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Acute and chronic changes of the brain tissue and the extracellular space
diffusion parameters in models of brain ischemia - the role of volume
regulation of glia and AQP4/TRPV4 complex

Akutní a chronické změny mozkové tkáně a difúzních parametrů
extracelulárního prostoru v modelech ischemie – úloha objemové regulace
glie a AQP4/TRPV4 komplexu

Dissertation Thesis

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Acute and chronic changes of the brain tissue and the extracellular space diffusion parameters in models of brain ischemia - the role of volume regulation of glia and AQP4/TRPV4 complex

ABSTRACT

The interaction of aquaporin 4 (AQP4) and transient receptor potential vanilloid isoform 4 (TRPV4) channels is crucial for volume regulation *in vitro*, but it remains unclear *in situ* or *in vivo*. We used the real-time iontophoretic method (RTI) *in situ* or *in vivo* to detect the extracellular space volume fraction (α) and tortuosity (λ), in several models of cytotoxic edema/ischemia, such as hypoosmotic stress, hyperkalemia, oxygen-glucose deprivation (OGD) and terminal ischemia. Magnetic resonance imaging was used to quantify the brain lesions one day (D1) and seven days (D7) after permanent middle cerebral artery occlusion (pMCAO). The results from mice lacking AQP4, TRPV4 or both channels (AQP4^{-/-}, TRPV4^{-/-}, AQP4^{-/-}/TRPV4^{-/-}, respectively) were compared with controls (Ctrl). Hypoosmotic stress or hyperkalemia did not evoke different responses between Ctrl and transgenic strains. During OGD, the α decrease was reduced in TRPV4^{-/-} and absent in AQP4^{-/-}/TRPV4^{-/-} mice. AQP4 or TRPV4 deficit slows down the α decrease during acute terminal ischemia *in vivo*. One day after pMCAO, the deficit of AQP4 or TRPV4 exacerbates the tissue damage, while the deletion of both channels mitigates it; this difference disappeared at D7. We conclude that the AQP4/TRPV4 complex plays a crucial role in acute ischemic swelling. However, dissimilar responses to ischemia induction in different transgenic strains or ischemic models indicate that both channels can also collaborate with other effector proteins.

Keywords

AQP4, cerebral ischemia, cytotoxic edema, diffusion parameters, extracellular space, TRPV4

Akutní a chronické změny mozkové tkáně a difúzních parametrů extracelulárního prostoru v modelech ischemie – úloha objemové regulace glie a AQP4/TRPV4 komplexu

ABSTRAKT

Interakce mezi aquaporinem 4 (AQP4) a kanály s přechodným receptorovým potenciálem vaniloidní izoformy 4 (TRPV4) je zásadní pro regulaci objemu *in vitro*, ale zůstává nejasná *in situ* nebo *in vivo*. Iontoforetickou metodu v reálném čase (RTI) *in situ* a *in vivo* jsme měřili hodnoty objemové frakce extracelulárního prostoru (α) a tortuozity (λ) v modelech cytotoxického edému/ischemie, jako je hypoosmotický stres, hyperkalémie, kyslíkovo-glukózová deprivace (OGD) a terminální ischemie. Magnetickou rezonancí jsme kvantifikovali rozsah mozkových lézí den (D1) a sedm dní (D7) po trvalé okluzi střední cerebrální arterie (pMCAO). Výsledky získané u myši deficitních pro AQP4, TRPV4 nebo oba proteiny (AQP4^{-/-}, TRPV4^{-/-}, AQP4^{-/-}/TRPV4^{-/-}, v tomto pořadí) jsme srovnávali s odpovědí kontrolních myši (Ctrl). Hypoosmotický stres a hyperkalémie neodhalily rozdílné odpovědi mezi Ctrl a transgenními kmeny. Během OGD byl pokles α u TRPV4^{-/-} myši menší a zcela chyběl u AQP4^{-/-}/TRPV4^{-/-} myši. Deficit AQP4 nebo TRPV4 zpomalil rychlost vzniku edému během terminální ischemie *in vivo*. Poškození tkáně den po pMCAO bylo u zvířat deficitních pro AQP4 nebo TRPV4 horší, ale delece obou proteinů vedla naopak k lepšímu výsledku; tento rozdíl není v D7. Uzavíráme, že AQP4/TRPV4 komplex hraje klíčovou roli v akutním ischemickém edému buněk, avšak odlišné reakce na indukci ischemie v různých transgenních kmenech nebo ischemických modelech ukazují, že oba kanály mohou spolupracovat i s jinými efektorovými proteiny.

Klíčová slova

AQP4, cerebrální ischemie, cytotoxický edém, difúzní parametry, extracelulární prostor, TRPV4

