PhD thesis

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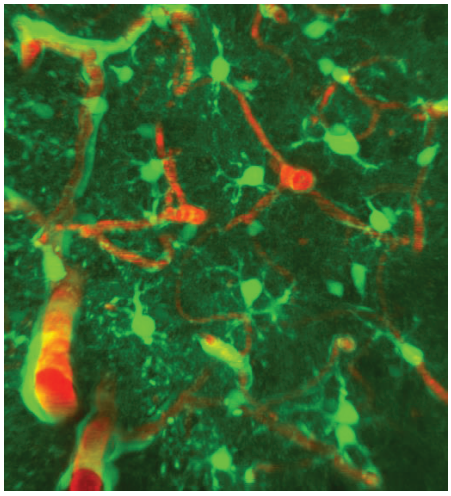

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Acknowledgements

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"The important thing is not to stop questioning."

- *Albert Einstein*

Oslo, May 2016

Abstract

This thesis is comprised of four papers that make up my doctoral degree thesis at the University of Oslo (UiO). These papers focus on the molecular and functional aspects of the brain-blood interface, with special attention on the astrocytes, the predominant glial cell in the brain. Water is essential for life, and water dyshomeostasis is a hallmark of many diseases that affect the central nervous system (CNS). Astrocytic processes at the brain-blood and the brain-liquor interfaces - coined endfeet - exhibit a dense expression of aquaporin-4 (AQP4) water channels. This thesis aims to provide a deeper insight into the roles of astrocytic endfeet in brain water transport and signaling.

In the first study (*Paper I*), we provided evidence that pericytes regulate AQP4 anchoring to perivascular astrocytic endfoot membranes.

In the second study (*Paper II*), we generated and characterized a glial-specific *Aqp4* knockout mouse line, in which the *Aqp4* gene is deleted specifically from astrocytes and ependymal cells. By characterizing this mouse line and comparing it with controls, we provided evidence that endothelial cells are devoid of AQP4. We found that deleting *Aqp4* from glial membranes reduced blood-to-brain water uptake as well as clearance of brain interstitial water. We concluded that the astrocytic endfoot sheath can serve as a barrier for water transport.

In the third study (*Paper III*), we investigated whether global and targeted removal of AQP4 from perivascular and ependymal membranes affected basal brain water content. We found that only global *Aqp4* deletion increased brain water content. Measurements of intracranial pressure during intracisternal infusion of tracer in wildtype and *Aqp4* mutant mice suggested that *Aqp4* gene deletion does not compromise extracerebral drainage pathways.

Finally, our last study (*Paper IV*) showed that astrocytes in acute hippocampal slices respond to Schaffer collateral stimulation with Ca^{2+} elevations. The stimulation-evoked Ca^{2+} signals were modulated by the intensity of the stimulation. Experiments in IP3R2 mutant mice revealed that the astrocytic Ca^{2+} response was dependent on Ca^{2+} release from internal stores.

Through these four projects, we have learned that astrocytes are important for the regulation of brain water, that there are different levels of involvement for the different pools of astrocytic water channels, and that astrocytic Ca^{2+} signaling occurs in response to neuronal signaling through release from internal stores – possibly triggered by cell swelling. Still, more studies are needed to unravel molecular targets in glia for novel treatment of neurological disorders with water dyshomeostasis.

Abbreviations

AQP4 – aquaporin-4 water channel protein

Aqp4 – gene coding for AQP4

α-DG – alpha-dystroglycan

β-DG – beta-dystroglycan

BBB – blood brain barrier

c*Aqp4* KO – conditional aquaporin-4 knockout

CD31 – cluster of differentiation 31 (platelet endothelial cell adhesion molecule (PECAM-1))

CNS – central nervous system

CSF – cerebrospinal fluid

DAPC – dystrophin-associated protein complex

EAAT1 – excitatory amino acid transporter 1

EAAT2 – excitatory amino acid transporter 2

EB – Evans Blue

eGFP – enhanced green fluorescent protein

GABA – γ-aminobutyric acid

GFAP – glial fibrillary acidic protein

GCaMP6f – genetically encoded calmodulin-binding calcium indicator, version 6

GFP – green fluorescent protein

ICP – intracranial pressure

iGluSnFR – intensity-based glutamate-sensing fluorescent reporter

K^+ - potassium

Kir4.1 – inwardly rectifying potassium (Kir) channel 4.1

KO – knockout

PAGE – polyacrylamide gel electrophoresis

PNS – peripheral nervous system

SDS – sodium dodecyl sulfate

WT – wildtype

1PLSM – one-photon laser scanning microscopy

2PLSM – two-photon laser scanning microscopy

Papers included in thesis

Paper I

Evidence that pericytes regulate aquaporin-4 polarization in mouse cortical astrocytes.

Gundersen GA, **Vindedal GF**, Skare O, Nagelhus EA. Brain Struct Funct. 2014 Nov;219(6):2181-6

Paper II

Glial-conditional deletion of aquaporin-4 (*Aqp4*) reduces blood-brain water uptake and confers barrier function on perivascular astrocyte endfeet.

Haj-Yasein NN, **Vindedal GF**, Eilert-Olsen M, Gundersen GA, Skare O, Laake P, Klungland A, Thoren AE, Burkhardt JM, Ottersen OP, Nagelhus EA. *Proc Natl Acad Sci USA.* 2011 Oct;108(43):17815-20

Paper III

Removal of aquaporin-4 from glial and ependymal membranes causes brain water accumulation.

Vindedal GF, Thoren AE, Jensen V, Klungland A, Zhang Y, Holtzman MJ, Ottersen OP, Nagelhus EA. *Resubmitted May 2016*

Paper IV

Origin and dynamics of astrocytic Ca^{2+} signals at activated hippocampal CA3-CA1 synapses of adult mouse.

Vindedal GF*, Tang W*, Hjukse JB, Jensen V, Nagelhus EA *both authors contributed equally. *Manuscript May 2016*

Papers not included in the thesis

Dynamics of ionic shifts in cortical spreading depression.

Enger R, Tang W*, **Vindedal GF***, Jensen V, Johannes Helm P, Sprengel R, Looger LL, Nagelhus EA *These authors contributed equally. Cereb Cortex. 2015 Nov;25(11):4469-76.

Molecular scaffolds underpinning macroglial polarization: an analysis of retinal Müller cells and brain astrocytes in mouse.

Enger R, Gundersen GA, Haj-Yasein NN, Eilert-Olsen M, Thoren AE, **Vindedal GF**, Petersen PH, Skare Ø, Nedergaard M, Ottersen OP, Nagelhus EA. Glia. 2012 Dec;60(12):2018-26.

Loss of Kir4.1 potassium channels in hippocampus of patients with mesial temporal lobe epilepsy.

Heuser K, Eid T, Lauritzen F, Thoren AE, **Vindedal GF**, Taubøll E, Gjerstad L, Spencer DD, Ottersen OP, Nagelhus EA, de Lanerolle N. J Neuropathol Exp Neurol. 2012 Sep;71(9):814-25.

IκB Kinase/Nuclear Factor κB-Dependent Insulin-Like Growth Factor 2 (Igf2) Expression Regulates Synapse Formation and Spine Maturation via Igf2 Receptor Signaling.

Schmeisser MJ, Baumann B, Johannsen S, **Vindedal GF**, Jensen V, Hvalby OC, Sprengel R, Seither J, Maqbool A, Magnutzki A, Lattke M, Oswald F, Boeckers TM, Wirth T. J Neurosci. 2012 Apr;32(16):5688-703.

Deletion of aquaporin-4 changes the perivascular glial protein scaffold without disrupting the brain endothelial barrier.

Eilert-Olsen M, Haj-Yasein NN, **Vindedal GF**, Enger R, Gundersen GA, Hoddevik EH, Petersen PH, Haug FM, Skare Ø, Adams ME, Froehner SC, Burkhardt JM, Thoren AE, Nagelhus EA. *Glia.* 2012 Mar;60(3):432-40

Evidence that compromised K^+ spatial buffering contributes to the epileptogenic effect of mutations in the human Kir4.1 gene (KCNJ10).

Haj-Yasein NN, Jensen V, **Vindedal GF**, Gundersen GA, Klungland A, Ottersen OP, Hvalby O, Nagelhus EA. Glia. 2011 Nov;59(11):1635-42.

INTRODUCTION

In 1846, Virchow discovered a new element in the central nervous system that seemed to embed the rest of the tissue. He called these elements "*nervenkitt*", later translated to *neuroglia*. In more recent years, studies have shown that glial cells actually outnumber neurons by a ratio of 1:1 up to 10:1, depending on the brain region, species and experimental technique (Bignami et al. 1991; Verkhratsky and Butt 2007; Azevedo et al. 2009).

Up until a few decades ago, glial cells were believed to have the sole function of providing structural support for neurons (Allen and Barres 2009). Today, with advances in technologies and new tools to study brain cell function, we know that glial cells serve many key regulatory, homeostatic and metabolic functions, and that they are involved in a number of pathological conditions (Kimelberg and Nedergaard 2010; Oberheim et al. 2012). The discovery of glial cell functions changed the field of neuroscience dramatically. However, glioscience is a research area with many controversies and unanswered questions, which need to be investigated in further detail for years to come.

1.1 Cell types of the brain

In addition to neurons, ependymal cells and vascular cells, the brain contains glial cells. Glial cells are divided into two groups: macroglia and microglia. Microglia are of mesenchymal origin and migrates to the brain during prenatal development, where they differentiate into the immune cells of the CNS (Kettenmann et al. 2011). Macroglia, on the other hand, are of ectodermal origin, and are divided into oligodendrocytes (Schwann cells in PNS) and astrocytes. The latter is the most numerous macroglial cell, and also the cell type of primary interest in this thesis.

1.1.1 Blood brain barrier

In 1885, the German physician and scientist Paul Ehrlich performed a set of experiments where he injected a dye intravenously, and discovered that every organ was colored – except the brain. 28 years later, Edwin Goldmann, one of Ehrlich´s students, injected dye into the cerebrospinal fluid (CSF), and found that the dye administered by this route easily stained the brain. This led to the conclusion that there must exist a barrier between the blood and brain compartments – which later became known as the blood brain barrier (BBB) (Wolburg et al 2009[a]).

The BBB acts as a selective barrier formed by the endothelial cells that line cerebral microvessels. It acts as a physical barrier because complex tight junctions between adjacent endothelial cells force most molecular traffic to take a transcellular route across the BBB, rather than moving paracellularly through the junctions, as in most other endothelia. Tight junction proteins between endothelial cells restrict paracellular diffusion of water-soluble substances from blood to brain. These tight junctions are intricate complexes of both transmembrane proteins (like occludin and claudins) and cytoplasmic proteins (like zonula occludens-1), linked to the actin cytoskeleton of the endothelial cells (Hawkins and Davis 2005). Small gaseous molecules, such as O_2 and CO_2, can diffuse freely through the lipid membranes, which is also a route of entry for small lipophilic agents, like drugs (e.g. barbiturates) and ethanol. The presence of specific transport systems on the luminal and abluminal membranes regulates the transcellular traffic of small hydrophilic molecules, which provides a selective transport barrier, permitting or facilitating the entry of required nutrients, and excluding or effluxing potentially harmful compounds (Abbott, Rönnbäck and Hansson 2006).

The traditional definition of the BBB was for many years that it is comprised solemnly of the brain capillaries´ specialized endothelial cell tight junction proteins. This definition, however, has been shown in several studies to be insufficient. To date, the general consensus is that the BBB does not solemnly refer to brain endothelial cells – which are the barrier proper – but also that to pericytes, the basal lamina and astrocytes (Figure 1) (Abbott, Rönnbäck and Hansson 2006; Wolburg et al. 2009[b]; Cardoso, Brites and Brito 2010; Abbott 2013). The basal lamina is a uniform and narrow matrix-like basement membrane

sandwiched between the vessel wall and the astrocyte foot processes ensheathing the cerebral microvessel (Li, Yuan and Fu 2010).

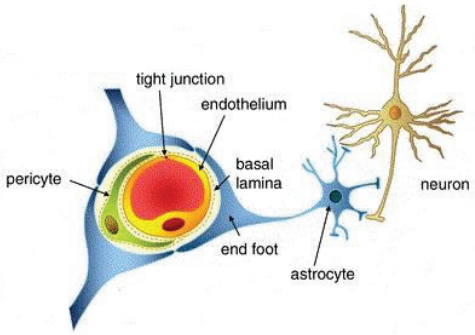

Figure 1. Illustration of the components making up the blood brain barrier (from Abbott 2013).

1.1.2 Pericytes

Pericytes are contractile, multipotent cells that surround the capillary circumference – covered by basal lamina on all sides. Pericytes are interspersed along the abluminal side of the endothelial cells in pre-capillary arterioles, capillaries, and the post-capillary venules – between the endothelial cells and the astrocytic endfeet (Figure 1) (Krueger and Bechmann 2010; Abbott 2013).

The density of pericytes varies along the vessel tree in different tissues, but in the brain the pericytes-to-endothelia ratio has been estimated to 1:3 (Dalkara, Gursoy-Ozdemir and Yemisci 2011). Pericytes communicate with endothelial cells via a series of signaling mechanisms, and are believed to serve several important physiological roles; amongst others within BBB formation, regulation of BBB function, formation of tight junctions, transendothelial vesicle transport, CNS homeostasis, phagocytosis, angiogenesis, vascular maintenance, and blood flow regulation on a capillary level (Daneman et al. 2010; Krueger and Bechmann 2010; Winkler, Bell and Zlokovic 2011; Sà-Pereira, Brites and Brito 2012).

Pericytes-deficient mice exhibit an increased BBB permeability (with an increased endothelial transcytosis), and pericytes seem to work at the BBB in at least two ways; by regulating BBB-specific gene expression patterns in endothelial cells, and by inducing polarization of astrocyte endfeet surrounding CNS blood vessels (Armulik et al. 2010). Little is known about how interactions between blood vessels and astrocytic endfeet are established and maintained.

1.1.3 Astrocytes

The term *astrocyte* was first used in the late 19th century, simply because the cells had a star-shaped appearance with the staining tools scientists had available. Later it became clear that there are different subgroups of astrocytes displaying a variety of shapes and functions; the spherically bushy form (protoplasmic astrocyte, grey matter) and the less bushy process-bearing form (fibrous astrocyte, white matter) (together called the stellate astrocytes), and the elongated form (Bergmann glia, cerebellum; Müller cells, retina) (called radial astrocytes) (Kimelberg and Nedergaard 2010). The bushy appearance of protoplasmic astrocytes is clearly shown when the cells are patched and loaded with fluorescent dye - as visualized in Figure 2 – which allows these astrocytes to form distinct domains in the brain.

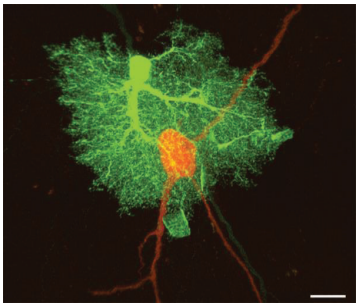

Figure 2. Micrograph displaying a protoplasmic astrocyte (in green) enveloping the cell body and the processes of a neuron (in red). Scale bar 10 µm (from Allen and Barres 2009).

Astrocytes hold important functions in the brain, and have been shown to be involved in buffering of extracellular K^+, control of pH, control of cerebral blood flow, metabolic coupling, lactate production, water and ion homeostasis, and uptake of and de novo synthesis of neurotransmittors (Glu, GABA) (Kimelberg and Nedergaard 2010; Parpura and Verkhratsky 2012[b]; Seifert, Schilling and Steinhäuser 2015). Uptake of glutamate is mediated by two glia-specific transporters —EAAT1 and EAAT2, which in rodents are known as glutamate–aspartate transporter (GLAST) and glutamate transporter 1 (GLT1), respectively.

