Glutamate can cause intracellular calcium increase by activation of mGluRs, mainly attributed to the calcium release from internal stores (Ben Achour et al. 2010). Calcium pumps and calcium-binding proteins can rapidly buffer increases of intracellular calcium, thereby inducing intracellular calcium increases as calcium spike(s) in astrocytes (Meyer and Stryer 1991). CaMKII can be activated by spike frequency-dependent calcium events (Hudmon and Schulman 2002). Besides calcium, the maximal activation of CaMKII requires the calmodulin binding (Katoh and Fujisawa 1991). An interesting nature of CaMKII is that the kinase can undergo multiple autoregulation, which is independent on its initial activators (Hudmon and Schulman 2002). That is, CaMKII not only behaves as a 'switch-like' manner, but also can be regulated by substrate phosphorylation and autophosphorylation (Le Vine et al. 1986). Therefore, the signaling cascade can be sustained or even enhanced after the initial activation of the kinase. The increase of astrocyte AQP4 water permeability was observed after incubation of glutamate or DHPG, indicating a physiological relevance of this regulation. To be exact, high extracellular glutamate needs to be confined within a very short period to avoid excitotoxicity (Danbolt 2001). Based on

the finding that CaMKII is involved in the regulation, it may be speculated that the opening of AQP4 is sustained after glutamate uptake in astrocytes (See 4.1.1). Taken together, the present evidence shows that glutamate regulation of astrocyte AQP4 is dependent on intracellular calcium/CaMKII signaling. The signaling cascades may continue without further glutamate stimulation. This strategic signal transduction suggests a role for dynamic regulation of AQP4 in response to neuronal activity.

4.1.3 The AQP4 gating effect is controlled by Serine 111

Corresponding to the serine 115 in spinach aquaporin, serine 111 of AQP4 is a potential phosphorylation site for the opening of the water channel. The residue is suggested to be a putative site for several serine/threonine protein kinases. Our results from *in vitro* phosphorylation showed that protein kinase G (PKG) directly phosphorylated a residue corresponding to the wild type AQP4 serine 111. In addition, neither glutamate nor DHPG had any significant effect on water permeability in cells expressing GFP-AQP4 S111A (serine 111 mutated to alanine). This evidence fosters the perception that AQP4 water permeability can be regulated by phosphorylation of serine 111. Furthermore, there was no change in the subcellular distribution of AQP4 observed during the measurements in GFP-AQP4 (+) cells, suggesting that the increase in water permeability was caused by channel gating rather than protein trafficking of AQP4.

Two ways of modulating the function of water channels have been proposed: channel trafficking and channel gating. The phenomenon of AQP4 trafficking and potential regulators have been reported and PKC, ERK1/2, PKA have been suggested to be involved in the process (Carmosino et al. 2007; Gan et al. 2012; Moeller et al. 2009; Ratelade et al. 2011). Interestingly, activation of CaMKII, the key mediator for glutamate regulation of AQP4 gating in our study, has been suggested to cause trafficking of AQP4 to the plasma membrane in manganese-treated cultured astrocytes (Rao et al. 2010). Together with our findings (Paper I and II), it cannot be excluded that both channel trafficking and gating can be induced by the same activator in astrocytes. It should be noted that a plasmid of GFP-AQP4-M23 was used in our study. Differences in N-terminus of AQP4 contribute to membrane trafficking or assembly of arrays (Neely et al. 1999). It has been reported that AQP4-M1 is completely internalized by neuromyelitis optica-IgG treatment, i.e. autoantibodies against AQP4, but the AQP4-M23 resists internalization (Hinson et al. 2012). Even though AQP4-

M23 has been found to be internalized with a longtime PKC stimulation (30min~60min) in *Xenopus* oocytes (Fenton et al. 2010; Moeller et al. 2009), any short term response of this regulation has never been shown. Even though it is difficult to isolate a gating effect in primary astrocytes, no exo/endocytosis of GFP-AQP4 was observed in transfected cells in response to mGluR activation. This strongly indicates that gating of AQP4 is taking place rather than recruitment of new molecules. Indeed, phosphorylation of AQP4 Serine 111, but not serine 180, has been shown to be crucial for single channel permeability (Mitsuma et al. 2010; Silberstein et al. 2004). In summary, GFP-AQP4 is dominantly distributed in the plasma membrane in our studies. Regarding the functional implications of serine 111, it is plausible and probable that AQP4 gating accounts for the 30~40% increase in AQP4 water permeability occurring within few minutes.

4.2 POTASSIUM DEPENDENT REGULATION OF ASTROCYTE WATER PERMEABILITY

4.2.1 Elevated potassium regulates astrocyte water permeability

We found that elevations in potassium increased astrocyte water permeability and that prolonged upregulation of the water permeability was dependent on Kir-channel function (paper II). The modulation of AQP4 water permeability was actively but not exclusively involved in this regulation, as elevated potassium also increased water permeability of GFP-AQP4 (-) cells to a small degree. According to the reported range of physiological and pathological $[K^+]_o$ (Walz 1987), 10mM and 35mM potassium were used to investigate astrocyte water permeability response to changes in extracellular potassium. We found that two distinct signal pathways were activated, depending on the different increases in potassium concentrations. Further results indicated that membrane depolarization is counteracted by Kir-channels when $[K^+]_o$ is moderately elevated. The function of Kir-channel is thus coupled to the dynamic regulation of astrocyte water permeability.