LIST OF ABBREVIATIONS

[Ca²⁺]_i intracellular calcium concentration

[K⁺]_e extracellular potassium concentration

[Na⁺]_i intracellular sodium concentration

2VO two-vessel occlusion

AA arachidonic acid

aCSF artificial cerebrospinal fluid

ADC apparent diffusion coefficient

ADC_w apparent diffusion coefficient of water

ADP adenosine diphosphate

ALDH1L1 folate enzyme aldehyde dehydrogenase 1 family member L1

AMPA α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor

AQP aquaporin

ASIC acid-sensing ion channels

ATP adenosine triphosphate

ATPase ATP hydrolase

BBB blood-brain barrier

BDNF brain-derived neurotrophic factor

BKCa calcium-activated big conductance potassium channels

Bral1 brain link protein 1

CCAL common carotid artery ligation

CCAO common carotid artery occlusion

ClC voltage-dependent chloride ion channel

CNS central nervous system

CSF cerebrospinal fluid

CSPGs chondroitin sulfate proteoglycans

Ctrl control

Ctrl1 cartilage link protein-1

Cx43 connexin 43

DAG diacylglycerol

DNA deoxyribonucleic acid

DW-MRI diffusion weighted magnetic resonance imaging

EAA excitatory amino acids

EAAT excitatory amino-acid transporter

ECM extracellular matrix

ECS extracellular space

EET epoxyeicosatrienoic acid

EGFP enhanced green fluorescent protein

ER endoplasmic reticulum

FOV field of view

GABA γ -aminobutyric acid

GAT γ -aminobutyric acid transporter

GFAP glial fibrillary acidic protein

GLAST glutamate transporter

GLT-1 glutamate transporter-1

GLUT glucose transporter

H-100 hypotonic solution 200 mOsmol/l

H-50 hypotonic solution 250 mOsmol/l

Hapln hyaluronan and proteoglycan link protein

i.p. intraperitoneal injection

Iba1 ionized calcium binding adaptor molecule 1

IKCa calcium-activated intermediate conductance potassium channels

IOI integrative optical imaging

IOS intrinsic optical signalling

IP3R inositol trisphosphate receptor

ISF interstitial fluid

ISMs ion-selective microelectrodes

KCa calcium-activated potassium channels

KCC chloride potassium co-transporter

Kir inwardly rectifying potassium channels

LP link protein

MCA middle cerebral artery

MCAO middle cerebral artery occlusion

MCT monocarboxylate transporter

MDMs magnetic dipole moments

mGluR metabotropic glutamate receptor

miRNA micro ribonucleic acid

MMP matrix metalloproteinase

MR magnetic resonance

MRI magnetic resonance imaging

mRNA messenger ribonucleic acid

mtDNA mitochondrial deoxyribonucleic acid

NA number of acquisitions

NC_{Ca-ATP} nonselective cation channel activated by ATP

NF- κ B nuclear factor kappa-light-chain-enhancer of activated B cells

NG2 neuronal-glial antigen 2

NGF nerve growth factor

NHE sodium–proton exchanger

Na⁺/K⁺-ATPase sodium–potassium ATP hydrolase (sodium–potassium pump)

NKCC Na⁺–K⁺–2Cl[–] cotransporter

NMDA N-methyl-D-aspartate

NO nitric oxide

NOS nitric oxide synthase

NVC neurovascular coupling

OAPs orthogonal arrays of particles

OGD oxygen-glucose deprivation

OPCs oligodendrocyte precursor cells

P2Y purinergic G protein-coupled receptor

PBS phosphate buffered saline

PCR polymerase chain reaction

PDGF- β platelet-derived growth factor- β

PDZ post synaptic density protein

pHe extracellular pH

pHi intracellular pH

PKC protein kinase C

PLA2 phospholipase A2

PLC phospholipase C

pMCAO permanent middle cerebral artery occlusion

PNNs perineuronal nets

RA reactive astrocytes

RARE rapid acquisition with a relaxation enhancement

RF radiofrequency

RNA ribonucleic acid

RNS reactive nitrogen species

ROS reactive oxygen species

RT room temperature

RTI real-time iontophoretic method

RT-VT roamer type of volume transmission

RVD regulatory volume decrease

RVI regulatory cell volume increase

RyR ryanodine receptor

SEM standard error of the mean

SGLT sodium-glucose transport protein

SKCa calcium-activated small conductance potassium channels

SNARE soluble N-ethylmaleimide-sensitive factor attachment protein receptor

STAT3 signal transducer and activator of transcription 3

STED stimulated emission depletion

SUR1 sulfonylurea receptor 1

SUSHI super-resolution shadow imaging

SWCNT single-walled carbon nanotubes

TE echo-time

TEA tetraethylammonium

TGF- β transforming growth factor- β

TM transmembrane

TMA tetramethylammonium

TN tenascin

TNF α tumor necrosis factor alpha

TNT tunnelling nanotube

TR repetition time

TREK TWIK-related potassium channel

TR-FAIM time-resolved fluorescence anisotropy imaging

TRP transient receptor potential channel

TRPA transient receptor potential ankyrin channel

TRPC transient receptor potential canonical channel

TRPM transient receptor potential melastatin channel

TRPML transient receptor potential mucolipin channel

TRPN transient receptor potential no mechanoreceptor potential C channel

TRPP transient receptor potential polycystin channel

TRPV transient receptor potential vanilloid channel

TTC 2,3,5-Triphenyltetrazolium Chloride

TWIK two-pore domain weak inwardly rectifying potassium channel

VEGF vascular endothelial growth factor

VRAC volume-regulated anion channel

WFA wisteria floribunda agglutinin

α -syn α -syntrophin

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1 INTRODUCTION

The brain is a complex tissue that contains both cellular and extracellular compartments. The extracellular space (ECS) provides a microenvironment consisting of interstitial fluid (ISF), ions, and secreted (or membrane-bound) molecules (Hrabetova et al., 2018). This microenvironment is indispensable for proper cell homeostasis and cellular communication (Agnati et al., 2006; Colbourn et al., 2019). The ECS is a dynamic compartment that changes significantly in pathophysiological conditions; however, its changes are also observed during physiological brain activity or sleep.

Brain cells are represented by neurons and glial cells, where each cell type has an irreplaceable role in physiological brain functions. Neurons are essential for precise and rapid communication over long distances. Glial cells play a more supporting role, creating a microenvironment that enables the proper activity of neurons. Different types of glial cells perform specialised functions essential for brain health. Oligodendrocytes are primarily responsible for myelination, ensuring efficient neuronal signal transmission, while microglia serve as the brain's immune defenders, responding to injury and clearing debris. Astrocytes, however, have a broader scope of activity, which includes, among others, the regulation of neuronal transmission, the maintenance of homeostasis, and the control of brain water balance. Their role in water and volume regulation is particularly critical, as astrocytes may actively adjust their cell volume to maintain extracellular fluid balance. This is achieved through various membrane proteins, such as aquaporins and ion channels, which facilitate the movement of water and ions across the cell membrane, allowing astrocytes to respond dynamically to changes in the brain's microenvironment. However, during pathological conditions, these mechanisms may cease to be sufficient, leading to the development of cytotoxic edema (Ho et al., 2012).

The development of cytotoxic and/or vasogenic edema resulting from ion and water shifts between brain compartments is one of the main features of ischemic injury. Although it is generally accepted that astrocytes play a major role in cytotoxic brain edema formation (Liang et al., 2007), it was demonstrated that neurons also markedly change their volume in response to brain ischemia (Hellas and Andrew, 2021; Steffensen et al., 2015). Changes in cell volume during their swelling are compensated for by a reciprocal change of the ECS volume, leading to a higher concentration of potentially neurotoxic substances and increased intracranial pressure. The formation of cerebral edema is a major cause of death in stroke patients (Yao et al., 2021). Unfortunately, current therapies focus on preventing the

consequences of elevated intracranial pressure rather than targeting specific molecular pathways that would help prevent the formation of edema (Yao et al., 2021). Therefore, studying the molecular mechanisms of the development of cytotoxic edema is crucial for the development of novel therapeutics.