Astrocytes are also believed to contribute to information processing and neurovascular coupling in addition to their trophic and supportive roles, and the term *tripartite synapse* recognizes the perisynaptic astrocytic processes as a third active member of the synapse (Araque et al. 1999; Haydon and Carmignoto 2006; Santello et al. 2012). The theory is that astrocytes can sense and respond to synaptic activity with an increase in intracellular Ca^{2+} levels, and even release signaling molecules called "gliotransmitters" that in turn can influence or modulate the adjacent pre- and/or post-synaptic neurons (Kimelberg and Nedergaard 2010; Bergersen et al. 2012; Gundersen, Storm-Mathisen and Bergersen 2015). A seminal observation that led investigators to believe that astrocytes dynamically interact with synapses by signaling to neurons through the Ca^{2+}-dependent release of glutamate, was done by Haydon and colleagues in 1994 (Seifert, Schilling and Steinhäuser 2015). These authors studied astrocyte-neuron co-cultures, and found thatincreasing intracellular Ca^{2+} concentration in astrocytes induced release of glutamate – which in turn was found to increase the Ca^{2+} levels of adjacent neurons (Parpura et al. 1994).

The traits of astrocytes increase in size and complexity from lower to higher species (Figure 3). Recent studies have shown that the human brain may harbor not only more complex astrocytes, but also some distinct astrocytes that cannot be recognized in lower species and non-human primates (Oberheim et al. 2009). These data have led to speculations if highly developed astrocytes are necessary for higher-order neuronal networks to function properly (Oberheim et al. 2009; Kimelberg and Nedergaard 2010).

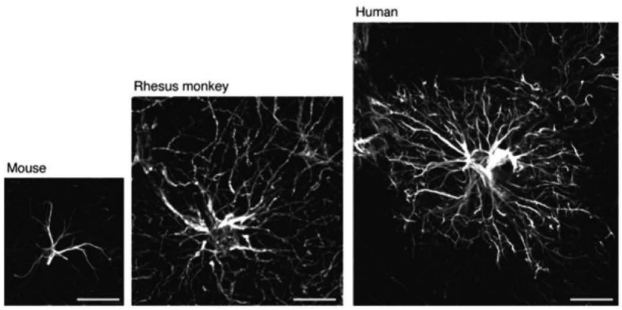

Figure 3. The size of astrocytes increases with increasing complexity of brain function over the species. GFAP-labeled protoplasmic astrocyte from mouse, Rhesus monkey and human. Scale bar 50 µm (from Kimelberg and Nedergaard 2010).

An increasing number of studies are showing that astrocytes most likely are highly involved in several pathological processes – such as cerebral edema, stroke, gliomas, epilepsy and neurodegenerative diseases (Manley et al. 2000; Amiry-Moghaddam and Ottersen 2003; Seifert, Schilling and Steinhäuser 2006; Gunnarson et al. 2009; O´Brien, Howarth and Sibson 2013; Coulter and Steinhäuser 2015; Phatnani and Maniatis 2015; Pekny et al. 2016), mainly through disturbances in clearance of molecules that can be harmful if allowed to accumulate in the extracellular space – like glutamate, K^+, or even water. An improved understanding of astrocyte biology, heterogeneity and physiology, as well as the involvement of these cells in pathogenesis can potentially offer development of novel strategies to treat neurological disorders (Verkhratsky et al. 2015).

1.1.3.1 Polarization

Astrocytes are strategically located in the brain - between the neurons and the brain´s blood supply. They are highly polarized cells with specialized membrane domains tabutting either synapses (i.e., in perisynaptic processes), blood vessels (perivascular endfoot processes), or processes of neighboring astrocytes. The latter domain contains gap junctional proteins that serve to connect astrocytes into a syncytium. The perisynaptic processes are considered important for the regulation of K^+ (by local uptake and spatial

buffering) and glutamate released into the synaptic cleft during neuronal activity, as astrocyte perisynaptic membranes express Kir4.1 potassium channels, glutamate transporters, Na^+/K^+-ATPase, NKCC1, as well as AQP4 water channels (Figure 4) (Kofuji and Newman 2004; Kimelberg and Nedergaard 2010; Nagelhus and Ottersen 2013).

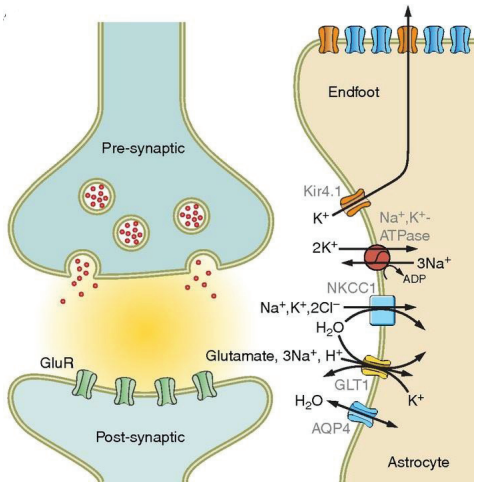

Figure 4. Illustration of key channels and transporters expressed in the astrocytic membranes. These molecules are believed to hold important functions in maintaining water and ion homeostasis (from Nagelhus and Ottersen 2013).

1.1.3.1.1 The perivascular astrocytic endfeet

Astrocytic endfeet cover approximately 99% of the brain´s capillaries (Mathiisen et al. 2010), making them important structures in regard to movement of fluid and molecules between blood and the brain and vice versa.

Studies of astrocytic endfoot membranes with immunogold labelling and electron microscopy revealed a very prominent feature of the astrocytic endfeet; namely that the endfoot membrane densely expresses AQP4 water channels, which are co-localized with Kir4.1 potassium channels (Figure 4) (Nagelhus et al. 1999; Nagelhus, Mathiisen and Ottersen 2004).

1.1.3.1.2 Astrocytic Ca^{2+} signals

Astrocytes have been shown to exhibit two distinct forms of Ca^{2+}-signals; intercellular Ca^{2+} waves and spontaneous Ca^{2+} oscillations in individual cells (Matyash and Kettenmann 2009). The initiating event that leads to intracellular Ca^{2+} waves in astrocytes appear to primarily derive from the activation of metabotropic G-protein-coupled receptors, activation of phospholipase C, and the production of a second messenger inositol 1,4,5-trisphosphate (IP3), which following IP3 receptor type 2 (IP3R2) activation leads to Ca^{2+} release from the endoplasmic reticulum (ER) (Scemes and Giaume 2006) – where the Ca^{2+} concentration is ~1000 times higher than in cytoplasm. The Ca^{2+} waves propagate either via functional gap junctions or by paracrine ATP-release (Verkhratsky, Rodríguez and Parpura 2012), while the mechanisms behind the spontaneous Ca^{2+} oscillations are more enigmatic. Astrocytic Ca^{2+} signals during osmotic swelling have been shown to be significantly reduced by *Aqp4* deletion, as shown by Thrane et al. (2011). Astrocytic Ca^{2+} signals are also reduced by general anesthesia (Thrane et al. 2012; Ding et al. 2013). Intracellular Ca^{2+} is tightly regulated in all mammalian cell types, as a rapid increase in this ion is, among other things, used to mediate programmed cell death (Parpura and Verkhratsky 2012[a]).

Despite intense research on astrocytic Ca^{2+} signals the last decades, the physiological importance of astrocyte Ca^{2+} signaling is still poorly understood. Pioneering discoveries in the early 1990ies showed that astrocytes express glutamate-sensitive ion channels that respond to locally applied glutamate by generating a spreading wave of elevated intracellular Ca^{2+} – a finding that pointed to a previously unrecognized role in signaling for these cells (Cornell-Bell, Finkbeiner and Smith 1990). Following these discoveries were studies showing that cultured astrocytes (Cornell-Bell, Thomas and Caffrey 1992), as well as dye-loaded organotypically cultured hippocampal slices (Dani, Chernjavsky and Smith 1992) and acute hippocampal slices (Porter and McCarthy 1996), responded to glutamate with elevations of cytoplasmic Ca^{2+}. These propagating waves of Ca^{2+} suggested that networks of astrocytes may constitute a signaling system within the brain, which communicate through the release of Ca^{2+} from intracellular stores and propagates as waves within the cytoplasm of

individual astrocytes and between adjacent astrocytes (Cornell-Bell, Finkbeiner and Smith 1990).

Initial studies were carried out in cell culture preparations using synthetic Ca^{2+} dyes. Subsequently, Ca^{2+} imaging experiments were performed on acute brain slices. In slice experiments, the slices were largely prepared using tissue from pups, as slices from adult animals proved to be difficult to load. Recently it was shown that the expression of astrocytic receptor believed to mediate the astrocytic Ca^{2+} signals, differs in young mice as opposed to adult mice (Sun et al. 2013). Furthermore, bulk dye loading is a rather inadequate method for visualizing the fine astrocytic processes, as seen in Figure 5 (Reeves, Shigetomi and Khakh 2011). Bulk loading mainly allows visualization of the cell soma and the thickest branches, leaving >90% of the astrocytic territory unsampled. The limitations of synthetic Ca^{2+} dyes have been solved by the development of genetically encoded Ca^{2+} indicators (Shigetomi et al. 2013; Tong et al. 2013).

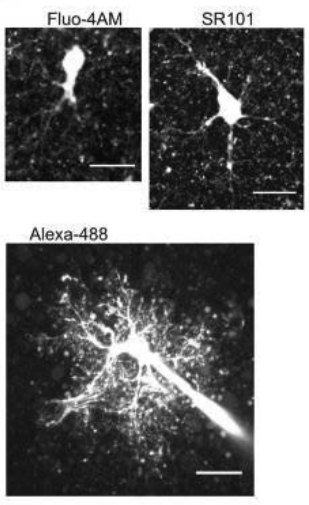

Figure 5. Examples of bulk loaded astrocytes (Fluo-4AM and SR101), as opposed to patch pipette loaded astrocytes (Alexa-488). Scale bars 15 µm (from Reeves, Shigetomi and Khakh 2011).

Mice deficient in IP3R2, which is enriched in astrocytes and - once stimulated by IP3 - mediates Ca^{2+} release from the ER, have been used to assess the consequences of removing

astrocytic Ca^{2+} signaling (Zhang et al. 2014). However, a recent study revealed that Ca^{2+} signals in astrocytic processes are largely preserved in IP3R2 knockout (KO) mice (Srinivasan R et al. 2015).

1.1.3.2 Aquaporins

Water can cross cell membranes by different means; by slow diffusion or through cotransporters and uniporters (Figure 6) (Agre 2004; MacAulay, Hamann and Zeuthen 2004).

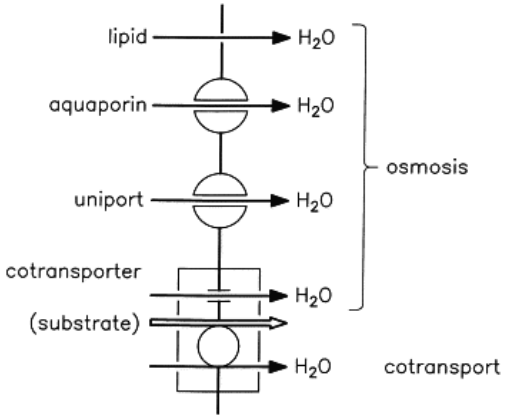

Figure 6. The different means by which water can cross the hydrophilic plasma membrane (from MacAulay, Hamann and Zeuthen 2004).

Cotransporters are highly expressed in the brain. In neuropil, we find KCC (K^+,Cl^-), NKCC1 (Na^+,K^+,$2Cl^-$), MCT (lactate and H^+, enriched in endothelial cells), GAT (GABA and Na^+,Cl^-) and EAAT (glutamate and Na^+,H^+,K^+) (MacAulay and Zeuthen 2010). The latter two are particularly enriched in perisynaptic astrocyte processes (Danbolt et al. 1998). The glucose transporter GLUT1 is also densely expressed in the brain, especially in capillaries (Pardridge, Boado and Farrell 1990; Kawamoto et al. 1995). Because of its ability to transport water, the GLUT1 could play a role in water transport across the BBB.

Water also crosses cell membranes through aquaporin water channels (Agre 2004; MacAulay, Hamann and Zeuthen 2004). Aquaporins (AQPs) are a family of proteins that form pores through the plasma membrane, and mediate rapid transmembrane transport of

water. The first aquaporin was discovered in 1992 (Preston et al. 1992), and the discovery defined a new subset of transmembrane pores. To this day, 13 aquaporins have been identified in mammals (namely AQP0-AQP12), and over 200 in plants and microbials (Johansson et al. 2000; Agre et al. 2002; Santoni et al. 2003). Some aquaporins (AQP0, 1, 2, 4, 5, 6 & 8) are selective to water molecules, while other aquaporins, like AQP3, AQP7, AQP9 and AQP10, transport small uncharged solutes such as glycerol, ammonia and urea in addition to water molecules, and are termed aquaglyceroporins (Agre et al. 2002). It is worth to mention that the endothelial walls of blood vessels in extracerebral organs express AQP1, making them highly permeable to water. This is not the case for blood vessels in the brain.

Three aquaporins are present in the rodent brain; AQP1 (choroid plexus epithelial cells) (Nielsen et al. 1993; Oshio et al. 2005), AQP4 (astrocytes and ependymal cells) (Frigeri et al. 1995; Nielsen et al. 1997) and AQP9 (expression controversial) (Tait et al. 2008). This thesis will focus on AQP4, which selectively mediates transport of water and is concentrated at the interface between brain and the major fluid compartments of the CNS (Nielsen et al. 1997; Nagelhus et al. 1998).

1.1.3.2.1 Aquaporin-4

In 1994, cloning and molecular characterization of a brain aquaporin (aquaporin-4; AQP4) was reported in two independent studies (Hasegawa et al. 1994; Jung et al. 1994). Subsequently, the AQP4 protein was found to be widely expressed throughout the neuropil, localized to glial and ependymal cells (Frigeri et al. 1995; Nielsen et al. 1997). Although AQP4 is present in all astrocytic processes, including the processes that envelope synapses, AQP4 has been found to be highly concentrated in astrocyte endfeet at the interface between brain tissue and the main fluid compartments of the CNS (Nielsen et al. 1997; Nagelhus et al. 1998). In fact, AQP4 expression is tenfold higher in the perivascular endfoot processes abutting onto blood vessels and facing pia limitans externa, than the synaptic glial processes (Nielsen et al. 1997; Nagelhus et al. 1998; Nagelhus et al. 2004). Thus, astrocytes are highly polarized cells, functionally as well as anatomically.

AQP4 has been shown to be absent from neurons, oligodendrocytes and microglia. Some studies have indicated AQP4 protein expression in endothelial cells (Kobayashi et al. 2001; Amiry-Moghaddam, Frydenlund and Ottersen 2004), in contrast to earlier reports (Nielsen et al. 1997; Nagelhus et al. 1999).

AQP4 is anchored to the perivascular membrane via the dystrophin-associated protein complex (DAPC), through interaction with α-syntrophin (Figure 7).

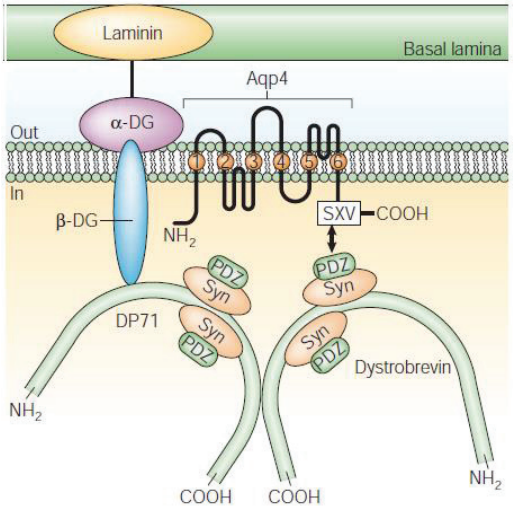

Figure 7. The DAPC binds the transmembrane protein β-dystroglycan (β-DG), which connects to the laminin/agrin-binding protein α-DG. On the cytoplasmic side of the complex, dystrophin binds to α-dystrobrevin. Each dystrophin and α-dystrobrevin molecule can bind up to two syntrophins. α-syntrophin is the main syntrophin expressed in astrocytic endfeet, and is thought to be responsible for the clustering of AQP4 in endfoot membranes (from Amiry-Moghaddam and Ottersen 2003).