Given that potassium kinetics can be slowed down in AQP4 knock-out mice (Binder et al. 2006; Strohschein et al. 2011), the upregulation of astrocyte water permeability in response to rapid elevation of $[K^+]_o$, may be interpreted to facilitate potassium handling in the CNS. The accelerated potassium kinetics may be predicted to eventually contribute to maintain brain water and ion homeostasis. In addition, for activity-dependent astrocyte swelling and cell volume restoration, the instant regulation of

astrocyte water permeability should be essential for dynamic changes in cell volume. Considering the potassium regulation of AQP4, our results showed that the increased AQP4 water permeability significantly dropped from 1min to 5min with 10mM $[K^+]_o$ incubation (Paper II, Fig 1E). Also, AQP4 water permeability was previously found not to be affected after long term incubation with different $[K^+]_o$ solutions (Fenton et al. 2010). This evidence indicates that dynamic changes of $[K^+]_o$, rather than absolute values of $[K^+]_o$ is activating the upregulation of AQP4 water permeability.

Within the pathological levels of $[K^+]_o$, astrocyte water permeability was only increased in the shorter time range (<5min). The abolished water permeability increase after minutes with pathologically high $[K^+]_o$ may further restrain potassium clearance from the ECS in abnormal conditions, prolong the depolarization of astrocytes and consequently attenuate glutamate uptake. However, it is too early to claim that pathologically high $[K^+]_o$ has a detrimental effect via the regulation of astrocyte water permeability. Reduced astrocyte water permeability has been reported to attenuate the development of brain edema following ischemic stroke (Verkman 2002). In line with this, cortical spreading depression, which can be triggered by abnormal high $[K^+]_o$, has been suggested to induce neuroprotective effects for brain injuries or ischemia (Chow et al. 2002; Gupta 2005; Kiss et al. 2004).

4.2.2 Evidence in favor of phosphorylation of AQP4

4.2.2.1 High potassium increases astrocyte water permeability via PKA-dependent regulation

As found in glutamate regulation of AQP4 water permeability, 10mM $[K^+]_o$ increased wild type AQP4 water permeability but had no significant effect on AQP4 S111A water permeability. cAMP production was increased by high potassium and a selective PKA inhibitor abolished the increase of astrocyte water permeability caused by 10mM $[K^+]_o$. Further, the result of *in vitro* phosphorylation supports the concept that PKA can phosphorylate AQP4 on the residue serine 111.

The AQP4 Serine 111 of has previously been recognized as a putative PKA phosphorylation site (Silberstein et al. 2004; Wood et al. 1996). Further, an AQP4 mutant, mimicking constitutive phosphorylation of serine 111, showed the characteristic of channel gating (Silberstein et al. 2004). The evidence presented in our study supports the concept of potassium regulation of AQP4 gating via PKA

phosphorylation. We cannot exclude that phosphorylation of other residues in AQP4 or other mechanisms besides channel gating are involved in potassium regulation of astrocyte water permeability. PKA phosphorylation of AQP4-M1 is also suggested to be involved in retaining the water channel in vesicle-recycling compartment after protein internalization (Carmosino et al. 2007). There is however a report showing contradicting results that PKA cannot phosphorylate astrocyte AQP4 (Nicchia et al. 2008). One technical explanation for this negative result has been rendered by the same department. Due to the fact that some of the putative consensus sequences for AQP4 phosphorylation are located at the C-terminus (Zelenina 2010), the available antibodies against AQP4 C-terminus therefore cannot recognize phosphorylated AQP4 (Carmosino et al. 2007). Accordingly, the phosphorylated AQP4 cannot be obtained by immunoprecipitation with anti-(C-terminus) AQP4 antibodies in that study (Nicchia et al. 2008), providing the possibility of false negative results. Nevertheless, although our results contribute to this question by investigating this regulation, the regulation of AQP4 by phosphorylation in intact preparations still calls for further studies.

4.2.2.2 Highly elevated potassium abolishes the increase of astrocyte water permeability via calcium mediated signaling

Consistent with a previous report (Duffy and MacVicar 1994), we found that intracellular calcium signaling was triggered by 35mM $[K^+]_o$ but not by 10mM $[K^+]_o$. Inspired by the findings of PKA-dependent regulation of astrocyte water permeability, we explored the role of calcineurin, a calcium dependent protein phosphatase, in this regulation. When calcium dependent dephosphorylation was attenuated by an inhibitor of calcineurin, astrocyte water permeability was increased after 5min incubation with 35mM potassium. In addition, this positive response demonstrated that the cells were able to increase water permeability even following 35 mM $[K^+]_o$ induced cell swelling (~9% fluorescence decrease), a phenomenon discussed in the original paper (paper II).

As opposed to calcium signaling triggered by activation of mGluRs, the high potassium-induced calcium response was dependent on extracellular calcium (data not shown). It has been shown that such calcium response is dependent on voltage-dependent calcium channels and the sodium calcium exchanger (Duffy and MacVicar 1996; Paluzzi et al. 2007; Rutledge and Kimelberg 1996). Interestingly, consistent with our studies (paper I & paper II), glutamate has been proposed to inhibit calcium influx by the activation of mGluRs and consequently cause downregulation of calcium

dependent dephosphorylation (Rodnight et al. 1997). Calcium/calcineurin dependent dephosphorylation has been reported to occur in astrocytes (Vinade et al. 1997). In addition, PKA and calcineurin can be coupled together via association with a common anchor protein and thereby regulate the phosphorylation state of key substrates (Coghlan et al. 1995). Together with our result of putative phosphorylation on AQP4 serine 111, we investigated the role of calcium/calcineurin in dephosphorylation and confirmed its function in potassium regulation of astrocyte water permeability. The details of this regulation, and whether calcineurin can dephosphorylate AQP4 directly, are still not resolved in our study. Nevertheless, the data showed that potassium-triggered calcium increase induces a negative signaling pathway regulating astrocyte water permeability.