1.1 Extracellular space

In brain tissue, the ECS occupies about 20 % of tissue in healthy adult animals (Nicholson and Sykova, 1998). The ECS contains ISF represented by a water solution of ions, gaseous molecules, and organic molecules, such as proteins, peptides, enzymes, or the floating chains of glycoproteins attached to the extracellular matrix (ECM). The source of ISF is still being investigated. Some studies have implied that it originates from cerebrospinal fluid (CSF), cell metabolism, and the vascular system (from the blood-brain barrier)(Ashok and Gabriele, 2020). Two patterns have been proposed for ISF outflow, direct ISF-CSF exchange (mainly through the glymphatic system into the subarachnoid space) and ISF outflow through the intramural periarterial drainage pathway (Kaur et al., 2021; Maloveska et al., 2022).

In addition to being an intimate cellular microenvironment, the ECS plays a significant role in communication between cells and information processing/integration (Lei et al., 2017). There are several types of intercellular communication, including the most precise and fastest type of cell communication, synaptic transmission. This type of neuronal communication depends on the presynaptic excitation, resulting in the opening of Ca^{2+} channels and release of mediator-loaded vesicles into the narrow synaptic cleft. The mediator molecules diffuse and reach their receptors on the postsynaptic membrane. The presence of astrocytic processes in the vicinity of the synapse modulates neuronal activity by uptake or release (Sancho et al., 2021; Schousboe, 2019). Some studies introduced a model of tripartite synapse, which incorporates astrocytic processes alongside pre- and postsynaptic element (Araque et al., 1999), while others even added the ECM as the fourth element and introduced a tetrapartite model of synapse (Bosiacki et al., 2019; Dityatev and Rusakov, 2011). Another well-studied form of intracellular transmission is communication via gap junctions that are found in some electrical synapses and in astrocytes (explained in detail in Chapter 1.4)(Eugenin et al., 2012). Interestingly, cells can form cytoskeletal protrusions, called tunnelling nanotubes (TNTs), which carry portions of the plasma membrane (smaller TNTs) or vesicles and organelles (larger TNTs) between cells (Agnati and Fuxe, 2014). Together,

these types of communication are presented as wired transmission (Marcoli et al., 2015)(Fig.1).

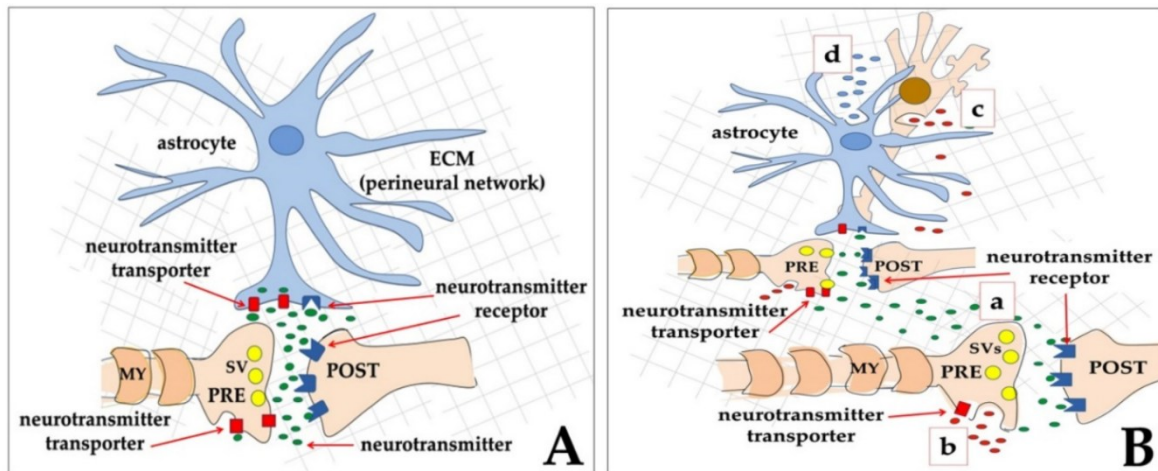


Fig. 1 Schematic drawing of two types of neurotransmission. (A) In the case of synaptic transmission, the presynaptic neuron (PRE) releases neurotransmitters (small green ovals) that bind to their receptors (blue) on the post-synaptic element (POST) or the astrocyte endfeet. Reuptake of neurotransmitter occurs on the PRE or astrocytic membrane, through specific transporters (red rectangles); (B) Examples of volume transmission are represented: (a), the PRE releases a neurotransmitters (small green ovals) that not only bind to its receptors (blue) on the POST, but also diffuses at different distances, thus reaching other synapses; (b) extrasynaptic release of signalling molecules (small red ovals) from the axon outside the synaptic cleft; (c) extrasynaptic release of signal molecules (small red ovals) from the soma of a neuron; (d) gliotransmitter release from an astrocyte (small blue ovals). From Schiera et al. (2019).

Expect from wired transmission, additional type of intercellular communication is represented by a volume (or extrasynaptic) transmission (Fig.1), which is mediated via the diffusion of neuroactive substances in non-defined pathway through the ECS and is much slower as compared to wired (synaptic) transmission (Agnati et al., 2006, 2010; Marcoli et al., 2015). Volume transmission plays an important role, especially in communication between independent synapses (“synaptic crosstalk”) and in modification of synaptic final output. In the case of synapses, that are poorly ensheathed by glial processes and the ECM (“open” synapses), the neurotransmitter may escape synaptic cleft in the process called “synaptic spillover” and then diffuses away from its release site and affects the neighbouring neurons via its interaction with the extrasynaptic high-affinity receptors (Kullmann et al., 1996). Synaptic spillover modulates processes such as long-term potentiation/depression, lactation, or the formation of fear memories (Asztely et al., 1997; Badia-Soteras et al., 2023;

Piet et al., 2004). On the contrary, ‘private’ synapses that are tightly ensheathed by glial processes and ECM molecules that form perineuronal nets, diminish the spill of neurotransmitters that intersynaptic communication (Sulzer and Pothos, 2000).

The source of ions or neuroactive molecules in the ECS can be represented by a synaptic or extrasynaptic release from vesicles, reverse action of several transporters, or release of gaseous molecules such as nitric oxide (NO)(Zoli et al., 1999). Volume transmission not only mediates the dialogue between different neurons or between neurons and glial cells, but also allows brain-body crosstalk, an extensive bidirectional communication between the nervous and the immune cells (Agnati et al., 2010; Agnati and Fuxe, 2014; Cossetti et al., 2012).