The interaction between AQP4 and α-syntrophin was shown in a study where it was demonstrated a significant loss of AQP4 immunogold labelling in α-syntrophin KO mice (Neely et al. 2001; Amiry-Moghaddam et al. 2004), quantified to 88% in the perivascular membrane (Amiry-Moghaddam et al. 2004).

1.1.3.2.1.1 Physiological and pathophysiological functions of AQP4

The discovery of clustered AQP4 expression in perivascular astrocyte endfeet opened a new field in molecular medicine, and it is now well established that AQP4 is involved in the pathophysiology of brain edema (Amiry-Moghaddam and Ottersen 2003; Papadopoulos and Verkman 2007). *Aqp4* gene deletion, as well as α-syntrophin gene deletion, reduces cytotoxic brain swelling and infarction volume in experimental stroke (Manley et al. 2000; Amiry-Moghaddam et al. 2003[a]). On the contrary, it has been demonstrated that *Aqp4* deletion aggravates vasogenic brain edema (Papadopoulos et al. 2004).

However, after more than a decade of intense research, the mechanisms underlying neuroprotection following *Aqp4* KO are not fully understood, and the physiological roles of AQP4 in brain water transport are still enigmatic. In the absence of an AQP4 inhibitor, studies assessing roles of AQP4 have largely relied on *Aqp4* KO mice and models where AQP4 anchoring to perivascular endfoot membranes has been disrupted.

From *Aqp4* KO studies, there is evidence of reduced seizure threshold and prolonged seizure activity (Binder et al. 2006). This also held true for studies in the model of loss of polarized AQP4 expression – the α-syntrophin knockout model – suggesting that functional integrity of perivascular endfeet is required to handle the excessive amounts of extracellular K^+, as increased $[K^+]_o$ tends to depolarize neurons and facilitate development of epileptiform seizures. However, the role of AQP4 in K^+ buffering is highly controversial (Zhang and Verkman 2008; Jin et al. 2013; Assentoft, Larsen and MacAulay 2015), and recent evidence points towards indirect effects of AQP4 through volume changes, as described below in Section 1.2.2 (Haj-Yasein et al. 2012; Haj-Yasein et al. 2015).

1.2 Fluid dynamics in the brain

The brain consists of ~80% water (Tait et al. 2008), and large water fluxes continuously take place between the different compartments of the brain, as well as between the brain parenchyma and the blood or cerebrospinal fluid (MacAulay and Zeuthen 2010). In order for the brain to function optimally, this transport and movement of fluid must not be compromised. Water and ion homeostasis is, under normal conditions,

maintained by transporters and channels localized in the plasma membrane of the cellular participants (Simard and Nedergaard 2004). Any dysregulation or lack of these membrane associates can cause the homeostasis to be compromised and may lead to disease.

The fluid in the brain moves between the major fluid compartments; the blood, the CSF, the interstitial fluid and the intracellular fluid. This movement is generally accepted to be a passive event, driven by hydrostatic and osmotic pressure gradients (Papadopoulos and Verkman 2007), although cotransporters and the glucose transporter GLUT1 have an inherent ability to transport water against an osmotic gradient (MacAulay and Zeuthen 2010).

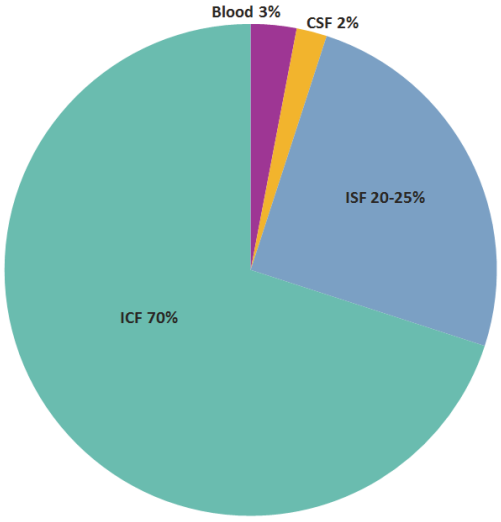

Figure 8. Illustration of volume fractions of the major fluid constituents in the brain as representation of the total brain volume: blood 3% (purple), cerebrospinal fluid (CSF) 2% (orange), interstitial fluid (ISF) 20-25% (blue), intracellular fluid (ICF) 70% (green) (illustration by Vindedal 2015, based on Lehmerkülher et al. 1993; Syková and Nicholson 2008; Akdogan et al. 2010).

1.2.1 Water homeostasis at the blood brain interface

In astrocytic endfeet, water can move bidirectionally through the abundantly expressed AQP4 water channels (Nielsen et al. 1997). The intrinsic water permeability of the

other layers of the BBB that do not express AQPs is largely unknown, but it is believed that water transport in these structures is co-transported with other molecules – such as electrolytes, amino acids, glutamate or lactate – through transporter proteins such as uniports or cotransporters, as mentioned above (Zeuthen and MacAulay 2002[a]; Zeuthen and MacAulay 2002[b]).

Water within the brain parenchyma can be cleared to the extra-cerebral systems via three routes; across the BBB into the blood stream, across the pial surface into the subarachnoid space, and across ependymal cells into the ventricles (Papadopoulos and Verkman 2007). A widely appreciated concept of fluid movement in the CNS claims that CSF is produced in choroid plexi in the lateral ventricles, flows through the 3rd and 4th ventricle before it enters the subarachnoid space and is resorbed by arachnoid granulations into venous sinuses (Abbott 2004). Yet, the precise mechanisms behind endogenous fluid production – as well as the flux routes and clearance pathways of CSF and excess interstitial fluid – remain poorly understood. The fact that the brain lacks a lymphatic circulation, and that all metabolically active tissues must dispose of waste, suggests that the brain must clear excess fluid and waste products by an alternative mechanism. Several studies have shown that CNS fluid can move along the perivascular space (Virchow-Robin space) surrounding cerebral blood vessels (Szentistványi et al. 1984; Rennels, Blaumanis and Grady 1990; Abbott 2004), but the findings are highly debated. In any case, it should be noted that the three routes by which fluid can be cleared to the extra-cerebral systems are all lined with glial cells that express AQP4 water channels (i.e., astrocytic endfeet, glia limitans and ependymal cells) (Papadopoulos and Verkman 2007).

1.2.2 Water homeostasis at the synaptic level

About thirty-five years ago, it was observed that when neurons are active, reversible changes occur in the intracellular and extracellular space, manifested as shrinkage of the extracellular space (ECS) and swelling of cellular components (Dietzel et al. 1980; Sykova and Nicholson 2008). The dynamic volume changes are thought to reflect redistribution of water

co-transported with ionic fluxes and transmitter uptake as a consequence to neuronal activity – such as K^+ and glutamate (MacAulay and Zeuthen 2010). The exact contribution of ion channels and transporters probably depend upon different parameters – such as stimulation strength, duration and brain region. In 2012, Haj-Yasein and colleagues showed a more pronounced activity-induced shrinkage of the ECS in *Aqp4* KO mice compared to wildtypes (WTs), indicating that AQP4 counteracts ECS shrinkage during neuronal firing. The data is consistent with the idea that water move into astrocytes through cotransporters along with the transported substrates and flows back into the ECS through AQP4, the latter pathway likely driven by the osmotic gradients set by the Na^+, K^+-ATPase.

1.3 Unresolved issues

The idea that astrocytes can be the target for future treatment of neurological disease is relatively new, as our knowledge about astrocytes and their functional significance is still rather limited and lags behind that of neurons. Recent technological advances, however, have greatly expanded the approaches to unravel the secrets of astrocytes. As these techniques are being developed, we keep seeing more sophisticated and complex astrocytic functions. Although brain water transport is disrupted in many neurological conditions, we are not yet familiar with the underlying mechanisms. Since the brain is the only organ in the body that does not have a lymphatic system, one intriguing question is how excess fluid is drained from the brain, and which molecules are involved. When we learn more about the molecular mechanisms that underlie this brain water transport, we will in turn be able to investigate the macroscopic fluid circulation of the brain and its regulation.

1.4 Overall aim of the thesis

With the studies included here, we wanted to identify important mechanisms behind regulation of brain fluid transport in and out of the brain, if and how astrocytic water channels are involved, and the regulation and kinetics of astrocytes Ca^{2+} signals. Firstly, we

wanted to investigate if adjacent cell types beyond the DAPC complex could be involved in the regulation of AQP4 anchoring to the perivascular endfoot membranes, specifically in regards to pericytes. We also wanted to establish once and for all if AQP4 is expressed in other cells associated with the BBB, or if in fact AQP4 expression in the gliovascular unit is exclusive to the astrocytic endfoot membranes. After investigating this, the next step was be to study if this perivascular pool of AQP4 had a functional role in water transport occurring at this site – i.e. in water transport from blood to brain. Naturally, this led us to wanting to study the exit routes of water transport as well – i.e. transport of water from brain to blood, and to establish any role for the different pools of AQP4 water channels. Finally, we wanted to investigate the functional aspects of mechanisms behind the astrocytic Ca^{2+} signals, more specifically to determine the predominant source of these signals, as well as the dynamics between neuronal and astrocyte activation – both in regards to the temporal dynamics and the glutamatergic involvement.

In more overall terms, the rationale behind the studies that constitute this thesis was to increase our understanding of astrocytes in brain fluid dynamics, specifically the involvement of astrocytic processes and the abundantly expressed glial AQP4, and in a signalling aspect.

2. MATERIALS AND METHODS

Gene knockout models and transgenic mice:

Gene knockout animal and transgenic animal techniques have proven extremely valuable in medical sciences, by allowing researchers to study the function of specific gene products (Gareriaux-Ruff and Kieffer 2007; Bockamp et al. 2008). To overcome the potential problem of a lethargic phenotype, it is also possible to knock down proteins using the cre-mediated LoxP-specific recombination, as we did in Paper II and III (conditional astrocyte-specific *Aqp4* KO and ependymal-specific *Aqp4* KO, respectively). By exclusively eliminating proteins of interest under a cell-specific promotor, we are able to pinpoint functional characteristics of single proteins in different cell populations.

In the projects included in this thesis, we have used both global KO mouse lines (*Aqp4* KO, α-syntrophin KO, IP3R2 KO), conditional KO mouse lines (c*Aqp4*-GFAP KO, c*Aqp4*-FoxJ1 KO), as well as a transgenic mouse line (GLT1-eGFP/NG2-DsRed double reporter mice). The GLT1-eGFP/NG2-DsRed transgenic double reporter mice were generated by crossing bacterial artificial chromosome (BAC) promoter reporter transgenic mice that express the fluorescent proteins eGFP (cf. Figure 8) and DsRed under the control of the natural GLT1 (Regan et al. 2007) and NG2 BAC promoters (Zhu et al. 2008), respectively.

None of these mouse models exhibit any abnormal growth, development, reduced survival or abnormal phenotype characteristics if not physiologically or artificially stressed.

Immunohistochemistry (IHC) for light microscope (LM) and immunogold cytochemistry for electron microscopy (EM):

Immunohistochemistry (IHC) allows for detection of specific proteins by introducing and recognizing antibodies specific to certain epitopes, and visualization by immunofluorescence – showing the distribution of proteins of interest within anatomical structures. We used indirect immunofluorescence, meaning that a primary antibody is linked

to the target-protein, and it recognized by a secondary antibody covalently attached to a fluorescent dye. When exposed to photons of the proper wavelength, the fluorescent dye emits light and is visualized in a confocal microscope (1PLSM). IHC protocols for labeling of AQP4, CD31 and DAPI were utilized in Paper I, II and III in this thesis.

The EM technique allows for detection of specific proteins at a subcellular level, demanding a resolution that confocal microscopy cannot fulfill due to limitations of light waves visible to the human eye. The procedure used in Paper I and II was postembedding immunogold cytochemistry, where a primary antibody is linked to the target-protein. Subsequently, a secondary antibody, which is connected to a gold particle, binds to the primary antibody. The gold particle is electron-dense and can therefore be visualized in the electron microscope. Immunogold cytochemistry was employed using antibodies against AQP4 and α-syntrophin.

Western Blotting (WB):

Western blotting is used to detect specific proteins in a biological sample by separating native or denaturated proteins by their molecular size by gel electrophoresis. To quantify the amount of expressed proteins, SDS-PAGE with subsequent immunoblotting was run on gels of an appropriate concentration. For this thesis, Western blots were run on AQP4, with β-actin as an internal control protein (Paper II).

To verify the conditional *Aqp4*$^{-/-}$ mice, quantitative SDS-PAGE/immunoblotting was run on homogenized whole-brain tissue samples from the respective mouse lines. After determining the total protein concentration for each homogenate, every loading sample was added 10 µg total protein from each sample and the gels were loaded. For the titration series, WT homogenate was diluted in a constitutive *Aqp4*$^{-/-}$ sample in set concentrations to determine the sensitivity of the immunoblotting.

Blots were blocked and then incubated with goat-anti-AQP4 antibody (Santa Cruz; sc-9888, 0.2µg/mL) at a dilution of 1:1000 in blocking solution overnight at room temperature, before washing and incubation with alkaline phosphatise-conjugated rabbit-anti-goat IgG at a dilution of 1:10.000 in blocking solution for 1h at room temperature. After scanning the blots, they were then reprobed using mouse-anti-β-actin (Abcam;ab-8226, 1.3µg/mL) in a dilution of 1:1000 in blocking solution overnight at room temperature. Finally, the blots were scanned for final results.

Evans Blue extravasation (EB):

EB (~0.96 kDa) is a chemical dye that binds to albumin (~68 kDa when bound) in the blood, which under normal circumstances will not cross the BBB. For this reason, systemic administration of EB can be used as an assay to evaluate the permeability of the BBB in pathological conditions. EB fluoresces at 680 nm when excited between 470-540 nm (Vise et al. 1975), and can therefore give a quantitative measure after homogenization and spectrophotometry. Freeze lesions were used as positive controls (Paper II), as such a cold injury will cause opening of the BBB (Ikeda et al. 1994).

Brain water content measurements and hypo-osmotic stress model:

The wet/dry brain weight method is a method where the brain is removed from the animal and freshly weighed, then weighed again after all liquid has evaporated in a vacuum for a certain time period (giving the dry weight), allowing for a calculation that states the percentage of water content (performed in Paper II and III). Animals were sacrificed by cervical dislocation and the brains were immediately dissected out intact in a standardized fashion to ensure a non-biased brain mass sample collection. Each brain sample was massed in a pre-weighed 10 mL glass vial, before being manually homogenized with a spatula against the inside of the same vial. The vial was then massed with the brain sample once again, and wet brain sample mass was calculated from the difference. The samples were dried in a Fistreem vacuum oven (Fistreem International) for 24 h at 80 °C and −1.000 mbar. After

drying, each vial with dried brain was again massed. Percentage brain water content was calculated as (wet mass − dry mass) × 100/(wet mass).

Brain water accumulation after hypo-osmotic stress was assessed for each genotype by comparing values for baseline brain water content with brain water content measured on mice decapitated 40 min after intraperitoneal injection of distilled water (150 mL/kg) (Paper II). Intraperitoneal injection of water lowers the blood´s concentrations of solutes, creating an osmotic pressure gradient between blood and brain, forcing water to be taken up by astrocytes and causing them to swell – thereby being a model for cytotoxic brain edema (Nase et al. 2008; Risher et al. 2009).