According to the range of increases in potassium concentrations, potassium appears to modulate astrocyte water permeability in both positive and negative ways, indicating that there should be a key mediator functioning as a 'sensor' for $[K^+]_o$.

4.2.3 Roles of Kir-channels

4.2.3.1 Kir-channels are involved in potassium regulation in astrocytes by modulation of calcium signaling

When Kir channels were blocked, we found that intracellular calcium also increased by exposure to 10mM $[K^+]_o$. The finding of a calcium response led us to hypothesize that the function of Kir channels is important for preventing a calcium response. Accordingly, Kir channels appeared to prevent the negative regulation triggered by calcium with moderate elevations of $[K^+]_o$. Indeed, using preincubation with barium blocking Kir channels, an intracellular calcium response was observed and 10mM $[K^+]_o$ no longer caused an increase in astrocyte water permeability in 5min.

The co-enrichment of AQP4 and Kir4.1 implicates a coupling of these two channels in brain water/potassium transport (Nagelhus et al. 1999), even though a direct protein-protein interaction has not been found (Puwarawuttipanit et al. 2006). Considering the functional interaction between these channels, there are reports contradicting this view (Ruiz-Ederra et al. 2007; Zhang and Verkman 2008). Water flux and potassium kinetics are tightly coupled (Horio 2001), but a functional coupling between AQP4 and Kir4.1 had not been demonstrated by dynamic potassium kinetics in previous studies (Ruiz-Ederra et al. 2007; Zhang and Verkman

2008). Based on our study, we propose that the cooperation of AQP4 and Kir4.1 will not be necessary unless there is a requirement for modulation of brain water and potassium homeostasis. However, when $[K^+]_o$ was elevated, a functional interaction between APQ4 and Kir channels could be established by the absence of intracellular calcium response.

A potassium threshold of voltage dependent calcium responses has been demonstrated in astrocytes (Duffy and MacVicar 1994). Kir channels are essential for astrocytes to keep their membrane potential (Djukic et al. 2007) and the conductance of Kir channel has been shown to be enhanced by increases of $[K^+]_o$ (Ransom and Sontheimer 1995). Thus, the function of Kir channels is crucial for membrane potential stabilization when extracellular potassium is elevated. Taken together, Kir channels may be related to the threshold of potassium for voltage dependent calcium responses, which in turn will give different effects on astrocyte water permeability as a response to distinct potassium kinetics.

4.2.3.2 Implications of Kir-channels function responding to potassium: a putative contribution to potassium buffering

To further explore the involvement of Kir function in the cellular responses to high $[K^+]_o$ in astrocytes, we measured changes in membrane potential caused by 10mM $[K^+]_o$, with and without Kir channel inhibition (data not included in paper II). By the use of a DiO/DPA membrane potential measurement kit (Bradley et al. 2009), we found that the change in astrocyte membrane potential following application of 10mM $[K^+]_o$, was more pronounced when Kir channels were blocked (Figure1, lower right panel). Actually, application of 10mM $[K^+]_o$ did not consistently induce depolarization of astrocytes (Figure1, lower left panel). Thus, Kir-channels appeared to stabilize the membrane potential. As expected, 35mM $[K^+]_o$ caused an immediate membrane depolarization (Figure1, lower left panel).

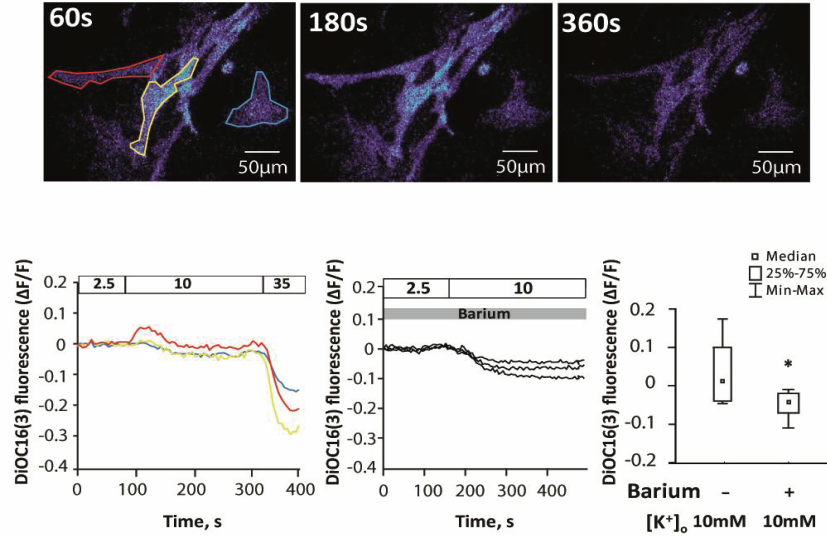


Figure 1 Upper panel shows images of primary cultured astrocytes loaded with the fluorescent membrane potential sensor DiO-C16(3). Images were acquired at the indicated time points. Three individual cells were marked corresponding to respective color. Lower left panel shows representative recordings of normalized fluorescence intensity during perfusion with indicated potassium concentrations (2.5mM, 10mM and 35mM, respectively). F = the mean value of fluorescence intensity for the first min (10 scans). Decreases in fluorescence intensity indicate depolarization (35mM potassium (~310-400s) or 10mM potassium+100μM barium (~150-300s)). Lower right panel shows summary data of fluorescence intensity changes induced by 10mM potassium with and without 100μM barium, respectively (8 plates, * $p < 0.05$).