In addition, it has been shown that the ECS also contains extracellular vesicles transporting packages of signaling molecules such as proteins, ribonucleic acids (RNA), lipids, metabolites, etc., to target cells (Cossetti et al., 2012; Schiera et al., 2019; Zappulli et al., 2016). Micro-vesicle migration is named roamer type of volume transmission (RT-VT) to distinguish it from the migration of chemical and physical signals (‘classical volume transmission’)(Agnati et al., 2014). Plasma membrane receptors (Canonico et al., 2012; Guescini et al., 2012; Luchetti et al., 2012), messenger RNA (mRNA)(Skog et al., 2008), microRNA (miRNA)(Valadi et al., 2007), deoxyribonucleic acid (DNA)(Balaj et al., 2011), and mitochondrial DNA (mtDNA)(Guescini et al., 2010) can be sent via micro-vesicles and diffuse through the ECS (Agnati et al., 2010; Raposo and Stoorvogel, 2013).

1.2 Extracellular matrix

The ECM is a collection of secreted or membrane-bound macromolecules that are present within the ECS (Fig.2). During development, the ECM plays a role, for example, in the proliferation and differentiation of neuronal progenitors, dendritic and axonal growth and guidance, migration, and synaptic plasticity (Soles et al., 2023). Additionally, reactive astrocytes excessively produce ECM molecules after spinal cord and brain injury (Fitch and Silver, 2008). Due to the high abundance of negative charge within these macromolecules, the ECM has the ability to harbour ions, secreted molecules (such as growth factors), and neuromodulatory agents (Dityatev and Schachner, 2006; Hrabetova et al., 2009). Due to its high hydration capacity, it maintains the volume affecting the normal brain activity (Tewari et al., 2022).

The macromolecules of the ECM are represented by several molecular groups or families:

- Glycosaminoglycans - the most abundant glycosaminoglycan in the tissues is hyaluronan. Several transmembrane and membrane-coupled are connected directly with the hyaluronan to anchor and stabilise the ECM (Frischknecht and Seidenbecher, 2008).
- Link proteins - the family of hyaluronan and proteoglycan link proteins (Hapln) has four distinct members: Crtl1 (Hapln1), Bral1 (Hapln2), Bral2 (Hapln4), and Lp3 (Hapln3). Link proteins are a key molecule in the formation and stabilisation of the condensed perineuronal matrix in the adult brain (Spicer et al., 2003; Oohashi et al., 2015).
- Proteoglycans - the heterogeneity of proteoglycans is based on differential expression and splicing of genes encoding core proteins as well as variations in the length and types of associated glycosaminoglycan chains. In the CNS, most of the proteoglycans carry either chondroitin or heparin sulfate side chains (Bandtlow and Zimmermann, 2000). Important chondroitin sulfate containing proteoglycans are, for example, lecticans, like aggrecan, versican, neurocan and brevican (Yamaguchi, 2000).
- Glycoproteins - the most abundant ECM glycoproteins in the CNS are tenascins. In the brain tissue, three types of tenascins (TN-C, TN-R, and TN-Y) have been identified (Joester and Faissner, 2001). TN-C is expressed mainly during early development, while, in adulthood, it is located in areas that exhibit neuronal plasticity, such as the hippocampus or hypothalamus (Tucic et al., 2021).

Fibrillary proteins such as collagen and elastin are located sparsely in the CNS, for example, in walls of blood vessels, meninges, and basal membranes (Rauch, 2004).

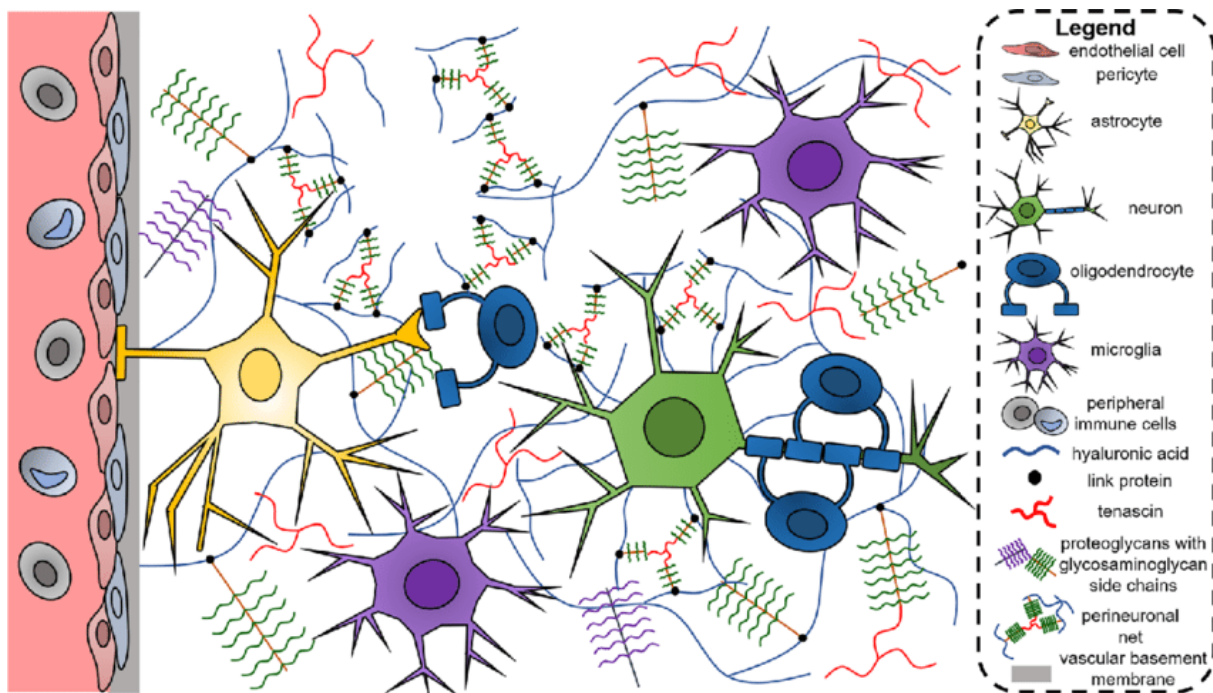


Fig. 2 The brain extracellular matrix. Hyaluronic acid can bind to chondroitin sulfate proteoglycans via link proteins and along with tenascins form perineuronal nets on the surfaces of neurons. The ECM, neurons and glial cells (e.g., astrocytes, oligodendrocytes, microglia) in the ECS are supported by basal membrane-surrounded blood vessels composed of vascular cells (e.g., endothelial cells and pericytes). The image depicts the astrocytic endfeet contacting blood vessels, with circulating peripheral immune cells contacting the myelinated neuron. From Ngo and Harley (2021).