Acute hippocampal brain slices:

For the slice experiments in Paper IV, the acute hippocampal slices were prepared as previously described (Haj-Yasein et al. 2015). WT mice were euthanized with Isoflurane (Baxter) and the brains quickly removed and placed in ice cold artificial cerebrospinal fluid (aCSF, 0°C, bubbled with 95 % O2 and 5 % CO2, containing (in mM): 124 NaCl, 2 KCl, 1.25 KH_2PO_4, 2 $MgSO_4$, 1 $CaCl^2$, 26 $NaHCO_3$ and 12 glucose). Coronal sections of 400 µm thickness were made from the dorsal half of each hippocampus perpendicular on the longitudinal axis. The slices were transferred to a humidified interface chamber. In the chamber the slices were continuously exposed to humidified gas at 32°C and perfused with aCSF (pH 7.3 and 2mM $CaCl^2$). 50 µM DL-2-amino-5-phosphonopentanoic acid (NMDA receptor antagonist DL-AP5 (Tocris)) was applied to the aCSF to inhibit glutamate binding to post-synaptic NMDA-receptors. After a period of rest, approximately one hour, single slices were transferred to the submerged recording chamber (32°C) for further experimentation. Extracellular synaptic responses were monitored by a glass electrode filled with aCSF (1-5MΩ), placed 200 µm from a similar constructed stimulation electrode. Following a period of stable synaptic responses (prevolley at ~1 mV), we selectively stimulated the Schaffer collaterals in stratum radiatum at different frequencies and durations.

Virus injection:

The basic concept of the viral transduction approach is to use virus as a media to deliver genes of interests into cells by virus infection (Robbins and Ghivizzani 1998; Verma and Weitzman 2005), such as the recombined adeno-associated virus (rAAV) in our study. With this method, the DNA sequences from our genes of interest could be included in the viral vectors, and further expressed in the specific target cell types in the mouse brain. The utilized indicators in Paper IV (iGluSnFR, jRGECO1a, GCaMP6f, respectively) were introduced under the glia-specific promotor glial-fibrillary acidic protein (GFAP) to target astrocytes or the neuronal promotor synapsin (Syn) to target neurons. The rAAV delivery approach allows the monitoring of cellular functions, observing the physiological states of specific cell populations (Davidson and Breakefield 2003). The corresponding rAAVs were delivered stereotactically into the hippocampus bilaterally, thereby providing a local, virus-mediated delivery of different sensors in aimed cell types. The stereotactic injection method also enables us to express both green fluorescent Ca^{2+} indicator GCaMP6f and red Ca^{2+} indicator jRGECO1a simultaneously, in order to monitor the interaction between these two cell types of interest; astrocytes and neurons respectively. The rAAVs were produced and purified as described before (Tang et al. 2015), and the viruses were stereotactically and bilaterally injected into the brains of deeply anesthetized (mixture of zolazepam (188 mg/kg body weight), tiletamine (188 mg/kg body weight), xylazine (4.5 mg/kg body weight) and fentanyl (26 µg/kg body weight)) 12 to 20 week old male C57BL/6J mice (Jackson Laboratories), as described previously (Tang et al., 2009). For virus transduction in adult mouse hippocampi, stereotactic coordinates relative to Bregma were: anteroposterior -2.0 mm, lateral ±1.5 mm. During each injection, a total of ~0.6 µl of purified rAAVs were delivered into each hemisphere, by a 2-step injection with 1.0 mm and 1.6 mm in depth, respectively.

Intracranial pressure measurements (ICP):

If water is allowed to accumulate in the brain – e.g. due to brain edema – high pressure builds up and compresses the brain within the rigid confines of the skull cavity. This can eventually lead to herniation and a potentially lethal outcome. Measuring the ICP in an experimental setting allows us to investigate how other manipulations affect the ICP.

In order to measure the intracranial pressure during intracisternal infusions (Paper III), C57BL/6J mice (n=8) and *Aqp4* KO mice (n=8) were anesthetized with an i.p. injection (10 mg/kg) of a zoletil cocktail (zolazepam (188 mg/kg), tiletamine (188 mg/kg), xylazine (4.5 mg/kg), fentanyl (26 µg/kg) body weight). Body temperature was monitored with a rectal probe and kept at 37°C by a temperature-controlled heating pad (Harvard Apparatus). Tracheostomy was performed and mice mechanically ventilated (model "SAR-1000" ventilator, CWE Inc.) with room air at 100 bpm, volume 0.25-0.35 ml/min (depending on size of the mouse). Blood gases, blood pressure and oxygen saturation were monitored using Mouseox (StarrLife Sciences) with thigh sensor for mice. Oxygen saturation was at all times above 90%. An incision in the neck region was made and muscle was bluntly dissected to localize the cisterna magna. A 30 GA needle connected to tubing and a 50 µl Hamilton syringe was inserted into the cisterna magna and fixed with tissue adhesive (LiquiVet Adhesive, Oasis). A small craniotomy (1mm in diameter, -3 mm Bregma, -2 mm lateral from midline) was made in the skull and the ICP catheter (SPR-1000, Millar) connected to a pressure transducer (PCU 2000) was inserted 2 mm under the dura (towards Bregma); above brain and underneath skull. After the pressure measurements stabilized, baseline values were recorded for 1 min, before an infusion of 2 µl/min of sterilized 0.9% saline was delivered by a syringe pump (kdScientific, model 100 series).

Two-photon laser scanning microscopy

Fluorescence imaging relies on the interaction between photon and molecule, raising the molecule to an *excited* state as it absorbs the energy from the photon. The molecule,

called a fluorochrome, lose the excess energy through mechanical vibration (phenomenon called *the Stokes shift*), and as a result it emits a new photon before returning to its basal *unexcited* state (Figure 9). The emitted photon is always of lower energy (red-shifted) compared to the excited molecule, i.e. it has a longer wavelength (Kimelberg and Nedergaard 2010). In a biological setting, fluorochromes are often bound to macromolecules to attain desired chemical properties (i.e. lipid solubility), and are then called *fluorophores* (Paredes et al. 2008). Fluorescent molecules are generally either chemicals (e.g. rhodamine dyes) or proteins (e.g. green fluorescent protein (GFP) from jellyfish).

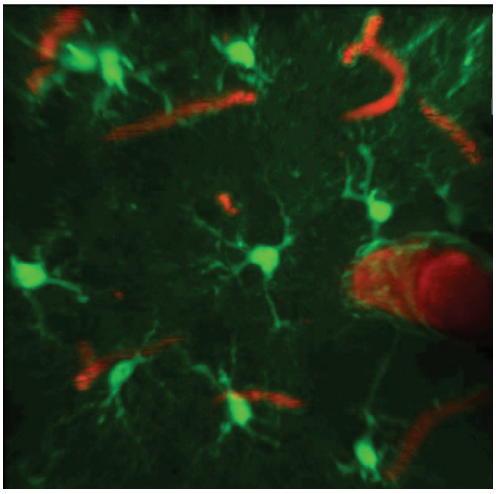

Figure 8. Two-photon image of cortical astrocytes (green: eGFP) with processes stretching into the neuropil, and several endfoot processes abutting onto capillaries (red: Texas Red injected i.v.). The black stripes in the vessels represent red blood cells. Scale bar 30 µm (Vindedal 2012, unpublished).

In vivo optical imaging is a relatively new technique that allows microscopic analyses of the living brain (Denk et al. 1990). Traditionally, in vivo imaging has been the domain of technologies such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET), which suffer from relatively poor anatomical resolution. In contrast, high resolution analyses typical of light microscopy and electron microscopy have been restricted to tissue sections or slices. In other words, high anatomical resolution at the light

microscopic level has been incompatible with analyses of living animals. This changed with the introduction of 2PLSM. With this technique, anatomical structures down to a size of 0.45 µm can be studied in living brains (Ottersen and Helm 2002).

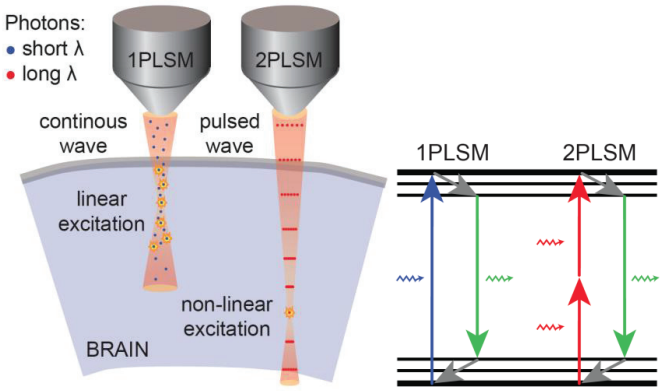

Figure 9. Sketch comparing one-photon laser scanning microscopy to two-photon laser scanning microscopy (Courtesy of Thrane and Thrane 2012).

3. SUMMARY OF FINDINGS

3.1 Paper I

Background: AQP4 water channels are concentrated in astrocytic endfoot membranes at the brain-blood and brain-cerebrospinal fluid interfaces. The mechanisms underpinning this polarized distribution of AQP4 are poorly understood.

Aims: The aim of Paper I was to investigate whether endfoot AQP4 expression depends on the adjacent cell type. Specifically, we wanted to provide evidence that pericytes regulate AQP4 anchoring to perivascular astrocytic endfoot membranes.

Methods: Cortical tissue from transgenic reporter mice and KO mouse models was transcardially fixed and underwent immunofluorescence protocols and quantitative immunogold experiments, before being analysed.

Results: AQP4 immunofluorescence of brain sections obtained from novel transgenic double reporter mice expressing enhanced green fluorescent protein (eGFP) in astrocytes and Discoma Red (DsRed) in pericytes revealed strong AQP4 signal in astrocytic processes adjacent to pericytes. Quantitative immunogold analysis of C57BL/6J mice showed that the AQP4 expression was higher in endfoot membranes abutting pericytes than in those facing endothelial cells. Similar findings were made for α-syntrophin, a member of the DAPC. The enrichment of α-syntrophin in membranes ensheathing pericytes persisted after *Aqp4* gene deletion.

Conclusion: Our data support the concept that pericytes are involved in the regulation of AQP4 polarization.

3.2 Paper II

Background: Our knowledge about the function of AQP4 has largely relied on the use of global *Aqp4* mutant mice, which do not allow conclusions on specific cellular and subcellular pools of AQP4.

Aims: The aims of Paper II were 1) to assess whether AQP4 is expressed in endothelial cells; 2) to investigate whether the astroglial pool of AQP4 controls water exchange across the blood-brain interface.

Methods: By using the *GFAP* promoter to drive gene excision we generated a glial-conditional *Aqp4* KO mouse line, which was then used for a battery of experiments. Immunofluorescence and immunogold quantitative electron microscopy analyses were performed on fixed cortical tissue sections from the glial-conditional *Aqp4* KO, the global *Aqp4* KO and WTs of C57BL/6J background. Whole-brain homogenates were blotted by gel electrophoresis from both KO models and compared. Brain water measurements were performed, as was a hypoosmotic stress model with injection of water i.p. with subsequent wet weight/dry weight measuring procedure. Evans Blue freeze lesion positive control experiments were performed to test the integrity of the BBB after gene deletion.

Results: Brains from glial-conditional *Aqp4* KOs were devoid of AQP4 as assessed by Western blots, ruling out the presence of a significant endothelial pool of AQP4. In agreement, quantitative analyses of ultrathin sections in electron microscope revealed no AQP4 signals in capillary endothelia. Compared with litter controls, glial-conditional *Aqp4* KO mice showed a 31% reduction in brain water uptake after systemic hypoosmotic stress and a delayed postnatal resorption of brain water. Deletion of astroglial *Aqp4* did not affect the barrier function to macromolecules.

Conclusion: We found no evidence for endothelial expression of AQP4 and conclude that glial AQP4 mediates drainage of brain water, as well as facilitates uptake of water, from blood. These conclusions were not confounded by altered vascular permeability to macromolecules. Taken together, the paper challenges the traditional concept of the BBB, and shows that the astrocytic covering of cerebral vessels is rate limiting to water movement.

3.3 Paper III

Background: We wanted to follow up on Paper II by looking further into the tissue- and cell-specific deletion of *Aqp4*. We generated an ependymal-specific *Aqp4* KO mouse model, and compared this model to the global *Aqp4* KO, to the loss-of-polarity model (α-syntrophin KO), and to WTs.

Aims: To investigate the exit routes of brain water and involvement of the different AQP4 pools in this process.

Methods: We measured brain water content by the wet weight/dry weight method on a novel ependymal-selective *Aqp4* KO mouse line, as well as on the global *Aqp4* KOs, the α-syntrophin KOs, and C57BL/6J WTs. We progressed with ICP measurements on the group that showed positive findings in brain water content and brain weight, i.e. the global *Aqp4* KOs, using C57BL/6J as a control group.

Results: We found that removal of AQP4 from ependymal cells did not affect basal brain water content, nor was the water content altered in α-syntrophin KO mice. We did, however, find that global deletion of *Aqp4* increased basal brain water content. This water accumulation was not due to extracerebral drainage problems, as global *Aqp4* KOs and WTs showed similar ICP responses to intracisternal fluid infusion.

Conclusion: Basal brain water content was increased in mice with global loss of AQP4, but not in mice with selective depletion of perivascular AQP4 or ependymal AQP4. Unique for the global *Aqp4* KO mice is the loss of the AQP4 pool subjacent to the pial membrane. By using basal brain water content as a proxy for the capacity of water exit in the mouse brain, we conclude that water accumulates in brain when subpial AQP4 is missing, pointing to a critical role of this pool of water channels in brain water drainage.

3.4 Paper IV

Background: Astrocytes are known to respond to neuronal activity by elevation of intracellular Ca^{2+}; however the kinetics and mechanisms of activity-evoked signals in astrocytic processes of mature mice are poorly understood. In this article, we performed

dual color 2-photon Ca^{2+} imaging of neurons and astrocytes in acute hippocampal slices during electrical stimulation.

Aims: The aims of Paper IV were 1) to determine the temporal relationship between Ca^{2+} signals in neurons and astrocytes during activation of CA3-CA1 glutamatergic synapses; 2) to reveal the source of the astrocytic Ca^{2+} signals; and 3) to determine the dynamics of extracellular glutamate levels following activation of the CA3-CA1 synapses.
Methods: C57BL/6J WTs, IP3R2 KOs and littermate control WTs were injected in the hippocampus bilaterally either with jRGECO1a and GCaMP6f Ca^{2+} indicators, or with iGluSnFR glutamate sensor under the GFAP promotor. Virus transduction was allowed to progress for two weeks, before the animal was sacrificed and the brain rapidly removed for preparation of acute hippocampal slices. The slices were allowed to recuperate for 1h, before two-photon laser scanning imaging was performed. Electrophysiological stimulation of neurons was performed simultaneously to evoke responses in astrocytes. To avoid induction of neuronal plasticity in the slices under stimulation paradigms, the NMDA antagonist DL-AP5 was added to the aCSF bath for the duration of the experiments.

Results: We report that the latency of astrocytic Ca^{2+} signals depended on the stimulation protocol, with shorter latencies during theta burst stimulation. Also, extracellular glutamate levels reached higher levels following theta bursts than after 20 Hz stimulation. The stimulation-evoked Ca^{2+} transients in all astrocytic compartments were prominently reduced in mice lacking the IP3R2 receptor.
Conclusion: We demonstrate that both the level of extracellular glutamate and the latency of the astrocytic Ca^{2+} signal depend on the intensity of neuronal activation, and that Ca^{2+} release from internal stores are the predominant source of astrocytic Ca^{2+} signals at activated CA3-CA1 synapses.

4. DISCUSSION

a. Impact of results

Recent studies have shown that astrocytes are a heterogeneous population of cells, across species, between brain regions, and that astrocytes in humans appear to be significantly more complex than in other model organisms, like rodents (Oberheim et al. 2009; Oberheim, Goldman and Nedergaard 2012). Also, it has come to light that when studying astrocytes, what occurs in the cell soma is not always representative for the whole cell, and that there is a need for studies of astrocytic microdomains (Tong et al. 2013) – like the endfeet. This thesis has provided new insight into the molecular and functional specialization of astrocytic endfeet, as discussed below.