Characterizations of astrocyte membrane potential have revealed properties of exclusive potassium conductance and that the behavior of the membrane potential deviates from a Nernst potential (Kimelberg et al. 1979; Walz et al. 1984). That is, with different $[K^+]_o$, astrocyte potassium permeability is not consistent. As a result, the Goldman equation can be applied for estimations of astrocyte membrane potential. The potassium conductance of astrocytes is largely due to Kir4.1 (Olsen and Sontheimer 2008). Therefore, it is plausible that the potassium permeability of Kir was changed by elevated $[K^+]_o$ in our study. The fact that knockdown or inhibition of Kir4.1 can induce remarkable astrocyte membrane depolarization (Chever et al. 2010; Kucheryavykh et al. 2007; Olsen and Sontheimer 2008) indicates that the current direction of potassium via Kir channels is potentially outward in resting status. Indeed, outward Kir currents (positive charge moving out of the cell) has been found to be regulated by single Kir-channel conductance (Liu et al. 2011). Notably, the current flow of Kir is not determined only by the membrane potential (potential difference) but also by the

concentration difference of potassium across the plasma membrane. The term ‘inward rectification’ refers to the nature of the channel’s current/voltage-relationship but not the actual current direction (Macaulay and Zeuthen 2012). Taken together, our data suggest that moderately elevated $[K^+]_o$ (10mM) may increase Kir channel potassium permeability, which will counteract membrane depolarization and consequently prevent intracellular calcium increase.

Theoretically, elevated $[K^+]_o$ will depolarize the cell, while according to the Goldman equation, enhanced potassium permeability of Kir channels could neutralize this effect, consistent with our results. Interestingly, Kir channels can be activated by cAMP/PKA and Kir channel dependent hyperpolarization has been reported (Bolton et al. 2006). We found that cAMP production was increased by elevation of $[K^+]_o$, thus it is possible that an increased Kir permeability and activation of cAMP/PKA could even induce membrane hyperpolarization. The proof-of-principle evidence has been shown in different models in the presence of Kir2.1 (Farr and David 2011; Filosa et al. 2006), which is also expressed in astrocytes (Kang et al. 2008). In fact, we observed membrane hyperpolarization in some cells during our measurements (Figure1, lower left panel and some data not shown). Membrane hyperpolarization suggests that there is potassium efflux from the astrocyte, which is dependent on the diffusion force preserved by the potassium gradient. Taken together, these speculations suggest an untraditional possibility that, during neuronal activity, the $[K^+]_o$ increase in the ECS may induce potassium release from the astrocyte. Such astrocyte potassium efflux will enhance extracellular potassium accumulation, activate sodium potassium ATPase within the tripartite synapse, and consequently accelerate the rebuilding of ion homeostasis in a later phase. It is plausible that there is no or very little ‘net’ potassium transferred from neurons to astrocytes, preventing potassium depletion from neurons. When $[K^+]_o$ is further elevated, as in intensive neuronal stimulation or in pathological conditions, the astrocyte membrane is eventually depolarized via potassium influx into astrocytes, at what time ‘classical’ potassium spatial buffering and potassium siphoning would occur (see Figure2). It should be noted that 10mM $[K^+]_o$ was used in our study, however the 10mM does not necessarily correspond to the same concentration *in vivo*. The ‘turning point’ from potassium efflux to potassium influx should be dependent on the local potassium kinetics and Kir channels expression. The topic of potassium homeostasis in astrocytes is exciting but needs further investigation.

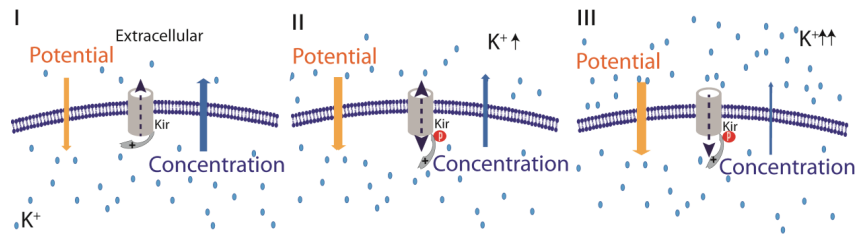


Figure 2 Graphical representation of a proposed function of Kir channels in response to elevated $[K^+]_o$. When $[K^+]_o$ was moderately elevated, we speculate that an increase of Kir permeability and phosphorylation of Kir induced by cAMP/PKA may cause potassium efflux driven by potassium concentration force (I \rightarrow II). When $[K^+]_o$ is further increased, potassium influx will occur (II \rightarrow III).

4.3 PROTECTIVE ROLE OF EPO IN BRAIN EDEMA

4.3.1 EPO prevents neurological dysfunction in acute brain edema

Water intoxication was induced by an injection of distilled water corresponding to 20% of body weight along DDAVP (400ng/kg) in mice. As a result of this, profound hyponatremia will occur and consequently induce cellular brain edema. In this model, application of EPO significantly attenuated neurological symptoms up to 180min after water loading.

Hyponatremia is associated with the accumulation of extracellular glutamate thought to be partly release by astrocytes (Haskew-Layton et al. 2008; Hyzinski-Garcia et al. 2011). Effects of EPO on glutamatergic signaling in astrocytes were therefore investigated (paper III). The study revealed that EPO abolished the effect of group I mGluRs (i.e. increase) on astrocyte water permeability. The result was further confirmed in hippocampal slices. An early transient increase of astrocyte water permeability by EPO was observed, however the mechanism of EPO-induced increase of astrocyte water permeability was not revealed. Interestingly, an upregulation of AQP4 protein expression has been observed in rats that received EPO, suggesting EPO may potentially increase astrocyte water permeability at least in later time point (Brissaud et al. 2010). Therefore, it should be valuable to investigate whether EPO-induced upregulation of AQP4 can benefit clearance of excessive brain water. Nevertheless, the effects of EPO in our study are short term rather than long term outcomes that require gene transcription and expressional changes. The results encouraged us to explore short term effects of EPO on astrocytes in other pathologies, such as ischemia, where the post-ischemic cellular edema can cause massive destruction of viable tissue in the penumbra (Feustel et al. 2004; Kimelberg 2005).