The ECM molecules could form a condensed structure, such as perineuronal/perinodal nets or axonal coats (Oohashi et al., 2002; Bekku et al., 2003), in whose lecticans are linked to hyaluronan by their N-terminal domains, while the attachment is further stabilised by Hapln proteins. The C-terminal of lecticans can mediate binding to other ECM proteins such as tenascins (Morawski et al., 2014; Suttkus et al., 2014). The presence of these complex ECM structures around neurons decreases synaptic plasticity, but also leads to an advantageous increase of the signal-to-noise ratio, or neuroprotection (Massey et al., 2006; Cabungcal et al., 2013; Fawcett et al., 2019). Perineuronal nets (PNNs) can be visualised by fluorescently labelled antibodies that bind to the core proteins or by lectins such as Wisteria floribunda agglutinin (WFA) that bind to the glycosaminoglycan side chains (Tewari and Sontheimer, 2019).

The composition of ECM is plastic and is maintained in a ‘steady state’ through a continuous production of ECM and proteases in neurons and glial cells, as well as through direct microglial uptake (Tewari et al., 2022). In several brain pathologies, such as traumatic brain injury, multiple sclerosis, and glioma, reactive astrocytes alter ECM expression levels of ECM as well as the matrix remodelling proteases. Consequently, the overall ECM is upregulated; however, PNNs are disrupted or lost (Tewari et al., 2022). The enzymes degrading the ECM include various families of proteases such as matrix metalloproteinases (MMPs), disintegrins and metalloproteases (ADAMs) or ADAMs with thrombospondin motif (ADAMTS), hyaluronidases/chondroitinases, and plasmin. MMP proteolytic activity is further controlled by specific protein inhibitors, the tissue inhibitors of metalloproteinases (TIMP)(Cabral-Pacheco et al., 2020). MMPs are involved in several physiological and pathophysiological processes, including angiogenesis, neurogenesis, axonal growth and regeneration, CNS injury, tumour metastasis, and neuroinflammation (Soles et al., 2023) and can be released by neurons, astrocytes, microglia, and endothelial cells (Tewari et al., 2022). MMP2 and MMP9 levels, which contribute to PNNs degradation increase significantly within hours after cerebral ischemia (Jickling et al., 2014). Degradation of PNNs can lead to reduced levels of growth factors, loss of neuroprotection, as well as perturbed expression of neurotransmitter receptors and ion channels that cause maladaptive neuroplasticity (Tewari et al., 2022).

1.3 Diffusion within the extracellular space

The diffusion is the primary mechanism governing the movement of ions and molecules through the ECS. In aqueous medium, small particles move randomly along their concentration gradient by Brownian motion as they collide with the water molecules surrounding them (Cecconi et al., 2005). This so-called free diffusion can be observed in water or diluted agar, where the diffusion of substances is described by Fick’s laws. In contrast, diffusion in brain is limited to the volume of the ECS and hindered by various diffusion barriers. In order to describe the diffusion within the ECS more accurately, the original Fick’s diffusion equations had been modified by introduction of following diffusion parameters (Nicholson and Phillips, 1981; Nicholson and Sykova, 1998)(see Chapter 3.1.1):

1. The extracellular volume fraction (α ; α = ECS volume/total tissue volume) represents the volume available for the diffusion of particles in the extracellular compartment. The ECS volume fraction in the healthy adult brain equals about 0.20-0.25, meaning that about 20-25% of brain tissue is occupied by the ECS.

2. Tortuosity (λ) represents the effect that is imposed by the diffusion barriers, which hinder the diffusion of ions and molecules (Tonnesen et al., 2023). It is a dimensionless parameter defined as $\lambda = (D/ADC)^{0.5}$, where D is the free diffusion coefficient and ADC is the apparent diffusion coefficient in the brain. Diffusion barriers arise mainly from:

- a) Geometry of the tissue structure together with viscosity of the ISF causing an additional delay for the diffusing ions and molecules in the ECS (Arizono et al., 2021; Paviolo et al., 2020)
- b) Dead-space microdomains that form between individual layers of myelin sheaths and at local enlargements of the ECS. In such microdomains, diffusing ions and molecules are being trapped at least for a while (Xiao et al., 2015)
- c) Specific binding sites on ECM cell membranes or on molecules (Thorne et al., 2008)
- d) Fixed negative charges present on the molecules of the ECM that affect the diffusion of charged molecules (Arranz et al., 2014; Hrabetova et al., 2009)

Due to diffusion barriers, the velocity of diffusion within the ECS in an adult healthy brain is approximately 2.5 times slower compared to a free medium which corresponds to a tortuosity value of about 1.5-1.6. Moreover, in certain brain regions, the values of tortuosity vary along different axes (Figley et al., 2022; Vorisek and Sykova, 1997a). This phenomenon is called diffusion anisotropy, and it is mainly observed in the white matter because of the parallel orientation of myelinated axon fibres. However, anisotropic diffusion can also be present in a subcortical grey matter within areas with a distinct organization of fine astrocytic processes, such as the cerebellum and the hippocampus (Mazel et al., 1998; Rice et al., 1993).

3. Non-specific uptake, k' , represents a loss of the diffusing substance into the cell, across the blood-brain barrier (BBB) or into the blood vessels and is concentration dependent. However, the diffusion of specific molecules (e.g., glutamate) is also affected by active transport across membranes. Substances with negligible k' value, e.g., tetramethylammonium ion (TMA^+), may be used as extracellular markers.

The volume of the ECS inversely reflects changes in the cell volume, which may alternate during various timescales, ranging from hours, as in the case of sleep-wake cycle (Xie et al., 2013), to seconds in the case of epilepsy (Colbourn et al., 2021). Circadian volume change rhythm facilitates metabolite clearance during sleep as part of the glymphatic system (Hablitz et al., 2020; Nedergaard, 2013; Xie et al., 2013). Importantly, an increase in cell water content leads to swelling of brain tissue and, due to a fixed volume of the skull, it can increase intracranial pressure (Wilson and Mongin, 2018).