Mechanisms underlying AQP4 polarization

It is well documented that AQP4 water channels are clustered in glial endfoot membranes around vessels. However, it is poorly understood how AQP4 polarization is induced and maintained. Could specific vascular cells regulate AQP4 anchoring to endfoot membranes? If so, it could be conjectured that endfoot membranes adjacent to the cell type in question exhibit particularly high levels of AQP4. To study mechanisms underlying AQP4 polarization, we in Paper I performed quantitative immunogold analysis of AQP4 expression in WT mice and found higher AQP4 density in endfoot membranes abutting pericytes than in those facing endothelial cells. Similar findings were made for α-syntrophin, and the clustering of α-syntrophin in the vicinity of pericytes persisted after *Aqp4* gene deletion. Thus, our study suggests that pericytes regulate AQP4 polarization through regulating the expression of the AQP4 anchoring molecule α-syntrophin. In line with our observation, Armulik and colleagues (2010) found that pericytes that were detached from endothelial cells showed intense lining with AQP4 and α-syntrophin immunostaining, indicating that pericytes express cues that mediate attachment of astrocytic endfeet. Also, gene array data

indicated down regulation of astrocyte markers in the pericyte-deficient brain microvasculature. The authors also found abnormal AQP4 distribution in pericyte-deficient mice; specifically, the perivascular AQP4 immunolabeling was weaker than in controls, indicating loss of AQP4 polarization to endfeet. Taken together, pericytes emerge as key regulators of AQP4 clustering in perivascular astrocytic endfeet. One could speculate that pial cells similarly regulate AQP4 expression in endfoot membranes along the cortical surface, but this has not yet been investigated.

Is there an endothelial pool of AQP4?

In order to assess the function of astroglial AQP4 by using mutant mouse models it is necessary to know the protein's cellular and subcellular distribution. To investigate AQP4's precise distribution at the blood-brain interface we constructed a novel glial-conditional c*Aqp4* KO mouse line (Paper II). The glial specificity of the KO was confirmed by immunoblots and immunohistochemistry, showing a lack of AQP4 immunoreactivity in brain, but not in other organs like muscles and kidneys. Earlier studies have indicated that endothelial cells may express AQP4 (Kobayashi et al. 2001; Amiry-Moghaddam, Frydenlund and Ottersen 2004), but as we show in Paper II, AQP4 is completely removed from brain when the GFAP promotor is used to drive gene excision. These findings indicate that AQP4 is exclusively expressed in astrocytes and ependymal cells - cells expressing the GFAP promoter. This finding is important as it would imply that altered brain function in global AQP4 mutants in most settings can be ascribed to perturbed function of astrocytes and ependymal cells. . Furthermore, it means that pharmaceutical compounds targeting AQP4 must be designed to cross the BBB and reach astrocytes and ependymal cell.

The finding that water uptake from blood during hypo-osmotic stress is slowed in mice lacking AQP4 in glial endfeet challenges the concept that transport is regulated at the level of endothelial cells. Our data indicate that astrocytic endfeet should be considered as a component of the BBB, as discussed below, being able to control water passage between blood and brain. The increased basal brain water content in mice lacking AQP4 suggests that

AQP4 is necessary for efficient clearance of brain water. Thus, loss of perivascular AQP4, as seen in Alzheimer's disease, stroke, traumatic brain injury and epilepsy (Yang et al. 2011; Steiner et al. 2012; Ren et al. 2013; Alvestad et al. 2013), could potentially be associated with brain water dyshomostasis.

Roles of distinct pools of AQP4

AQP4 is known to be anchored to the perivascular membrane by α-syntrophin, and deleting α-syntrophin has been shown to reduce AQP4 expression in the perivascular endfeet by 88% (Eilert-Olsen et al. 2012). To investigate the roles of the different pools of AQP4 on brain water homeostasis, we in Paper III employed different KO models; 1) the global *Aqp4* KO mouse model lacking AQP4 in all cells; 2) the α- syntrophin KO model, showing loss of AQP4 polarization to perivascular endfeet; and 3) a novel ependymal-specific KO model lacking AQP4 in the basolateral membrane domains of ependymal cells. The rationale for comparing these three KO models was that each of these three pools of AQP4 could be responsible for the effects observed in our glial-conditional *Aqp4* KO animals. Surprisingly, we found no change in basal brain water content in α- syntrophin KO mice. Neither did removal of AQP4 from ependymal membranes affect the brain water content. Taken together our data suggest that a large portion of brain water leave the brain across subpial endfoot membranes – containing a pool of AQP4 deleted only in the global *Aqp4* KO model.

Barrier properties of astrocytic endfeet

Astrocytes are considered to be important for inducing BBB properties in the endothelial cells (Janzer and Raff 1987), and for maintaining the integrity of the BBB. However, it has been shown that the BBB forms in the embryonic stage, before the formation of astrocytes, indicating that the functional barrier properties for other molecules than water actually establish in the neonatal stages (Daneman et al. 2010). Also, the integrity of the BBB is not compromised by deleting the perivascular pool of AQP4 (Eilert-Olsen et al 2012). However, it does affect the basal water content in the brain. Loss of AQP4 also leads

to widening of the extracellular space (Binder et al. 2004; Yao et al. 2008). Taken together, these findings indicate important physiological roles for AQP4 water channels; namely clearance of brain interstitial water and regulation of extracellular space volume.

Also, we show in Paper II that the increased brain water content first manifested itself around the time AQP4 expression starts, i.e. approximately postnatal day 15, and that it persisted throughout adulthood. These findings point to that a removal of AQP4 from endfeet gives rise to delayed water transport from blood to brain, but also from brain to blood/CSF.

Since astrocytic endfeet express AQP4, water can move bidirectionally through these processes (Nielsen et al. 1997). The finding in Paper II that endothelial cells are devoid of AQP4 suggests that other molecules mediate water transport across the the BBB. The glucose uniporter GLUT1 is likely involved, as it also conducts water and is abundantly expressed in both luminal and abluminal endothelial membranes (MacAulay and Zeuthen 2010. The intrinsic water permeability of CNS membranes that do not express AQPs remains unknown.

The animals that lack AQP4 have 1% higher basal brain water content (Paper II), without having a compromised BBB (Eilert-Olsen et al. 2012). Our finding in Paper III that the pressure profile following intracisternal ACSF injections is unaltered in *Aqp4* KO mice speaks against any involvement of AQP4 in drainage of CSF through arachnoid villi or any other efflux route downstream of the subarachnoidal space.

Several studies have shown that tracers that are injected intracortically or into the cisterna magna eventually ends up in cervical lymph nodes (Kida, Pantazis and Weller 1993; Plog et al. 2015), meaning that – one way or another – the tracer crosses over to the peripheral bloodstream. The exact mechanisms by which fluid clearance occurs are not known. The fact that the brain lacks a lymphatic system makes these questions even more intriguing, in particular since the brain is a highly metabolically active organ and thus in high need of an efficient waste removal system. Several studies have shown that brain fluid can move along the perivascular space (Virchow-Robin space) surrounding cerebral blood vessels (Szentistványi et al. 1984; Rennels, Blaumanis and Grady 1990; Abbott 2004), proposing that fluid enters the perivascular space along penetrating arteries from the cortical surface,

driven by arterial pulsations, and that the fluid enters either cells or the extracellular space, before being cleared through a gliovascular pathway to venules (Rennels et al. 1985; Kimelberg 2004). A recent paper from Iliff and colleagues (2012) supports these findings and implicates a role for AQP4 in paravascular fluid transport, CSF recycling and parenchymal solute clearance. The authors coined the drainage route for the *glymphatic pathway,* referring to its function analogue to that of lymphatic vessels and dependency on glial water channels. In fact, they show that *Aqp4* knockout mice exhibits a slowed CSF influx through this pathway, and a ~70% reduction in interstitial solute clearance, as well as a significantly reduced clearance of [^{3}H] mannitol (Iliff et al. 2012). However, there are conflicting data concerning this hypothesized pathway. In 1985, Rennels and colleagues showed that within 6 minutes of tracer infusion the entire microvasculature (arteries, capillaries and veins) was outlined by tracer. From the 1990s till present day, Weller and Carare with colleagues have – in several studies – provided data indicating that tracers exit the brain along the basement membranes of arteries Weller, Kida and Zhang 1992; Carare et al. 2008; Weller et al. 2008; Weller et al. 2009). Our finding in Paper III that intracisternal infusion of ACSF – 2 µl/min for 5 min; the same volume and speed as in Iliff et al., 2012 – temporary increases ICP could imply that the reported influx of CSF tracer along arteries is due to unphysiological pressures. Thus, more research is needed to fully understand paravascular fluid flow patterns and clearance routes for interstitial waste. Further, our claim in Paper III that a significant portion of water exits the brain across the pial surface, needs to be substantiated.

Astrocytic Ca^{2+} signals

Despite the growing realization of the importance of glial cells in brain signaling, we are still in the initial stages of understanding the physiological consequences of the intriguing bidirectional communication between glial cells and the brain´s blood vessels, and between glial cells and neurons.

Most of our knowledge on astrocytic Ca^{2+} signals derives from studies using cell cultures, brain slices or anesthetized (young) animals. The limitations of these techniques, as

well as the fact that young mice express a different subset of receptors than adult mice (Sun et al. 2013), raises the question what can we interpret from these data? What has emerged in later time is the definite need for studies that focus on the different microdomains of the astrocytes, which has become possible with the use of genetically encoded Ca^{2+} indicators. One of the proposed functions of astrocytic endfeet is that they are involved in or responsible for neurovascular coupling. It seems intuitively logical to assume there is some involvement of endfeet, as they are strategically located anatomically for such a function. However, any regulation of blood flow should be swift, to efficiently follow any change in physiological demand. The Ca^{2+} wave has been observed *in vitro* and *in vivo* to travel a lot slower than neuronal signals; 4-20 µm per sec, as opposed to 10-100 m per sec in axons (Hartline and Colman 2007). Conversely, one could argue that this fact implicates astrocytic Ca^{2+} signals to be significant for a modulatory effect over time and distance, which could be more relevant for neurovascular coupling. However, as we show in Paper IV with dual color imaging of activity-induced astrocytic Ca^{2+} signal latency, the kinetics of the astrocytic Ca^{2+} signals rely on the intensity of the neuronal stimulation, and can be significantly modified according to the strength of the input. This implies that the astrocytic Ca^{2+} signal potentially can have more impact in physiological settings.

Findings from several laboratories indicate that astrocytes only express IP3R2, and initially it was reported that knocking out IP3R2 completely obliterates the astrocytic Ca^{2+} responses (Petravicz, Fiacco and McCarthy 2008; Agulhon, Fiacco and McCarthy 2010; Agulhon et al. 2013). While the astrocyte Ca^{2+} signals appeared to have been completely abolished, the genetic manipulation had no effect on behavioral (Petravicz, Boyt and McCarthy 2014), neuronal (Petravicz, Fiacco and McCarthy 2008; Agulhon, Fiacco and McCarthy 2010) or vascular functions (Nizar et al. 2013; Takata et al. 2013; Bonder and McCarthy 2014) – leading investigators to conclude that astrocyte Ca^{2+} signals have no role in these functions. The preponderance of findings indicated that most, if not all, receptor dependent Ca^{2+} signals within astrocytes are due to release of Ca^{2+} through IP3 receptors in the endoplasmic reticulum (Petravicz, Boyt and McCarthy 2014). On the other hand, studies utilizing similar or complementary approaches have suggested that astrocyte Ca^{2+} signals are indeed involved in blood vessel and neuronal functions (Straub et al. 2006; Wang et al. 2012;

Srinivaran et al. 2015). Also, a general assumption has been that the loss of Ca^{2+} signals in the cell soma reflects a similar loss in astrocyte processes. However, when this was investigated by Srinivasan and colleagues (2015) using more sensitive tools than what has been available in earlier years, they found diverse types of Ca^{2+} signals in astrocytes, with most occurring in processes rather than in somata. These signals were preserved in IP3R2 knockout mice in brain slices and in vivo, and occurred in endfeet. These findings contribute to the current controversies in the field on the relevance of and the mechanisms behind Ca^{2+} signaling, and present a new form of Ca^{2+} signaling in astrocytes that are found to occur in the processes and that is, in fact, independent on IP3R2. An important take home message is that astrocytic Ca^{2+} signals are not necessarily uniform within the astrocytic territory but rather show variations within microdomains (Shigetomi et al. 2013).

In Paper IV, we investigated the Ca^{2+} signals in different astrocytic microdomains and found that they did indeed persist to some degree in the IP3R2 KO mice – also in perivascular endfeet, although the activity was reduced. This finding leads us to contemplate what other mechanisms are involved in astrocytic Ca^{2+} signaling, and if there are separate mechanisms present in the perivascular endfeet, like plasma membrane channels taking up Ca^{2+} from the extracellular space, which in turn leads to a Ca^{2+}-activated Ca^{2+} release. A previous study by Simard and colleagues in 2013 showed that neuronal stimulation can induce Ca^{2+} transients that spread along vessels. The involvement of astrocytic Ca^{2+} signals in neurovascular coupling was rejected after studies concluded that there were no Ca^{2+} signals in astrocytes after IP3R2 deletion. This has now been disproved in newer studies using more sensitive imaging tools, and there is a current hypothesis that Ca^{2+} signals in astrocytic endfeet most likely work through separate mechanisms than Ca^{2+} release in/near the soma - allowing us to revisit the hypothesis that these Ca^{2+} signals are involved in neurovascular modulation in the brain, perhaps even across a distance along the longitudinal axis of blood vessels. That being said, both vasodilatation and vasoconstriction as response mechanisms of astrocytic Ca^{2+} signals as controller of neurovascular coupling are under intense debate because different experimental paradigms and designs have generated contradictory results (Takano et al. 2006; Iadecola and Nedergaard 2007; Attwell et al. 2010). Others believe that pericytes are the cells that control cerebral blood flow (Hall et al. 2014). Another issue that

must be taken into consideration is that several anesthetizing agents show vasoconstricting or vasodilating properties, which in turn could have biased *in vivo* studies on blood flow regulation in anesthetized animals. Furthermore, astrocytic Ca^{2+} signals are severely suppressed by anasthetics (Thrane et al. 2012). Taking these findings into account, it seems the future lays in awake animal imaging as far as astrocytic Ca^{2+} signaling goes.

One most interesting question is whether activity-induced astrocytic Ca^{2+} signals – as those we demonstrate in Paper IV – are triggered by swelling and functionally coupled to volume regulation and brain water clearance. Notably, the delicate astrocytic processes around synapses have a large surface to volume ration and should easily swell during glutamate uptake. Another molecule that has to be cleared during neuronal activity is K^+. AQP4 is co-expressed with Kir4.1 – a potassium channel that is claimed to be involved in K^+ removal from the extracellular space during neuronal activity (Nagelhus et al. 1999). There is also evidence that this co-expression of AQP4 and Kir4.1 is due to a functional coupling, and that the two molecules are acting in concert by clearing activity-dependent K^+ fluxes accompanied by water (Amiry-Moghaddam et al. 2003[b]; Amiry-Moghaddam and Ottersen 2003). Also, it has been shown that adding K^+ to an astrocyte cell line transfected with AQP4 caused an immediate >40% increase in astrocyte water permeability, which sustained in 5 min (Song and Gunnarson 2012). When Kir-channels were blocked, however, the addition of K^+ also induced a Ca^{2+} increase in the astrocytes, and the water permeability increase no longer persisted. This is in line with earlier findings demonstrating that high neuronal activity is associated with shrinkage of the ECS, indicative of a redistribution of water – to which AQP4 is likely involved. Furthermore, a link has been created between brain swelling, astrocytic Ca^{2+} signals and AQP4, as shown by Thrane et al. in 2011. In a model for edema, they reported that brain swelling triggered Ca^{2+} signals in astrocytes, and that deleting the *Aqp4* gene led to a distinct reduction of these Ca^{2+} signals. Taken together, these data can be indicative of a role for astrocytic Ca^{2+} signals in volume regulation.