4.3.2 EPO interferes with calcium signaling

Pretreatment with EPO for 10 min caused an alteration in calcium oscillations in response to DHPG (paper III). There was a significant difference in the number of calcium peaks between EPO (-) and EPO (+) cells, indicating that mGluR-induced calcium signaling was interfered by EPO. EPO alone did not induce any calcium response in primary astrocyte cultures. In addition, we found that EPO attenuated extracellular signal-regulated kinases (ERK1/2) phosphorylation induced by mGluRs activation, whereas EPO alone enhanced the phosphorylation of ERK1/2 (data not published) (Figure3).

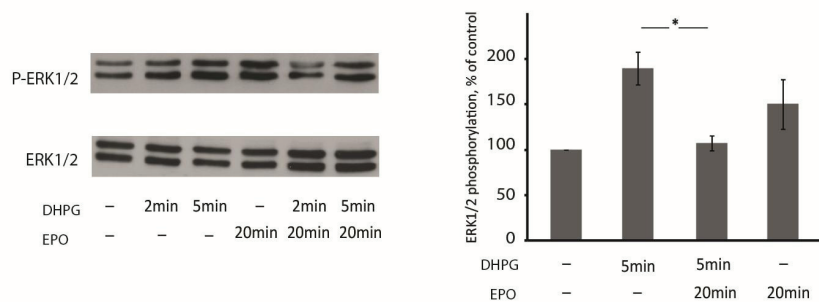


Figure 3 EPO attenuates the fraction of phosphorylated ERK1/2 caused by mGluR-activation in astrocytes. The phosphorylation of ERK1/2 and pERK1/2 was detected by western blot (left panel). The corresponding treatment time is labeled respectively. The right panel shows the summary of ERK1/2 phosphorylation, normalized by ERK1/2 expression (4 blots, * $p < 0.01$).

A high frequency of calcium spikes (calcium oscillations) can prevent the dissociation of calmodulin from CaMKII, thus increasing the probability that neighboring subunits will also bind calcium/calmodulin during successive spikes (Hudmon and Schulman 2002). Therefore, EPO may prevent signaling cascades via attenuating of calcium oscillations induced by mGluR activation. As discussed in our original paper, activation of PKC could be involved in EPO modulation of calcium oscillations, but the details remain to be elucidated.

One study has reported that CaMKII may be an upstream regulator of ERK phosphorylation (Choe and Wang 2001). Also, phosphorylation of ERK1/2 is involved in upregulation of AQP4 expression (Qi et al. 2011; Rao et al. 2010). We found that DHPG or EPO could enhance ERK1/2 phosphorylation in astrocytes separately, consistent with previous reports (Lee et al. 2004; Park et al. 2006; Peavy and Conn

1998). However, with pre-incubation of EPO, DHPG-induced ERK activation was down-regulated (Figure 3). Taken together, it is plausible that EPO dynamically can regulate AQP4 expression via ERK1/2 signaling by modulation of calcium signaling with/without glutamate. Such on/off regulation may benefit the control of water homeostasis in the different phases of brain edema and serve as long term effects in EPO neuroprotection. That is, it can be speculated that EPO can attenuate brain edema induced by hyponatremia via antagonizing glutamate-induced astrocyte water permeability increase. Eventually, when extracellular glutamate concentration is under control, EPO may upregulate AQP4 expression to facilitate the brain water clearance.

4.3.3 A double-edged sword and double opportunities

In study III, we showed that EPO antagonized glutamate-mediated water flux. The results also raise the possibility that EPO could attenuate brain injuries associated with pathological swelling of astrocytes, such as stroke and brain trauma. However, considering translation from experimental results to possible clinical applications of EPO, there are several issues I would like to discuss in this thesis.

First, AQP4 is a bidirectional water channel. Modulation of AQP4 will not determine brain water influx or efflux but only control the speed of water transport. During brain edema development, decreasing astrocyte AQP4 water permeability will slow down water influx into the brain and accordingly attenuate brain edema, in line with our findings and other reports (Manley et al. 2000; Vajda et al. 2002). However, it is reasonable to expect that blocking AQP4 also will hamper brain water clearance in edema resolution. Prolongation of brain edema will increase the risk of brain damage. Therefore, the modulation of AQP4 should be different depending on the time point in the course of edema. For instance, neurological symptoms caused by water overload peaked by 75 min in the water intoxication model. On the other hand, stroke-induced brain edema often occurs after hours and could last for several days (Berezcki et al. 2000; Lipton 1999). Secondly, it is not fully understood that how EPO passes through blood-brain barrier. The strategy of EPO application should be adjusted according to the integrity of blood-brain barrier, which is intact in water intoxication but could be disturbed in acute cerebral ischemia (Yang and Rosenberg 2011). Finally, brain water efflux is exceeding brain water influx due to the robust brain metabolism (see introduction 1.5). AQP4 blocking should have a negative impact in

brain edema combined with intensive neuron activity. Indeed, in a status epilepticus model, there are significantly greater tissue edema in AQP4 knockout mice compared to wild type, indicating that AQP4 dependent water efflux is crucial for edema clearance following intense seizure activity (Lee et al. 2012).

Taken together, as an attractive target for clinical applications, AQP4 should be dynamically modulated. At the level of brain tissue, inhibition of AQP4 will not always benefit the whole course of brain edema. There are at least two time points where regulation of AQP4 water permeability in opposite directions could be beneficial. The identification of ideal therapeutic time windows for AQP4 modulation is challenging, but should be feasible with imaging and monitoring of brain metabolism.