Long-term variations of the ECS diffusion parameters can be also affected by a prolonged loss or development of additional diffusion barriers, which result, for example, from axonal myelination, plastic rearrangement of the tissue geometry, or from alterations in the ECM content in various physiological or pathological conditions. These changes include the loss of the typical organisation of astrocytic processes in the hippocampus during ageing (Sykova et al., 2002) or structural changes of astrocytes in the hypothalamus during lactation (Piet et al., 2004), remodelling of the ECM in tumorous (Verkman, 2013; Zamecnik et al., 2004) or epileptogenic tissue (Zamecnik et al., 2012) and after ischemia (Anderova et al., 2011) influence diffusion in the ECS.

1.3.1 Methods used for diffusion measurements

Several techniques were implemented to date to determine geometry and diffusion properties of the ECS. Measurements of tissue impedance (Van Harreveld et al., 1971) and radiotracer methods (Fenstermacher and Kaye, 1988) belong to the first techniques utilised for detection of the ECS properties. The basis of the tissue impedance method is the high electrical resistance of the cell membranes compared to that of the extracellular medium. Using low-frequency rectangular current pulses, the measurements are recorded as a voltage drop across the series of electrodes (Matsuoka and Hossmann, 1982). The radiotracer method involves a compound with atom/s replaced by a radionuclide, which is injected into the ventricle and over time spreads into adjacent brain regions and could be detected after a chosen time in the fixed brain (Bohr et al., 2022). With the development of microscopy, magnetic resonance imaging, and other technologies, researchers can choose from a wide array of experimental techniques. The aim of the following list is to present an overview of the available techniques that allow researchers to determine the brain ECS properties, highlighting their individual strengths and weaknesses (comprehensive information can be found in Soria et al., 2020a):

1) Point-source techniques

a) Real-time iontophoretic method (RTI)

The RTI method implements an ion-selective microelectrode (ISM) to measure the concentration of the extracellular marker (usually TMA^+) that has spread after being locally introduced into the tissue via a glass capillary by real-time iontophoresis (RTI). The pair of electrodes is calibrated in aqueous agar prior to use. By a non-linear fitting of the obtained experimental diffusion curve, the ECS volume fraction, as well as tissue tortuosity is computed (Nicholson and Phillips, 1981)(see Chapter 3.1.1). An advantage of the RTI method is a possible implementation for repeated time-lapse measurements in the brain slices, as well as *in vivo*. The downside lies in the lack of visualisation of the ECS structure and the low spatial resolution, defined as the 50 to 150 μm distance between the RTI and the ISM tip (Soria et al., 2020).

b) Microfiber optical measurements

Method similar to RTI; however, it uses light instead of electrical current and a membrane-impermeant fluorophore as the diffusing agent (Zhang and Verkman, 2010). The microfiber is used for the excitation of the fluorophore and the collection of emitted fluorescence, while the fluorophore is delivered iontophoretically or by pressure injection from a nearby capillary (Soria et al., 2020). Microfiber imaging has not gained the popularity of the RTI method, and existing publications have emerged from a single lab. Its main drawback is the lack of structural geometric ECS data.

2) Magnetic resonance imaging

Magnetic resonance imaging (MRI) is a noninvasive method used widely for diagnostic, clinical, and experimental purposes. The technique utilises strong magnetic fields and radio waves to image protons in tissue. Basics of MRI are explained in Chapter 3.1.2. The downside of MRI for studying the ECS is that both extra- and intracellular water contribute to the signal. To address the ECS compartment more specifically, some MRI studies introduce an extracellular contrast agent (Han et al., 2014; Meriaux et al., 2018).

3) Microscopy-based techniques

a) Electron microscopy

Electron microscopy offers ECS images with nanoscale resolution (Kasthuri et al., 2015; Korogod et al., 2015). The major drawback is the need for tissue fixation.

b) Intrinsic optical signal (IOS) imaging

IOS is a label-free light microscopy technique that detects changes in tissue reflectance or transmission of light due to different refractive indices of cellular membranes and interstitial fluid (Frostig et al., 1995). However, the physics behind the observed signal is still not well understood (Tao et al., 2002). The advantages of IOS are its simplicity, its *ex vivo* compatibility, and the imaging speed to address changes in volume in cellular cells and ECS (Soria et al., 2020).

c) Integrative optical imaging (IOI)

IOI, which may be also classified as a point-source method, is based on the introduction of an inert fluorophore into the tissue by a pressure injection from a capillary tip and evaluation of the fluorophore distribution by fluorescence microscopy. The advantages lie in the relatively high specificity of the fluorescence signal to the ECS compartment and it is live cell compatible (Soria et al., 2020).

d) Two-photon and confocal microscopy

Two-photon and confocal microscopy can be used similarly to IOI, when the fluorophore is pressure-ejected from a capillary tip to investigate its distribution and spread in the ECS with higher resolution (Iliff et al., 2012). The resolution of these methods is not sufficient to resolve the entire geometric structure and imaging deeper inside the tissue is problematic, as the excitation wavelengths could not penetrate deeper into the tissue (Soria et al., 2020).

e) Time-resolved fluorescence anisotropy imaging (TR-FAIM)

TR-FAIM allows assessment of ISF viscosity by measuring the polarisation of the emitted fluorescence light relative to the polarization of the excitation light. Polarised excitation light will preferentially excite fluorophores with a certain orientation. However, during a few nanoseconds between excitation and emission, the fluorophore will spontaneously undergo molecular rotation so that the emission polarisation diverges from the excitation polarization; i.e., it is anisotropic (Soria et al., 2020). Accordingly, differences in viscosity will give rise to differences in anisotropy, and corresponding anisotropy map is produced (Le Grimmellec et al., 1982). So far, the two-photon TR-FAIM approach has only been used by a single group.

f) Super-resolution fluorescence microscopy

Super-resolution microscopy allows imaging beyond the diffraction barrier of light, which limits optical resolution in conventional microscopy approaches and thus provides better resolution (Tonnesen and Nagerl, 2013). Basically, stimulated emission depletion (STED) microscopy is based on a confocal or two-photon microscope, with an additional laser beam co-aligned with the excitation beam to enhance resolution. Specifically, super-resolution shadow imaging (SUSHI) is based on labelling of the ISF with a fluorophore that renders cellular structures visible as shadows. The method allows ECS to be analysed geometrically (Tonnesen et al., 2018) and can be used in living tissue. The main limitation of the SUSHI approach is the imaging depth.

g) Carbon Nanotube Localization Microscopy

Another super-resolution microscopy approach is based on imaging of near-infrared fluorescent nanometre-sized single-walled carbon nanotubes (SWCNTs) injected into the brain parenchyma *in vivo* and later observed in acute brain slices. Analysis of time-lapse images with single-particle tracking algorithms yield real-time data on local relative diffusivity (Godin et al., 2017; Paviolo et al., 2020). The method opens the possibility of *in vivo* studies of relatively large tissue areas. The strength of the technique lies in providing both topological and diffusional information of the ECS. However, the ECS areas of width smaller than the tubes will remain inaccessible, and tubes may get stuck. A recent variation of this method implemented fluorescent nanoparticles and multiple-particle-tracking algorithms to characterise diffusion in the ECS (McKenna et al., 2021).