<u>AQP4 as a potential therapeutic target</u>

The opposing effects of AQP4 on the two types of brain edema described above make it difficult to predict whether AQP4 inhibiting drugs will in fact be beneficial or harmful in clinical settings of brain injury. Also, any attempt to inhibit or block AQP4 to counteract edema development must take into account possible side effects relating to the role of AQP4 in maintaining K^+ homeostasis (Amiry-Moghaddam and Ottersen 2003) and clearing waste, as described earlier. Given the limited knowledge on AQP4 regulation, targeting the molecules anchoring AQP4 to the plasma membrane might also be an approach for therapeutic modulation of water transport.

We lack efficient treatment strategies based on a molecular understanding of brain water transport. Modulation of AQP4 could therefore prove to be therapeutically useful, e.g. to avoid the invasive surgical procedures used today to release pressure in the brain caused by water accumulation intracranially. In any case, any potential drug modulating AQP4 function must be able to cross the BBB, as endothelial cells do not express AQP4 (Paper II).

b. Methodological considerations

The use of genetically modified animal models or transgenic models is a vast benefit when investigating the potential function of specific proteins and structures. The possibility of knocking out or in proteins permits researchers to study the isolated effect of the knockout/knockin. However, we need to keep in mind that the brain is a plastic organ, and there is a possibility that genetically modifying the DNA of an animal model might trigger some compensatory effects. Some genetic modulations, like deleting Kir4.1, are not compatible with life, leading to a lethargic phenotype. In some of these cases, or simply in the cases where it is not favorable to lack a protein of interest throughout development, it can be wise to make use of the conditional knockout strategy with cre recombinase to overcome any such issue. By adding a cell- or tissue-specific promotor to the gene construct, the genetic modification can be pinpointed to the exact region of interest in the model organism.

A limitation of the immunohistochemistry technique is its spatial resolution on the micrometer scale, and that it is a snapshot of the tissue at the time it was fixated. Further, tissue processing inevitably leads to a change in tissue morphology. Also, membrane proteins are of such small size that EM cannot visualize them unless they are labeled with gold particles. For this reason, the resolution of immunogold cytochemistry is determined by the size of the antibody complex employed. This also means that the gold particle is not in the exact same position as the protein of interest. Furthermore, antibodies are not able to penetrate the resin that is used to embed the EM tissue before cutting. This means that, in all cases, there will be a limited number of epitopes available for the primary antibodies.

The success of all immuno-protocols is determined by the sensitivity and specificity of the antibodies used, making it extremely important to run control experiments and take steps to ensure antibody specificity (or run parallel experiments with knockout samples). Moreover, great care must be taken when preparing your samples, as the ultrastructure and biological composition can be compromised during the course of the treatment. The use of antibodies requires thorough testing to ensure that there is no cross-reactivity or unspecific binding. The antibodies we have used in Paper I-IV have all been extensively tested in earlier projects, and because we also have knockout models we had tissue from these models as negative control experiments.

A limiting factor of the EB method for assessing BBB integrity is that not all BBB opening may be associated with increased permeability to macromolecules (such as albumin). In principle, several dyes should be employed in order to let the dye bind to macromolecules of different molecular sizes. A drawback with the HRP technique is that it is not a quantitative measure, meaning that findings are based on gross, macroscopic analyses.

An issue to take into consideration for the brain water content measurements is to carefully extract the brain quickly, as water starts evaporating immediately after it is removed from the skull. However, allowing a large number of samples in each group and doing statistics and calculations in order to catch any fluctuations that might arise from inconsistencies caused by methodology, can secure reproducibility.

Slices are easy to work with, and one great advantage is that electrodes can be placed in a precisely chosen spot under visual control (Andersen 1981), in our case stratum radiatum – allowing us to stimulate axons of the pyramidal neurons, to evoke a response in adjacent astrocytes. Under normal experimental circumstances, acute brain slices can live for approximately 8-15 hours post-dissection with the correct care (Andersen 1981; Buskila et al. 2014). They can also easily be manipulated, e.g. by drugs applied into the aCSF. In our slice experiments we added the NMDA receptor antagonist DL-AP5 (Tocris), by these means altering the external milieu and ensuring a block of any neuronal plasticity. These slices also allow for investigation of neuronal and astrocytic signals without the influence of an anesthetic agent present. Although this in vitro slice technique can be very useful in some studies, extrapolation of findings to an in vivo situation should be done with caution. There are also several points to be aware of with this technique. The oxygen concentration which is continuously added to the aCSF is unnaturally high at 95% compared to the atmosphere. There is also the disadvantage that the cells in the slices are isolated from blood flow, the BBB, physiological ICP and the circuitry of the intact brain. Furthermore, morphological changes can be induced during incubation, like swelling and retraction of fine astrocytic processes (Fiala et al. 2003). In spite of this, the use of acute hippocampal brain slices is a well-known and highly characterized experimental model, and constituted valuable for us in Paper IV.

All interventions on tissue can cause damage. This is a limiting factor when working with the 2PLSM technique *in vivo*; the surgical procedures are complex and takes time to master. Insufficiently executed preparations will have detrimental effects – not only on the imaging quality, but also on physiological processes (such as increased Ca^{2+} signaling in unhealthy tissue). The same goes for making slices; unhealthy slices will not exhibit normo-physiological processes. For the in vivo experiments, it is also of great importance that the animal is kept at physiological parameters throughout the course of the experiment. For instance can suboptimal ventilation lead to hypoxic condition, which can trigger a pathological signaling cascade in the brain, which is prone to damage induced by hypoxic conditions. We avoided this issue by placing the mice on a ventilator maintaining a steady

and controlled respiration. Another technical issue is that removing a piece of the skull can lead to herniation of the brain through the craniotomy. This could potentially be of greater risk to the *Aqp4* KO animals, which we provided evidence for exhibit megalocephaly as compared to wild types and littermate controls (Paper III). Such herniation can compress vessels near the edges of the craniotomy, leading to a stop in the blood flow of these vessels near the surface. A possible solution to this problem would be to perform a thinned skull preparation instead of a craniotomy. Also, any bleed or even heat from the drill can cause damage to the cortical tissue, and it is not always evident from gross inspection. Therefore, the surgical procedures should be done as elegantly and quickly as possible.

As for the viral vector injections, the number of transduced cells after injection is limited by volume constraints; for example can only a volume of 1-5 µl be injected into mouse parenchyma (Davidson and Breakefield 2003). Furthermore, the titer of the viral vector and the efficiency of the vectors infection are limiting factors. Therefore, the efficiency of transfection, and also the expression of the fluorescent indicators in the brain, will depend on the quality of the injection. The degree of successful transfection and accuracy of injection site can differ somewhat between experiments.

2PLSM is based on the principle that if a pulsed laser emits a high enough concentration of coherent, monochromatic photons, two or more photons may excite the same molecule simultaneously (see right panel figure 10). Since multiple photons are used to excite the target, each individual photon can have a relatively low energy, i.e. a longer wavelength (Denk et al. 1990; Kimelberg and Nedergaard 2010). This has several advantages compared to conventional confocal microscopy; longer wavelengths (800-1000 nm) achieve deeper tissue penetration (500-1000 um) and exert much less photo damage, causing the fluorophores to generate fewer free radicals when stimulated, and thereby producing less phototoxicity (Kimelberg and Nedergaard 2010). Finally, the transfer of light energy in 2PLSM is non-linear, meaning that photons will only excite the tissue in the focal point, not in the entire light cone. There are some issues to consider when it comes to the 2PLSM technique. The resolution limit of 2PLSM is theoretically close to one-half the wavelength of the exciting light (Kimelberg and Nedergaard 2010), meaning that 2PLSM currently cannot

resolve structures smaller than ~0.45 um, which is still quite large compared to the resolution limit for e.g. transmission EM (Nedergaard, Ransom and Goldman 2003). In practice the resolution limit is often much higher due to other limitations, such as optical aberrations caused by the tissue. Furthermore, due to the denseness of biological tissue, 2PLSM is usually unable to image deeper than ~500 um below the tissue surface. Maximum imaging depth is a factor of the wavelength used to excite the specimen, meaning that the laser sets the limit for how deep it is possible to image. Not being able to image below ~layer IV of the cortical surface naturally creates limitations to which areas of the brain and what biological processes one is able to visualize. An example of this is Paper IV, where we investigate Ca^{2+} signals in astrocytes in the Schaffer collaterals. Because of the location of the hippocampal formation, the imaging depth is too far from the surface of the brain for an imaging strategy in intact animals, leaving us with the slice experimental approach. Also, 2PLSM has a technical limit on its temporal resolution, because 2PLSM (like confocal microscopy) relies on the movement of galvanometric mirrors to raster scan the laser beam across the tissue. These mirrors are not able to move on a millisecond timescale, making the temporal resolution a lot slower than many of the biological processes it is supposed to image; like the iGluSnFR glutamate sensor we used in Paper IV.

c. Ethical considerations

There are always ethical issues that must be taken into strict consideration in animal science, especially when the experimental protocols in question include *in vivo* procedures. Several key agreements, including Nuremberg (1947) and Helsinki (1974), have highlighted the need for an ethical framework to regulate the conduct of medical research. All experiments referred to in this thesis have been executed in a manner that complies with Norwegian laws, and all protocols were approved by the Animal Care and Use Committee of the Institute of Basic Medical Sciences, University of Oslo, Norway. Every protocol has been designed after the 3R-principle, stating that all experimental protocols involving research animals must be established under these three guidelines; *replacement*, *refinement* and *reduction* (Russell and Burch 1959; Laboratory Animal Handling course, UiO). In order to

investigate hypotheses dealing with the regulation of blood flow, BBB integrity, changes in ICP, paravascular transport and brain waste clearance, it is detrimental to study these phenomena in a healthy brain situated in an intact system. Therefore, we were not able to substitute the mouse models with other techniques for our experiments (replacement). The ethical handling of the research animals is always prioritized, and is essential for obtaining trustworthy and for setting the standard for basic medical research. When designing the experiments, we always kept the groups of animals to a bare minimum where statistical analyses are possible (reduction). This was obtained by careful planning in the initial stages of designing the studies. Also, by injecting animals with viral vectors instead of breeding them in colonies, we avoided having to sacrifice a large number of mice that would have been surplus due to incorrect genotype. By doing our last project in slices (Paper IV), we were able to reduce the number of animals even further, as each brain gave us several slices, thereby allowing the slice number to increase the "n" without using additional animals. Finally, we put a lot of effort into refining our experiments. We designed an optimal anaesthesia/analgesia paradigm, and perfected the surgical and termination techniques, so that all experiments were done as fast as possible with as little stress as possible for the research animals. Also, every measure was taken to ensure physiological parameters throughout the course of the experiment – including the surroundings – preventing any loud noises, bright lights, unnatural temperatures etc. that could evoke a response in the animals.

d. Philosophy of science

My projects aimed to test the validity of the neurophysiological hypotheses as described above, through the collection of empirical observations and the analysis of these observations. All hypotheses were designed to be testable and falsifiable, in line with the concept of *critical rationalism* introduced by Karl Popper (Faludi 1983), thereby determining the projects as scientific studies. By collecting and analyzing these observations, I have interpreted these data into credible inductive theories, which served to falsify or validate the hypotheses I started out with.

e. Future perspectives

Our results demonstrate that astrocytes perform homeostatic and signaling functions in the brain, optimizing the conditions in the brain. However, in experimental design there are always limitations and challenges to overcome, making it important to always evaluate the employed techniques for a continuous improvement of experimental design. One likely improvement for future experiments will be to eliminate the use for anaesthesia, which needless to say has a grave impact on the brain and its dynamic environment. Therefore, any future experiments allowing for the use of awake animal models will probably make important contributions to our knowledge of astrocytic functions in health and disease. Also, since the α-syntrophin knockout model does not exhibit AQP4 expression in perivascular endfeet without having increased basal brain water content, this model seems to be preferential in projects where the aim is to study the gliovascular unit.

5. CONCLUDING REMARKS

This thesis provides evidence that pericytes are involved in the regulation of AQP4 anchoring in the perivascular astrocytic endfoot membrane, and that AQP4 expression is exclusive to astrocytes and ependymal cells in the brain. Taking into consideration the abovementioned and the fact that removing AQP4 from perivascular endfeet does not significantly affect basal brain water content, we propose that an important exit route for excess brain fluid is transmembrane drainage across the subpial astrocytic membranes into the CSF. Knocking out the ependymal pool of AQP4 does not affect the basal brain water content. Furthermore, we show that removing AQP4from the astrocytic perivascular endfoot membrane slows uptake of water from blood to brain, implicating that the astrocytic endfeet act as a barrier for entry of water at the BBB, and that the perivascular endfeet should be considered as an integrated component of the BBB.

The astrocytic Ca^{2+} signals are in large part reduced in mice lacking IP3R2. Thus, Ca^{2+} release from internal stores (i.e. the ER) are the predominant source of astrocytic Ca^{2+} signals following stimulation of the Schaffer collateral system. We also provide findings that indicate that the response of these Ca^{2+} signals depended on the stimulation protocol, being modulated more strongly, and perhaps relying upon other mechanisms, at stronger stimulation paradigms. These response patterns were also reported for extracellular glutamate levels, which reached higher extracellular levels following stronger neuronal stimulations. The functional significance of the astrocytic Ca^{2+} signals remains unknown. We do, however, speculate if these signals can be involved in volume regulation and volume homeostasis.

THE CANDIDATE´S EXPERIMENTAL CONTRIBUTION

Paper I: "Evidence that pericytes regulate aquaporin-4 polarization in mouse cortical astrocytes".

- breeding of double transgenic reporter mice
- fixated all tissue for further experiments
- planned and performed the light microscopic part of the study
- wrote corresponding parts of the manuscript

Paper II: "Glial-conditional deletion of aquaporin-4 (*Aqp4*) reduces blood-brain water uptake and confers barrier function on perivascular astrocyte endfeet".

- breeding of astrocyte-specific Aqp4 knockout mouse model
- planned and performed all Western blot experiments
- contributed to basal brain water measurements
- contributed to Evans Blue experiments
- prepared "Figure 1" and wrote corresponding parts of the manuscript

Paper III: "Basal brain water content is increased in mice with global deletion of aquaporin-4, but not in mice with a selective loss of perivascular or ependymal aquaporin-4".

- breeding of the Aqp4 KO, α-syntrophin KO and Aqp4 FOXJ1 cre transgenic mouse line
- planned and performed basal brain water measurements and brain weight experiments
- planned and performed the light microscopic part of the study
- prepared "Figure 1"
- wrote corresponding parts of the manuscript

Paper IV: "Origin of astrocytic Ca^{2+} signals at activated hippocampal CA3-CA1 synapses of adult mouse".

- breeding of the IP3R2 knockout mouse line
- planned and performed in vivo virus injection experiments
- performed two-photon imaging and electrophysiology brain slice experiments
- wrote parts of the manuscript

REFERENCE LIST

Abbott NJ. Evidence for bulk flow of brain interstitial fluid: significance for physiology and pathology. Neurochem Int. 2004 Sep;45(4):545-52

Abbott NJ. Blood-brain barrier structure and function and the challenges for CNS drug delivery. J Inherit Metab Dis. 2013 May;36(3):437-49

Abbott NJ, Rönnbäck L, Hansson E. Astrocyte–endothelial interactions at the blood–brain barrier. Nat Rev Neurosci. 2006 Jan;7:41-53

Agre P. Aquaporin water channels (Nobel Lecture). Angew Chem Int Ed Engl. 2004 Aug 20;43(33):4278-90

Agre P, King LS, Yasui M, Guggino WB, Ottersen OP, Fujiyoshi Y, Engel A, Nielsen S. Aquaporin water channels--from atomic structure to clinical medicine. J Physiol. 2002 Jul 1;542(Pt 1):3-16

Agulhon C, Fiacco TA, McCarthy KD. Hippocampal short- and long-term plasticity are not modulated by astrocyte Ca2+ signaling. Science. 2010 Mar 5;327(5970):1250-4.