4.4 THE EFFECT OF EPO ON ASTROCYTE METABOLISM IN ISCHEMIA

4.4.1 EPO enhances astrocyte metabolism in ischemia

4.4.1.1 Astrocyte alkalization induced by EPO is dependent on the sodium hydrogen exchanger (NHE) in OGD

We found that EPO caused a gradual alkalization of astrocytes and that pH_i reached a plateau level in 5~10min in OGD, a cellular model of ischemia (paper IV). The effect was abolished by inhibition of NHE. Also, in the presence of $\text{CO}_2/\text{HCO}_3^-$ in the solution, astrocytes baseline pH_i of was stable in the OGD buffer during measurements.

As an acid extruding transporter, NHE is one of the key players regulating astrocyte pH_i . We did not observe any significant pH_i decrease in astrocytes exposed to OGD. This could be due to the presence of $\text{CO}_2/\text{HCO}_3^-$ and the continuous OGD condition, which is not the case in a previous study showing different results (Kintner et al. 2004). It has been reported that EPO can stimulate hematopoietic cell proliferation via activation of NHE (Rich et al. 1998). In addition, NHE can be activated via phosphorylation of ERK1/2 in astrocytes (Mandal et al. 2009), which interestingly relates to our previous results (see 4.3.2). Notably, inhibition of NHE at 10min, 24h, and 48h after neonatal hypoxia/ischemia has been shown to be neuroprotective (Cengiz et al. 2011). Considering the finding that EPO effects can be mediated via NHE, there may be a concern about the possible risk of EPO application in certain phases of ischemia.

4.4.1.2 *The outcomes of increased lactate release in OGD*

EPO significantly increased both NADH fluorescence and lactate release in astrocytes. The enhanced lactate release was abolished by inhibition of NHE with DMA. The evidence indicates increased glycolysis and astrocyte metabolism as a result of intracellular alkalinization.

As an end product of anaerobic glycolysis, lactate can be accumulated in the brain in ischemia. Severe lactic acidosis is generally considered to be destructive (Chesler 2005). On the other hand, as a potential energy resource, lactate itself is unlikely to be harmful and is actually a preferred fuel for many cells including neurons (Pellerin and Magistretti 1994; Vernon and LeTourneau 2010). There are also reports that lactate has a neuroprotective role both *in vitro* and *in vivo* after cerebral ischemia (Berthet et al. 2009). Besides, extracellular lactate can regulate brain blood flow via vasodilatation under low concentration of oxygen (Gordon et al. 2008), which should be beneficial in the ischemic condition. However, sufficient supply of oxygen needs to be concerned for lactate to be neuroprotective. Without rebuilding of circulation, lactate released by astrocytes cannot be utilized by neurons for aerobic metabolism. On the contrary, with insufficient oxygenation, lactic acidosis can result in inhibition of cell metabolism. High lactate level is found in the acute phase of stroke patients (Henriksen et al. 1992). Thus, repeated EPO applications during the acute phase of ischemic stroke may further increase local production of lactate, which could have a negative impact on the tissue. But it also should be noted that, in the very early phase of ischemic stroke, an instant elevation of astrocyte metabolism in the penumbra cannot be simply described as negative. The fact that maintained astrocyte function can adjust potassium kinetics, stabilize the membrane potential and control extracellular concentration of transmitters, theoretically should prevent or attenuate neuronal apoptosis (also see 4.4.2 and 4.4.3).

4.4.2 EPO boosts glutamate uptake in ischemic astrocytes

4.4.2.1 EPO restored astrocyte acidification induced by glutamate

In paper IV, we found that glutamate caused a rapid acidification in astrocytes under OGD conditions and that EPO completely reversed this acidification. Inhibition of NHE abolished pH_i restoration caused by EPO. Coincubation with the NHE inhibitor DMA attenuated the effect, but EPO could still cause a small but significant pH_i increase, probably due to incomplete inhibition or NHE independent mechanisms.

Astrocyte glutamate transporters carry one proton into the cell for every cycle of transport, inducing intracellular acidification. The effect has been observed both in acute brain slices and cultured astrocytes (Amato et al. 1994; Azarias et al. 2011). Notably, the glutamate uptake-induced acidification is also found in mitochondria, indicating limited aerobic metabolism in astrocytes responding to synaptic activity (Azarias et al. 2011). In ischemia, the sustained level of extracellular glutamate should exacerbate cellular acidosis, eventually inhibiting glycolysis. Therefore, restoration of intracellular pH as by EPO may maintain glycolysis and temporarily supply energy needed for local homeostasis. There are, however, two aspects I would like to point out. First, under 'pure' OGD condition the major source of glycolysis comes from glycogen stores in astrocytes. In principle, an enhanced glycolysis cannot persist after glycogen depletion. In ischemia, the actual time window for a beneficial effect of EPO-induced restoration of pH is dependent on the local blood flow. Thus, with more local blood flow, as distant from the ischemic core, the local homeostasis can be maintained by EPO for a longer time. Secondly, without adequate oxygen supply the anaerobic glycolysis will lead to accumulation of extracellular lactate, which may be not neuroprotective in a situation of low oxygen/lactate ratio.

4.4.2.2 EPO modulation of glutamate uptake in ischemia is dependent on Na,K-ATPase

EPO increased [^3H] D-aspartate uptake in OGD in 5min both with and without glutamate stimulation. This upregulation depended on astrocyte alkalization via NHE, as coincubation of DMA abolished this effect. Further results showed that the increased glutamate uptake was dependent on Na,K-ATPase function, in line with a coupling between Na,K-ATPase function and glutamate uptake in astrocytes as suggested by others (Rose et al. 2009).