1.4 Astrocytes

The cellular compartment of the brain contains neurons and glial cells; the latter represents a heterogeneous group of cells, generally classified into astrocytes, oligodendrocytes, microglia, ependymal cells, tanycytes, radial glia, and NG2 glia. Each cell type could potentially change its volume when exposed to osmotic stimuli; however, astrocytes play a pivotal role in regulation the homeostasis of extracellular fluid homeostasis through various membrane proteins responsible for the active and passive transport of ions, organic osmolytes, and water (Ochoa-de La Paz and Gullias-Canizo, 2022). In addition, astrocytes are strategically located between blood vessels and neurons, with a polarized distribution of membrane proteins, a feature that is considered essential for astrocytic functions (Fig.3).

Distinct morphological types of astrocytes have been described in the brain. In grey matter, protoplasmic astrocytes have complex arborization, whereas in the white matter fibrous astrocytes have fewer, but longer processes (fibrous). Interlaminar astrocytes reside in cortical layer 1 and exhibit long processes that terminate in layers 2–4. In addition, varicose projection astrocytes are observed only in humans and other apes in layers 5–6 and in the white matter of the cortex (Degl’Innocenti and Dell’Anno, 2023). The processes of protoplasmic astrocytes envelop synapses, while the processes of fibrous astrocytes contact nodes of Ranvier (Araque et al., 1999; Kohler et al., 2021). The heterogeneity of astrocytes is especially evident when molecular differences are considered. For example, a single-cell RNA sequencing identified five transcriptomically distinct astrocyte subtypes in the adult mouse cortex and hippocampus recently (Batiuk et al., 2020).

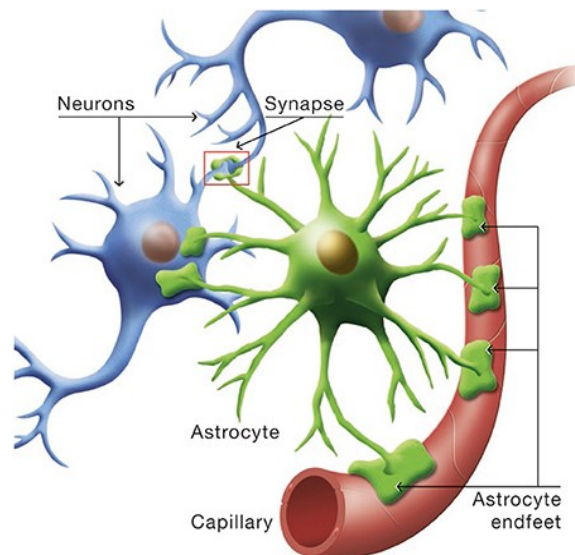


Fig. 3 Location and contacts of astrocytes. Astrocytes are located between brain capillaries and neurons, which they contact with numerous endfeet. From Demetrius et al. (2014).

Common markers of mature astrocytes are glial fibrillary acidic protein (GFAP), vimentin, nestin, excitatory amino acid transporter, folate enzyme aldehyde dehydrogenase 1 family member L1 (ALDH1L1)(Jurga et al., 2021). The markers that are used to label astrocytes are often not selective only to astrocytes. Sometimes it is just a matter of expression level when compared to the other cell groups and between heterogenic astrocyte

subpopulations. The astrocytic structural marker protein is GFAP, which is present in most CNS astrocytes and represents an intermediate filament type III protein (Jurga et al., 2021).

Important structural and functional distinction of astrocytes is the presence of membrane specializations – gap junctions and orthogonal arrays of particles (OAPs). Through gap junctional coupling, astrocytes form a multicellular network providing exchange of metabolites, signalling molecules, and ionic currents (Pannasch et al., 2011). Gap junctions are made of transmembrane proteins, known as connexons; each of them is further constructed out of six connexin molecules (McCutcheon et al., 2020). The connexons on adjacent cells join and create channels that allow the interchange of ions and small molecules. Other membrane specialisations, OAPs, are observed only in freeze-fracture preparations, mostly on the astrocytic endfeet facing and surrounding blood vessels and on the astrocytic processes facing the pia mater. Thus, they are believed to function in the transport of substances between astrocytes and blood/CSF. Recently, it has been shown that most of the water transporting channels aquaporin type 4 (AQP4) are organised into OAPs under physiological conditions (De Bellis et al., 2021; Lisjak et al., 2017).

1.4.1 Functions of astrocytes

Exceptional position of astrocytes between neurons and blood vessels, their multiple contacts with other glial cells, and the presence of specific channels and transporters on the astrocytic membrane yield them unique properties and functions in the CNS that may be divided into several groups.

1.4.1.1 Developmental function

Adult neurogenesis has been best described in the subgranular zone and the subventricular zone (Jurkowski et al., 2020). In contrast to neurogenesis, gliogenesis occurs everywhere in the adult brain (Rusznak et al., 2016). The role of astrocytes in neurogenesis is the secretion/exposition of molecular boundaries/cues important for axonal guidance and synaptogenesis (Christopherson et al., 2005). Astrocytes are also important for synaptic maturation and elimination (Verkhratsky and Nedergaard, 2018).

1.4.1.2 Vascular function

Astrocytes form extensive contacts with blood vessels in the CNS. Their endfeet are part of the BBB which is composed of capillary endothelial cells (connected by tight junctions) surrounded by a basal lamina and perivascular pericytes (Langen et al., 2019). By releasing prostaglandins, NO and arachidonic acid (AA), astrocytes can regulate

microcirculation by increasing or decreasing blood flow (Gordon et al., 2007; Verkhratsky and Nedergaard, 2018).