Agulhon C, Boyt KM, Xie AX, Friocourt F, Roth BL, McCarthy KD. Modulation of the autonomic nervous system and behaviour by acute glial cell Gq protein-coupled receptor activation in vivo. J Physiol. 2013 Nov 15;591(Pt 22):5599-609

Akdogan I, Kiroglu Y, Onur S, Karabuluti N. The volume fraction of brain ventricles to total brain volume: a computed tomography stereological study. Folia Morphol (Warsz). 2010 Nov;69(4):193-200

Allen NJ, Barres BA. Neuroscience: Glia - more than just brain glue. Nature. 2009 Feb 5;457(7230):675-7

Alvestad S, Hammer J, Hoddevik EH, Skare Ø, Sonnewald U, Amiry-Moghaddam M, Ottersen OP. Mislocalization of AQP4 precedes chronic seizures in the kainate model of temporal lobe epilepsy. Epilepsy Res. 2013 Jul;105(1-2):30-41 . doi: 10.1016/j.eplepsyres.2013.01.006. Epub 2013 Jan 26.

Amiry-Moghaddam M, Frydenlund DS, Ottersen OP. Anchoring of aquaporin-4 in brain: molecular mechanisms and implications for the physiology and pathophysiology of water transport. Neuroscience. 2004 ;129(4):999-1010

Amiry-Moghaddam M, Otsuka T, Hurn PD, Traystman RJ, Haug FM, Froehner SC, Adams ME, Neely JD, Agre P, Ottersen OP, Bhardwaj A. An α-syntrophin-dependent pool of Aqp4 in astroglial end-feet confers bidirectional water flow between blood and brain. Proc. Natl Acad. Sci. USA 2003[a] 100, 2106–2111

Amiry-Moghaddam M, Ottersen OP. The molecular basis of water transport in the brain. Nat Rev Neurosci. 2003 Dec;4(12):991-1001

Amiry-Moghaddam M, Williamson A, Palomba M, Eid T, de Lanerolle NC, Nagelhus EA, Adams ME, Froehner SC, Agre P, Ottersen OP. Delayed K^+ clearance associated with aquaporin-4 mislocalization: phenotypic defects in brains of α-syntrophin-null mice. Proc. Natl Acad. Sci. USA 2003[b] 100, 13615–13620

Andersen, P. Brain slices – a neurobiological tool of increasing usefulness. Trends in Neurosciences. 1981 March 161:53-56

Araque A, Parpura V, Sanzgiri RP, Haydon PG. Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci. 1999 May;22(5):208-15

Armulik A, Genové G, Mäe M, Nisancioglu MH, Wallgard E, Niaudet C, He L, Norlin J, Lindblom P, Strittmatter K, Johansson BR, Betsholtz C. Pericytes regulate the blood-brain barrier. Nature. 2010 Nov 25;468(7323):557-61

Assentoft M, Larsen BR, MacAulay N. Regulation and Function of AQP4 in the Central Nervous System. Neurochem Res. 2015 Dec;40(12):2615-27

Attwell D, Buchan AM, Charpak S, Lauritzen M, Macvicar BA, Newman EA. Glial and neuronal control of brain blood flow. Nature. 2010 Nov 11;468(7321):232-43

Azevedo FA, Carvalho LR, Grinberg LT, Farfel JM, Ferretti RE, Leite RE, Jacob Filho W, Lent R, Herculano-Houzel S. Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. J Comp Neurol. 2009 Apr 10;513(5):532-41

Bergersen LH, Morland C, Ormel L, Rinholm JE, Larsson M, Wold JF, Røe AT, Stranna A, Santello M, Bouvier D, Ottersen OP, Volterra A, Gundersen V. Immunogold detection of L-glutamate and D-serine in small synaptic-like microvesicles in adult hippocampal astrocytes. Cereb Cortex. 2012 Jul;22(7):1690-7

Bignami A, Asher R, Perides G. Brain extracellular matrix and nerve regeneration. Adv Exp Med Biol. 1991 296:197-206

Binder DK, Papadopoulos MC, Haggie PM, Verkman AS. In vivo measurement of brain extracellular space diffusion by cortical surface photobleaching. J Neurosci. 2004 Sep 15;24(37):8049-56

Binder DK, Yao X, Zador Z, Sick TJ, Verkman AS, Manley GT. Increased seizure duration and slowed potassium kinetics in mice lacking aquaporin-4 water channels. Glia. 2006 Apr 15;53(6):631-6

Bockamp E, Sprengel R, Eshkind L, Lehmann T, Braun JM, Emmrich F, Hengstler JG. Conditional transgenic mouse models: from the basics to genome-wide sets of knockouts and current studies of tissue regeneration. Regen Med. 2008 Mar;3(2):217-35

Bonder DE, McCarthy KD. Astrocytic Gq-GPCR-linked IP3R-dependent Ca2+ signaling does not mediate neurovascular coupling in mouse visual cortex in vivo. J Neurosci. 2014 Sep 24;34(39):13139-50

Buskila Y, Breen PP, Tapson J, van Schaik A, Barton M, Morley JW. Extending the viability of acute brain slices. Sci Rep. 2014 Jun;16(4):5309, 1-7

Carare RO, Bernardes-Silva M, Newman TA, Page AM, Nicoll JA, Perry VH, Weller RO. Solutes, but not cells, drain from the brain parenchyma along basement membranes of capillaries and arteries: significance for cerebral amyloid angiopathy and neuroimmunology. Neuropathol Appl Neurobiol. 2008 Apr;34(2):131-44

Cardoso FL, Brites D, Brito MA. Looking at the blood-brain barrier: molecular anatomy and possible investigation approaches. Brain Res Rev. 2010 Sep 24;64(2):328-63

Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science. 1990 Jan 26;247(4941):470-3

Cornell-Bell AH, Thomas PG, Caffrey JM. Ca2+ and filopodial responses to glutamate in cultured astrocytes and neurons. Can J Physiol Pharmacol. 1992;70 Suppl:S206-18

Coulter DA, Steinhäuser C. Role of astrocytes in epilepsy. Cold Spring Harb Perspect Med. 2015 Mar 2;5(3):a022434

Dalkara T, Gursoy-Ozdemir Y, Yemisci M. Brain microvascular pericytes in health and disease. Acta Neuropathol. 2011 Jul;122(1):1-9

Danbolt NC, Chaudhry FA, Dehnes Y, Lehre KP, Levy LM, Ullensvang K, Storm-Mathisen J. Properties and localization of glutamate transporters. Prog Brain Res. 1998;116:23-43

Daneman R, Zhou L, Kebede AA, Barres BA. Pericytes are required for blood-brain barrier integrity during embryogenesis. Nature. 2010 Nov 25;468(7323):562-6

Dani JW, Chernjavsky A, Smith SJ. Neuronal activity triggers calcium waves in hippocampal astrocyte networks. Neuron. 1992 Mar;8(3):429-40

Davidson BL, Breakefield XO. Viral vectors for gene delivery to the nervous system. Nat Rev Neurosci. 2003 May;4(5):353-64

Denk W, Strickler JH, Webb WW**.** Two-photon laser scanning fluorescence microscopy. Science. 1990 Apr 6;248(4951):73-6

Dietzel I, Heinemann U, Hofmeier G, Lux HD. Transient changes in the size of the extracellular space in the sensorimotor cortex of cats in relation to stimulus-induced changes in potassium concentration. Exp Brain Res. 1980;40(4):432-9

Ding F, O'Donnell J, Thrane AS, Zeppenfeld D, Kang H, Xie L, Wang F, Nedergaard M. α1-Adrenergic receptors mediate coordinated Ca2+ signaling of cortical astrocytes in awake, behaving mice. Cell Calcium. 2013 Dec;54(6):387-94

Eilert-Olsen M, Haj-Yasein NN, Vindedal GF, Enger R, Gundersen GA, Hoddevik EH, Petersen PH, Haug FM, Skare Ø, Adams ME, Froehner SC, Burkhardt JM, Thoren AE, Nagelhus EA. Deletion of aquaporin-4 changes the perivascular glial protein scaffold without disrupting the brain endothelial barrier. Glia. 2012 Mar;60(3):432-40

Faludi A. Critical Rationalism and Planning Methodology. Urban Studies. 1983 20;2:265-78

Fiala JC, Kirov SA, Feinberg MD, Petrak LJ, George P, Goddard CA, Harris KM. Timing of neuronal and glial ultrastructure disruption during brain slice preparation and recovery in vitro. J Comp Neurol. 2003 Oct 6;465(1):90-103

Frigeri A, Gropper MA, Turck CW, Verkman AS. Immunolocalization of the mercurial-insensitive water channel and glycerol intrinsic protein in epithelial cell plasma membranes. Proc Natl Acad Sci U S A. 1995 May 9;92(10):4328-31

Frigeri A, Gropper MA, Umenishi F, Kawashima M, Brown D, Verkman AS. Localization of MIWC and GLIP water channel homologs in neuromuscular, epithelial and glandular tissues. J Cell Sci. 1995 Sep;108 (Pt 9):2993-3002

Frigeri A, Nicchia GP, Nico B, Quondamatteo F, Herken R, Roncali L, Svelto M. Aquaporin-4 deficiency in skeletal muscle and brain of dystrophic mdx mice. FASEB J. 2001 Jan;15(1):90-98

Gundersen V, Storm-Mathisen J, Bergersen LH. Neuroglial Transmission. Physiol Rev. 2015 Jul;95(3):695-726

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

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

Haj-Yasein NN, Vindedal GF, Eilert-Olsen M, Gundersen GA, Skare O, Laake P, Klungland A, Thoren AE, Burkhardt JM, Ottersen OP, Nagelhus EA. Glial-conditional deletion of aquaporin-4 (Aqp4) reduces blood-brain water uptake and confers barrier function on perivascular astrocyte endfeet. Proc Natl Acad Sci USA. 2011 Oct;108(43):17815-20

Haj-Yasein NN, Jensen V, Østby I, Omholt SW, Voipio J, Kaila K, Ottersen OP, Hvalby Ø, Nagelhus EA. Aquaporin-4 regulates extracellular space volume dynamics during high-frequency synaptic stimulation: a gene deletion study in mouse hippocampus. Glia. 2012 May;60(6):867-74. doi: 10.1002/glia.22319. Epub 2012 Mar 14.

Haj-Yasein NN, Bugge CE, Jensen V, Østby I, Ottersen OP, Hvalby Ø, Nagelhus EA. Deletion of aquaporin-4 increases extracellular K(+) concentration during synaptic stimulation in mouse hippocampus. Brain Struct Funct. 2015 Jul;220(4):2469-74

Hall CN, Reynell C, Gesslein B, Hamilton NB, Mishra A, Sutherland BA, O'Farrell FM, Buchan AM, Lauritzen M, Attwell D. Capillary pericytes regulate cerebral blood flow in health and disease. Nature. 2014 Apr 3;508(7494):55-60

Hasegawa H, Ma T, Skach W, Matthay MA, Verkman AS. Molecular cloning of a mercurial-insensitive water channel expressed in selected water-transporting tissues. J Biol Chem. 1994 Feb 25;269(8):5497-500

Hawkins BT, Davis TP. The blood-brain barrier/neurovascular unit in health and disease. Pharmacol Rev. 2005 Jun;57(2):173-85

Haydon PG, Carmignoto G. Astrocyte control of synaptic transmission and neurovascular coupling. Physiol Rev. 2006 Jul;86(3):1009-31

Iadecola C, Nedergaard M. Glial regulation of the cerebral microvasculature. Nat Neurosci. 2007 Nov;10(11):1369-76

Ikeda Y, Wang M, Nakazawa S. Simple quantitative evaluation of blood-brain barrier disruption in vasogenic brain edema. Acta Neurochir Suppl (Wien). 1994;60:119-20

Iliff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, Benveniste H, Vates GE, Deane R, Goldman SA, Nagelhus EA, Nedergaard M. A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid β. Sci Transl Med. 2012 Aug 15;4(147):1-11

Janzer RC, Raff MC. Astrocytes induce blood-brain barrier properties in endothelial cells. Nature. 1987 Jan 15-21;325(6101):253-7

Jin BJ, Zhang H, Binder DK, Verkman AS. Aquaporin-4-dependent K(+) and water transport modeled in brain extracellular space following neuroexcitation. J Gen Physiol. 2013 Jan;141(1):119-32

Johansson I, Karlsson M, Johanson U, Larsson C, Kjellbom P. The role of aquaporins in cellular and whole plant water balance. Biochim Biophys Acta. 2000 May 1;1465(1-2):324-42

Jung JS, Bhat RV, Preston GM, Guggino WB, Baraban JM, Agre P. Molecular characterization of an aquaporin cDNA from brain: candidate osmoreceptor and regulator of water balance. Proc Natl Acad Sci USA. 1994 Dec 20;91(26):13052-6

Kawamoto S, Goto H, Watanabe M, Shimada M. GLUT-1 glucose transporter is localized heterogeneously within the microvessels of the mouse hippocampus. Acta Histochem. 1995 Jan;97(1):105-11.

Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. Physiology of microglia. Physiol Rev. 2011 Apr;91(2):461-553

Kida S, Pantazis A, Weller RO. CSF drains directly from the subarachnoid space into nasal lymphatics in the rat. Anatomy, histology and immunological significance. Neuropathol Appl Neurobiol. 1993 Dec;19(6):480-8

Kimelberg HK. Water homeostasis in the brain: basic concepts. Neuroscience. 2004;129(4):851-60

Kimelberg HK, Nedergaard M. Functions of astrocytes and their potential as therapeutic targets. Neurotherapeutics. 2010;7(4):338-53

Kobayashi H, Minami S, Itoh S, Shiraishi S, Yokoo H, Yanagita T, Uezono Y, Mohri M, Wada A. Aquaporin subtypes in rat cerebral microvessels. Neurosci Lett. 2001 Jan 19;297(3):163-6

Krueger M, Bechmann I. CNS pericytes: concepts, misconceptions, and a way out. Glia. 2010 Jan 1;58(1):1-10

Kofuji P, Newman EA. Potassium buffering in the central nervous system. Neuroscience. 2004;129(4):1045-56

Lehmenkühler A, Syková E, Svoboda J, Zilles K, Nicholson C. Extracellular space parameters in the rat neocortex and subcortical white matter during postnatal development determined by diffusion analysis. Neuroscience. 1993 Jul;55(2):339-51

Li G, Yuan W, Fu BM. A model for the blood-brain barrier permeability to water and small solutes. J Biomech. 2010 Aug 10;43(11):2133-40

Lumeng CN, Hauser M, Brown V, Chamberlain JS. Expression of the 71 kDa dystrophin isoform (Dp71) evaluated by gene targeting. Brain Res. 1999 May 29;830(1):174-8

MacAulay N, Hamann S, Zeuthen T. Water transport in the brain: role of cotransporters. Neurosci. 2004;129(4):1031-44

MacAulay N, Zeuthen T. Water transport between CNS compartments: contributions of aquaporins and cotransporters. Neuroscience. 2010 Jul 28;168(4):941-56

Manley GT, Fujimura M, Ma T, Noshita N, Filiz F, Bollen AW, Chan P, Verkman AS. Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke. Nat Med. 2000 Feb;6(2):159-63

Matyash V, Kettenmann H. Heterogeneity in astrocyte morphology and physiology. Brain Res Rev. 2010 May;63(1-2):2-10

Muntoni F, Torelli S, Ferlini A. Dystrophin and mutations: one gene, several proteins, multiple phenotypes. Lancet Neurol. 2003 Dec;2(12):731-40

Nagelhus EA, Horio Y, Inanobe A, Fujita A, Haug FM, Nielsen S, Kurachi Y, Ottersen OP. Immunogold evidence suggests that coupling of K+ siphoning and water transport in rat retinal Müller cells is mediated by a coenrichment of Kir4.1 and AQP4 in specific membrane domains. Glia. 1999 Mar;26(1):47-54

Nagelhus EA, Mathiisen TM, Ottersen OP. Aquaporin-4 in the central nervous system: cellular and subcellular distribution and coexpression with KIR4.1. Neuroscience. 2004;129(4):905-13

Nagelhus EA, Ottersen OP. Physiological roles of aquaporin-4 in brain. Physiol Rev. 2013 Oct;93(4):1543-62

Nagelhus EA, Veruki ML, Torp R, Haug FM, Laake JH, Nielsen S, Agre P, Ottersen OP. Aquaporin-4 water channel protein in the rat retina and optic nerve: polarized expression in Müller cells and fibrous astrocytes. J Neurosci. 1998 Apr 1;18(7):2506-19

Nase G, Helm PJ, Enger R, Ottersen OP. Water entry into astrocytes during brain edema formation. Glia. 2008 56:895–902

Nedergaard M, Ransom B, Goldman SA. New roles for astrocytes: redefining the functional architecture of the brain. Trends in neurosciences. 2003;26(10):523-30

Neely JD, Amiry-Moghaddam M, Ottersen OP, Froehner SC, Agre P, Adams ME. Syntrophin-dependent expression and localization of Aquaporin-4 water channel protein. Proc Natl Acad Sci USA. 2001 Nov 20;98(24):14108-13

Nico B, Paola Nicchia G, Frigeri A, Corsi P, Mangieri D, Ribatti D, Svelto M, Roncali L. Altered blood-brain barrier development in dystrophic MDX mice. Neuroscience. 2004;125(4):921-35.