The Na,K-ATPase function was enhanced by EPO within the first 5min in OGD both with and without glutamate stimulation. However, with prolonged glutamate and EPO treatment, a pilot study showed that the pump function was actually attenuated after 15min (data not shown), compared to the control incubated only with glutamate (200 μM). A possible explanation is that the astrocyte glycogen stores have been decreased by 15min preincubation of glutamate. With co-treatment of EPO, the glycogen consumption can be further increased due to enhanced glycolysis. As Na,K-ATPase function is strongly dependent on ATP production (Silver and Erecinska 1997),

pump function may be restricted by the low level of glycogen after 15min in our measurements. In figure 4, the proposed effect of EPO on Na,K-ATPase function in the presence of glutamate are rendered. The amount of rubidium uptake, as a measure of Na,K-ATPase activity, is represented by the area under the curve in the different time windows, respectively.

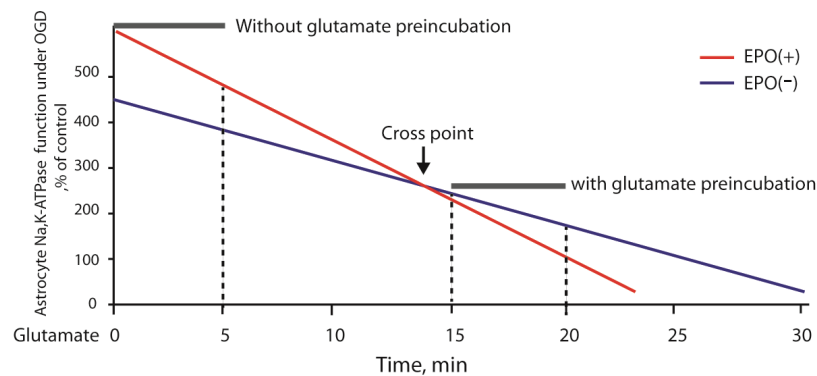


Figure 4 Graphical representation of a proposed relationship between the effect of EPO on Na,K-ATPase function in OGD with or without preincubation with glutamate (200μM). The graph is based on experimental data referred to in the thesis text (see 4.4.2.2). Red line: with EPO. Blue line: without EPO. Areas under the curve represent astrocyte Na,K-ATPase activity as measured by ouabain dependent rubidium uptake. Horizontal bars represent the 5min measurement window for rubidium uptake (together with glutamate treatment and with 15min glutamate preincubation, respectively). EPO was found to increase Na,K-ATPase activity in the first 5min together with glutamate exposure (area under the red curve is larger than area under the blue curve). When glutamate and EPO preincubation was prolonged to 15min, Na,K-ATPase activity was decreased in the measurement window (area under the red curve is smaller than area under the blue curve), interpreted as a result of glycogen depletion.

In summary, based on our *in vitro* results, it can be speculated that EPO application after some time accelerates energy depletion in OGD, especially when the extracellular glutamate concentration is pathologically increased. In areas of more blood flow distant from the ischemic core as in the penumbra *in vivo*, the ‘cross point’ (see figure 4) induced by EPO application can be postponed, buying more time for neuroprotection. To conclude, the result of EPO treatment in ischemic stroke will probably be determined by the degree of reestablished local circulation. Local blood flow can be improved by treatment with tissue plasminogen activator (tPA), currently used therapeutically in ischemic stroke, which however has a narrow time window for application (Hatcher and Starr 2011). When planning for the use of EPO in combination with other pharmacological agents as therapy in stroke, also the optimal timing for administration of EPO must be considered.

4.4.3 Neuroprotection in ischemia: speculations on selection and protection

Several complex processes will be triggered after the onset of an ischemic stroke (Krakauer 2007). During reperfusion following ischemia, deleterious biochemical responses can make the situation even more complicated (Aronowski et al. 1997; Hafeez et al. 2007). However, the definition of ischemic stroke can be simplified as ‘insufficient brain energy and oxygen supply for metabolic demands’. So theoretically, there are only two basic ways to achieve neuroprotection in ischemia: restoration of energy supply or downregulation of brain metabolism. Inspired by the effects of EPO on astrocytes in this study, I would like to speculate on a third option for ischemic neuroprotection.

In the penumbra close to the ischemic core, where blood flow is remarkably limited (15%–40% of normal blood flow), the metabolism of neurons could eventually be inhibited by lactacidosis. Further, glutamate uptake will be severely disturbed after glycogen is depleted in astrocytes. Indeed, considerable lesion growth has been observed in stroke models and in patients presenting with ischemic stroke (Back and Schuler 2004). Consistently, a typical delayed neuronal death following ischemic stroke onset has been observed and widely discussed (Lipton 1999; Wideroe et al. 2007)(also see 1.6). It could take days or even weeks for the dying neurons to undergo programmed cell death (Lipton 1999). The traditional approach for neuroprotection in ischemia has focused on strategies against delayed apoptosis, but substantial experimental evidence shows that modulation of apoptosis may not stop the entire pathological development (Yakovlev and Faden 2004). With limited energy supply, the dying cells could be a burden for the system by consuming oxygen for their inevitable death. In other words, without modulation of metabolism, neuroprotection in ischemia is hard to envision without sufficient oxygen supply. On the other hand, by enhancing anaerobic metabolism in astrocytes, EPO can increase local lactate release and accelerate the breakdown of glycogen. When glutamate uptake is upregulated by EPO, this should prevent neuronal apoptosis in the penumbra area distant from the ischemic core. Concomitantly, it is plausible that EPO may accelerate the destructive processes in the area close to the core because of lactacidosis and glycogen depletion, leading to immediate neuronal death. Such ‘selective’ neuronal death, induced by EPO, could be beneficial for other neurons at risk, if limited oxygen can be ‘saved’ for surviving cells.