Nielsen S, Nagelhus EA, Amiry-Moghaddam M, Bourque C, Agre P, Ottersen OP. Specialized membrane domains for water transport in glial cells: high-resolution immunogold cytochemistry of aquaporin-4 in rat brain. J Neurosci. 1997 Jan 1;17(1):171-80

Nielsen S, Smith BL, Christensen EI, Agre P. Distribution of the aquaporin CHIP in secretory and resorptive epithelia and capillary endothelia. Proc Natl Acad Sci U S A. 1993 Aug 1;90(15):7275-9

Nizar K, Uhlirova H, Tian P, Saisan PA, Cheng Q, Reznichenko L, Weldy KL, Steed TC, Sridhar VB, MacDonald CL, Cui J, Gratiy SL, Sakadzić S, Boas DA,Beka TI, Einevoll GT, Chen J, Masliah E, Dale AM, Silva GA, Devor A. In vivo stimulus-induced vasodilation occurs without IP3 receptor activation and may precede astrocytic calcium increase. J Neurosci. 2013 May 8;33(19):8411-22

Oberheim NA, Goldman SA, Nedergaard M. Heterogeneity of astrocytic form and function. Methods Mol Biol. 2012;814:23-45

Oberheim NA, Takano T, Han X, He W, Lin JH, Wang F, Xu Q, Wyatt JD, Pilcher W, Ojemann JG, Ransom BR, Goldman SA, Nedergaard M. Uniquely hominid features of adult human astrocytes. J Neurosci. 2009 Mar 11;29(10):3276-87

O'Brien ER, Howarth C, Sibson NR. The role of astrocytes in CNS tumors: pre-clinical models and novel imaging approaches. Front Cell Neurosci. 2013 Apr 16;7:40

Oshio K, Watanabe H, Song Y, Verkman AS, Manley GT. Reduced cerebrospinal fluid production and intracranial pressure in mice lacking choroid plexus water channel Aquaporin-1. FASEB J. 2005 Jan;19(1):76-8

Ottersen OP, Helm PJ. How hardwired is the brain? Nature. 2002 Dec 19-26;420(6917):751-2

Papadopoulos MC, Manley GT, Krishna S, Verkman AS. Aquaporin-4 facilitates reabsorption of excess fluid in vasogenic brain edema. FASEB J. 2004 Aug;18(11):1291-3

Papadopoulos MC, Verkman AS. Aquaporin-4 and brain edema. Pediatr Nephrol. 2007 Jun;22(6):778-84

Pardridge WM, Boado RJ, Farrell CR. Brain-type glucose transporter (GLUT-1) is selectively localized to the blood-brain barrier. Studies with quantitative western blotting and in situ hybridization. J Biol Chem. 1990 Oct 15;265(29):18035-40.

Paredes RM, Etzler JC, Watts LT, Zheng W, Lechleiter JD. Chemical calcium indicators. Methods. 2008 Nov;46(3):143-51

Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG. Glutamate-mediated astrocyte-neuron signalling. Nature. 1994 Jun 30;369(6483):744-7

Parpura V, Verkhratsky A. Homeostatic function of astrocytes: Ca(2+) and Na(+) signalling. Transl Neurosci. 2012 [a] Dec;3(4):334-344

Parpura V, Verkhratsky A. Astrocytes revisited: concise historic outlook on glutamate homeostasis and signaling. Croat Med J. 2012 [b] Dec;53(6):518-28

Pekny M, Pekna M, Messing A, Steinhäuser C, Lee JM, Parpura V, Hol EM, Sofroniew MV, Verkhratsky A. Astrocytes: a central element in neurological diseases. Acta Neuropathol. 2016 Mar;131(3):323-45

Petravicz J, Fiacco TA, McCarthy KD. Loss of IP3 receptor-dependent Ca2+ increases in hippocampal astrocytes does not affect baseline CA1 pyramidal neuron synaptic activity. J Neurosci. 2008 May 7;28(19):4967-73

Petravicz J, Boyt KM, McCarthy KD. Astrocyte IP3R2-dependent Ca(2+) signaling is not a major modulator of neuronal pathways governing behavior. Front Behav Neurosci. 2014 Nov 12;8:384

Phatnani H, Maniatis T. Astrocytes in Neurodegenerative Disease. Cold Spring Harb Perspect Biol. 2015 Apr 15. [Epub ahead of print]

Plog BA, Dashnaw ML, Hitomi E, Peng W, Liao Y, Lou N, Deane R, Nedergaard M. Biomarkers of traumatic injury are transported from brain to blood via the glymphatic system. J Neurosci. 2015 Jan 14;35(2):518-26

Porter JT, McCarthy KD. Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. J Neurosci. 1996 Aug 15;16(16):5073-81

Preston GM, Carroll TP, Guggion WB, Agre P. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science. 1992 Apr;256(5055):385-7

Reeves A, Shigetomi E, Khakh ES. Bulk loading of calcium indicator dyes to study astrocyte physiology: key limitations and improvements using morphological maps. J Neurosci. 2011 Jun 22; 31(25): 9353–9358.

Ren Z, Iliff JJ, Yang L, Yang J, Chen X, Chen MJ, Giese RN, Wang B, Shi X, Nedergaard M. 'Hit & Run' model of closed-skull traumatic brain injury (TBI) reveals complex patterns of post-traumatic AQP4 dysregulation. J Cereb Blood Flow Metab. 2013 Jun;33(6):834-45

Rennels ML, Blaumanis OR, Grady PA. Rapid solute transport throughout the brain via paravascular fluid pathways. Adv Neurol. 1990;52:431-9

Rennels ML, Gregory TF, Blaumanis OR, Fujimoto K, Grady PA. Evidence for a 'paravascular' fluid circulation in the mammalian central nervous system, provided by the rapid distribution of tracer protein throughout the brain from the subarachnoid space. Brain Res. 1985 Feb 4;326(1):47-63

Risher WC, Andrew RD, Kirov SA. Real-time passive volume responses of astrocytes to acute osmotic and ischemic stress in cortical slices and in vivo revealed by two-photon microscopy. Glia. 2009 57:207–221

Russell WMS, Burch RL. The Principles of Humane Experimental Technique. 1959. Johns Hopkins University: USA

Santoni V, Vinh J, Pflieger D, Sommerer N, Maurel C. A proteomic study reveals novel insights into the diversity of aquaporin forms expressed in the plasma membrane of plant roots. Biochem J. 2003 Jul 1;373(Pt 1):289-96

Saadoun S, Tait MJ, Reza A, Davies DC, Bell BA, Verkman AS, Papadopoulos MC. AQP4 gene deletion in mice does not alter blood-brain barrier integrity or brain morphology. Neuroscience. 2009 Jul 7;161(3):764-72

Santello M, Calì C, Bezzi P. Gliotransmission and the tripartite synapse. Adv Exp Med Biol. 2012;970:307-31

Sá-Pereira I, Brites D, Brito MA. Neurovascular unit: a focus on pericytes. Mol Neurobiol. 2012 Apr;45(2):327-47

Scemes E, Giaume C. Astrocyte calcium waves: what they are and what they do. Glia. 2006 Nov 15;54(7):716-25

Seifert G, Schilling K, Steinhäuser C. Astrocyte dysfunction in neurological disorders: a molecular perspective. Nat Rev Neurosci. 2006 Mar;7(3):194-206

Shigetomi E, Bushong EA, Haustein MD, Tong X, Jackson-Weaver O, Kracun S, Xu J, Sofroniew MV, Ellisman MH, Khakh BS. Imaging calcium microdomains within the entire astrocyte territories and endfeet with GCaMPs expressed using adeno-associated viruses. J Gen Physiol. 2013 May;141(5):633-47

Simard M, Arcuino G, Takano T, Liu QS, Nedergaard M. Signaling at the gliovascular interface. J Neurosci. 2003 Oct 8;23(27):9254-62

Simard M, Nedergaard M. The neurobiology of glia in the context of water and ion homeostasis. Neuroscience. 2004;129(4):877-96

Song Y, Gunnarson E. Potassium dependent regulation of astrocyte water permeability is mediated by cAMP signaling. PLoS One. 2012;7(4):e34936

Srinivasan R, Huang BS, Venugopal S, Johnston AD, Chai H, Zeng H, Golshani P, Khakh BS. Ca2+ signaling in astrocytes from Ip3r2-/- mice in brain slices and during startle responses in vivo. Nat Neurosci. 2015 Apr 20. doi: 10.1038/nn.4001. [Epub ahead of print]

Steiner E, Enzmann GU, Lin S, Ghavampour S, Hannocks MJ, Zuber B, Rüegg MA, Sorokin L, Engelhardt B. Loss of astrocyte polarization upon transient focal brain ischemia as a possible mechanism to counteract early edema formation. Glia. 2012 Nov;60(11):1646-59. doi: 10.1002/glia.22383. Epub 2012 Jul 10.

Straub SV, Bonev AD, Wilkerson MK, Nelson MT. Dynamic inositol trisphosphate-mediated calcium signals within astrocytic endfeet underlie vasodilation of cerebral arterioles. J Gen Physiol. 2006 Dec;128(6):659-69

Sun W, McConnell E, Pare JF, Xu Q, Chen M, Peng W, Lovatt D, Han X, Smith Y, Nedergaard M. Glutamate-dependent neuroglial calcium signaling differs between young and adult brain. Science. 2013 Jan 11;339(6116):197-200

Syková E, Nicholson C. Diffusion in brain extracellular space. Physiol Rev. 2008 Oct;88(4):1277-340

Szentistványi I, Patlak CS, Ellis RA, Cserr HF. Drainage of interstitial fluid from different regions of rat brain. Am J Physiol. 1984 Jun;246(6 Pt 2):F835-44

Tait MJ**,** Saadoun S**,** Bell BA**,** Papadopoulos MC. Water movements in the brain: role of aquaporins**.** Trends in Neurosciences. 2008 Jan. 31(1), 37-43

Takano T, Tian GF, Peng W, Lou N, Libionka W, Han X, Nedergaard M. Astrocyte-mediated control of cerebral blood flow. Nat Neurosci. 2006 Feb;9(2):260-7

Takata N, Nagai T, Ozawa K, Oe Y, Mikoshiba K, Hirase H. Cerebral blood flow modulation by Basal forebrain or whisker stimulation can occur independently of large cytosolic Ca2+ signaling in astrocytes. PLoS One. 2013 Jun 13;8(6):e66525

Thrane AS, Rangroo Thrane V, Zeppenfeld D, Lou N, Xu Q, Nagelhus EA, Nedergaard M. General anesthesia selectively disrupts astrocyte calcium signaling in the awake mouse cortex. Proc Natl Acad Sci U S A. 2012 Nov 13;109(46):18974-9

Thrane AS, Rappold PM, Fujita T, Torres A, Bekar LK, Takano T, Peng W, Wang F, Thrane VR, Enger R, Haj-Yasein NN, Skare Ø, Holen T, Klungland A, Ottersen OP, Nedergaard M, Nagelhus EA. Critical role of aquaporin-4 (AQP4) in astrocytic Ca2+ signaling events elicited by cerebral edema. Proc Natl Acad Sci USA. 2011 Jan 11;108(2):846-51

Tong X, Shigetomi E, Looger LL, Khakh BS. Genetically encoded calcium indicators and astrocyte calcium microdomains. Neuroscientist. 2013 Jun;19(3):274-91

Verkhratsky A, Butt A. Glial Neurobiology. 2007. John Wiley & Sons Ltd: England

Verkhratsky A, Rodríguez JJ, Parpura V. Calcium signalling in astroglia. Mol Cell Endocrinol. 2012 Apr 28;353(1-2):45-56

Verkhratsky A, Steardo L, Parpura V, Montana V. Translational potential of astrocytes in brain disorders. Prog Neurobiol. 2015 Sep 16. pii: S0301-0082(15)30026-5. doi: 10.1016/j.pneurobio.2015.09.003. [Epub ahead of print]

Vise WM, Liss L, Yashon D, Hunt WE. Astrocytic processes: A route between vessels and neurons followingblood-brain barrier injurt. JNEN. 1975;34(4):324-34

Weller RO, Kida S, Zhang ET. Pathways of fluid drainage from the brain-morphological aspects and immunological significance in rat and man. Brain Pathol. 1992 Oct;2(4):277-84

Weller RO, Subash M, Preston SD, Mazanti I, Carare RO. Perivascular drainage of amyloid-beta peptides from the brain and its failure in cerebral amyloid angiopathy and Alzheimer's disease. Brain Pathol. 2008 Apr;18(2):253-66

Weller RO, Djuanda E, Yow HY, Carare RO. Lymphatic drainage of the brain and the pathophysiology of neurological disease. Acta Neuropathol. 2009 Jan;117(1):1-14

Winkler EA, Bell RD, Zlokovic BV. Central nervous system pericytes in health and disease. Nat Neurosci. 2011 Oct 26;14(11):1398-405

Wolburg H, Noell S, Mack A, Wolburg-Buchholz K, Fallier-Becker P. Brain endothelial cells and the glio-vascular complex. Cell Tissue Res. 2009[a] Jan;335(1):75-96

Wolburg H, Noell S, Wolburg-Buchholz K, Mack A, Fallier-Becker P. Agrin, aquaporin-4, and astrocyte polarity as an important feature of the blood-brain barrier. Neuroscientist. 2009[b] Apr;15(2):180-93

Yang J, Lunde LK, Nuntagij P, Oguchi T, Camassa LM, Nilsson LN, Lannfelt L, Xu Y, Amiry-Moghaddam M, Ottersen OP, Torp R. Loss of astrocyte polarization in the tg-ArcSwe mouse model of Alzheimer's disease. J Alzheimers Dis. 2011;27(4):711-22. doi: 10.3233/JAD-2011-110725.

Yao X, Hrabetová S, Nicholson C, Manley GT. Aquaporin-4-deficient mice have increased extracellular space without tortuosity change. J Neurosci. 2008 May 21;28(21):5460-4

Zeuthen T, MacAulay N. Passive water transport in biological pores. Int Rev Cytol. 2002[a];215:203-30

Zeuthen T, MacAulay N. Cotransporters as molecular water pumps. Int Rev Cytol. 2002[b];215:259-84

Zhang H, Verkman AS. Aquaporin-4 independent Kir4.1 K+ channel function in brain glial cells. Mol Cell Neurosci. 2008 Jan;37(1):1-10

Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keeffe S, Phatnani HP, Guarnieri P, Caneda C, Ruderisch N, Deng S, Liddelow SA,Zhang C, Daneman R, Maniatis T, Barres BA, Wu JQ. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. J Neurosci. 2014 Sep 3;34(36):11929-47

Zhou J, Kong H, Hua X, Xiao M, Ding J, Hu G. Altered blood-brain barrier integrity in adult aquaporin-4 knockout mice. Neuroreport. 2008 Jan 8;19(1):1-5