Preserved astrocyte function, such as sufficient glutamate uptake, can improve the local homeostasis to avoid initiation of apoptosis. Taken together, without reperfusion, the neuroprotective effect of EPO can be considered as a process of selection rather than pure protection in the penumbra.

In the early phase of acute ischemic stroke, the microenvironment in the ischemic area determines the outcomes of ischemic neurons (see introduction 1.6). In line with this, clinical trials have shown that tPA is only beneficial within the first 3~4.5h after stroke onset (Davis and Donnan 2009; Levi 2004). Considering application of EPO, it is likely that the optimal time window for EPO treatment is also within the first few hours of the acute phase of stroke. Longer EPO treatment could increase metabolic demands, augment lactate accumulation and perhaps boost oxidative stress. In the latest clinical trial of EPO treatment in acute ischemic stroke, EPO was in addition applied at 24 and 48 hours after the onset of symptoms. The overall death rate in the EPO treatment group was 80% higher than in the placebo group (Ehrenreich et al. 2009). However, as mentioned, EPO may provide protection for neurons with restored astrocyte function after circulation is reestablished. Besides, EPO may also facilitate brain edema clearance in a later phase (see 4.3.3). Thus, EPO treatment should not be confined to the limited time period as for tPA. The pathological process of ischemic stroke can last months. Hence, EPO may benefit stroke recovery after the acute phase. To achieve a successful clinical protocol for application of EPO in ischemic stroke, further investigations are needed. In conclusion, with increasing understanding of the effects of EPO, the traditional neuroprotection in ischemia could be redefined as other cell types besides neurons must be taken into consideration.

5 GENERAL DISCUSSION

In the human brain, the billions of neurons are either performing signal transduction or waiting for it. Not surprisingly, neurons will need other cell types to accomplish complex and accurate brain activity. In this thesis, I have been focusing on the roles of astrocytes in aspects of neuron-glia interaction. Short term regulation of astrocyte water permeability was studied and mechanisms for this regulation were revealed. Both physiological and pathological implications of the insights into astrocyte functions and regulation have been discussed. It has been an exciting experience to explore the effects of EPO on astrocytes. With the current knowledge, it is too early to conclude about guidelines of clinical use of EPO in treatments. Hopefully, however, the findings and speculations in this thesis can contribute to the strategies for further studies or even clinical trials of EPO, which is the aspiration of this thesis.

In support of neuron-astrocyte interaction, both glutamate and elevation of potassium was shown to short term regulate astrocyte water permeability via AQP4. Studies of mechanisms showed that the regulation is dependent on protein phosphorylation and that AQP4 water permeability can be modulated by channel gating. Our data suggest that astrocyte water permeability is upregulated dynamically to serve rapid neuronal activities. The opening of AQP4 will facilitate water transport following the osmotic gradient in the tripartite synapse and accordingly maintain water and ion homeostasis. Therefore, theoretically, the upregulation of astrocyte water permeability is beneficial for the restoration of the microenvironment in subcellular compartments. On the other hand, the ECS will decrease substantially in response to neuronal activates due to swelling of astrocytes, which consequently ameliorates the accumulation of local ions and neurotransmitters. The swelling of astrocytes is intimately correlated with water permeability of the plasma membrane. Hence, the dynamic management of astrocyte water permeability should be crucial for neuronal function. With regard to pathological processes, the regulation of astrocyte water permeability could be complicated by multiple factors, such as calcium, intercellular acidification or other cellular events. For the treatment of brain edema, it is difficult to speculate about the pathological role of AQP4 without comprehensive information concerning the pathogen, stages and type of disorder. Nevertheless, AQP4 should be considered as a potential target for development of new therapeutic strategies in brain edema. Both downregulation and upregulation of astrocyte water permeability via AQP4 may have protective effects

depending on the time point of the intervention. In addition, my speculation regarding the role of astrocyte Kir channels in the modulation of potassium kinetics may contribute to the potassium spatial buffering theory (see 4.2.3.2).

As an efficient neuroprotective agent, EPO has been reported to be an attractive candidate drug for acute or chronic brain injuries. We found that preincubation with EPO could attenuate symptoms of water intoxication. The effect was interpreted to be a consequence of the documented modulations of astrocyte water permeability. Water intoxication has been a matter of concern in endurance sports and diseases such as cirrhosis, kidney and heart failure. In these clinical situations, application of EPO or EPO analogs could be an option to prevent the effects of water overload. However, the long term effects of EPO on astrocyte water permeability are not fully clarified. The efficiency and the safety profile for prolonged treatments with EPO still needs to be obtained in experimental models and clinical trials. On the other hand, for interventions of ischemic stroke, a role for EPO is strongly supported both experimentally and clinically. But, as in many interventional clinical trials, the use of EPO failed to show to be positive in a clinical trial involving 522 patients with acute ischemic stroke. This huge gap between experimental models and clinical outcomes may be partly filled by data from research on astrocytes. Indeed, increasing evidence suggests that astrocytes are intimately involved in CNS energy metabolism. It is reasonable and necessary to study the roles of astrocytes in neuroprotection in general as in the use of EPO in the treatment of ischemic stroke. In addition, the positive effect of EPO on glutamate uptake also inspires me to speculate about the potential for EPO applications in neurodegenerative diseases.

As highlighted throughout my thesis, I believe that the understanding of the brain gets more comprehensive when astrocyte-neuron interactions are included. For therapeutic strategies in brain metabolic crisis, focusing only on neurons is like helping fish without water. Astrocytes are designed to maintain neuronal homeostasis and facilitate neuron activities, so that the brain can work as an integrated system rather than clusters of neurons. The masterwork of astrocytes is turning billions of neuronal voices into the melody of the brain, which is the beauty of these star-shaped glial cells.

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