1.4.1.3 Metabolic function

Astrocytes are the main storage sites of glycogen in the CNS with the greatest accumulation in areas of high synaptic density (Falkowska et al., 2015). After glucose uptake through a glucose transporter 1 (GLUT1), glucose is phosphorylated to glucose-6-phosphate that acts as a substrate for glycogen synthesis (Walls et al., 2009; Falkowska et al., 2015; Schousboe, 2019). Astrocytes secrete lactate (arising metabolically from glycolysis), which is utilised by neurons for their metabolism (Falkowska et al., 2015).

1.4.1.4 Signalling function

Astrocytes express many receptors that are observed in neurons, such as - ionotropic and metabotropic glutamate, purinergic, γ -aminobutyric acid (GABA), glycine, acetylcholine and even dopamine, serotonin, histamine, or opioid receptors, etc. (Verkhratsky and Nedergaard, 2018). Intracellular Ca^{2+} transients, which are evoked by different neurotransmitters, are fundamental for the astrocytic release of gliotransmitters (Bazargani and Attwell, 2016) that differently modulate synaptic transmission or long-term synaptic plasticity (Lia et al., 2021). The phospholipase C (PLC) signalling pathway of these receptors (or direct opening of a channel) triggers Ca^{2+} elevations. The Ca^{2+} elevations occur on the very fine processes and can either remain restricted locally or eventually propagate to the main processes and soma (Pasti et al., 1997). Gliotransmitters are primarily glutamate, purines, GABA, D-serine, tumour necrosis factor alpha ($\text{TNF}\alpha$), prostaglandins, brain-derived neurotrophic factor, reactive oxygen species (ROS), etc. (Perea and Araque, 2010). Research showed that the release of gliotransmitters is likely dependent on Ca^{2+} and SNARE (an acronym derived from “SNAP [Soluble N-ethylmaleimide-sensitive factor Attachment Protein] Receptor”) protein in vesicles (Zorec et al., 2012, 2016). However, Fiacco and colleagues (Fiacco and McCarthy, 2018) have questioned the concept of gliotransmission and argue that it is not significant under physiological conditions (Savtchouk and Volterra, 2018).

The Ca^{2+} signals may spread along the cell and to neighbouring astrocytes in the form of Ca^{2+} waves. Calcium waves are a unique type of intercellular communication in astrocyte networks with transmission of Ca^{2+} and IP_3 through gap junctions that allow the propagation of the signal. Moreover, astrocytes are equipped with adenosine triphosphate (ATP) release

mechanisms and ATP receptors inducing $[Ca^{2+}]_i$ increase, securing an extracellular portion of the calcium wave propagation (Fujii et al., 2017). In addition to the release of gliotransmitters, calcium signals may affect vasoconstriction of blood vessels in the brain (Deitmer and Araque, 2009).

1.4.1.5 Neurotransmitter uptake

Astrocytes express GABA, glycine and glutamate transporters that enable the uptake of these neurotransmitters from the ECS (Mahmoud et al., 2019; Minelli et al., 1996, 1995; Weber and Barros, 2015). Uptake of glutamate is especially important because of its potential excitotoxicity. Glutamate is co-transported into the cell together with Na^+ via glutamate aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) and recycled via glutamate-glutamine cycle (Schousboe, 2019).

1.4.1.6 Ion homeostasis

The distribution and concentration of ions are finely balanced to maintain the membrane potential of cells. An increase in $[K^+]_e$ (during action potential) can result in the inactivation of Na^+ channels and inhibition of neuronal activity by a negative feedback mechanism that includes acidification of the ECS. To keep continuous neuronal activity, astrocytes redistribute excessive K^+ from the ECS by a local K^+ uptake and K^+ spatial buffering into the regions with a lower $[K^+]_e$ (Beckner, 2020). To preserve electroneutrality, K^+ influx into the cell is accompanied by either influx of anions, such as Cl^- , or efflux of cations such as Na^+ .

Astrocytes regulate $[K^+]_e$, particularly around neurons, in order to secure the ionic balance necessary for continuous neuronal activity. Their homeostatic function for potassium is ensured by the presence of diverse potassium ion channels and transporters (Verkhratsky and Nedergaard, 2018), such as:

- Ca^{2+} -dependent K^+ channels (K_{Ca}), which are activated at depolarisation by a subsequent influx of Ca^{2+} into the cells
- Inward rectifying channels (Kir). Kir4.1, a weak rectifying channel with the highest conductance in astrocytes, allowing bidirectional movement of K^+ depending on the transmembrane gradient of this cation. This enables K^+ reuptake from the ECS after increased neuronal activity

- Voltage-independent two-pore domain weak inwardly rectifying potassium channel (TWIK-1) and TWIK-related potassium channel (TREK-1) both playing an essential part in the regulation of resting membrane potential contributing significantly to passive conductance of mature hippocampal astrocytes (Zhou et al., 2009)
- Voltage-gated potassium channels (K_v), activated of membrane potential changes, and they themselves were suggested to play a role in modulating of membrane potential; blockade of K_v channels in cortical astrocytes diminished their ability to repolarize (Wu et al., 2015)
- K^+/Cl^- (KCC) and $Na^+/K^+/2Cl^-$ cotransporter (NKCC) that mediate electrically neutral movement of either K^+ and Cl^- or Na^+ , K^+ and 2 Cl^- across the cell membrane, respectively
- Na^+/K^+ - ATP hydrolase (ATPase) that exchanges the extracellular K^+ for the intracellular Na^+ using ATP as energy substrate

The initial uptake of extracellular K^+ prior to its redistribution throughout the glial syncytium is mediated through many subtypes of K^+ -permeable channels, including both voltage-dependent (i.e., inward-rectifying and K_v families) and independent channels (McNeill et al., 2021). Potassium ions are also transported into astrocytes by Na^+/K^+ -ATPase, KCC, and NKCC cotransporters (Kofuji and Newman, 2004). The main K^+ channels assisting the spatial buffering are inwardly rectifying channels, mainly of Kir4.1 type (Verkhratsky and Nedergaard, 2018) that are located in the perisynaptic and perivascular membrane, where the excess of intracellular K^+ is shunted to the vascular system (Dunn and Nelson, 2010).

In contrast to neurons, where sodium channels are essential for proper neuronal action potential generation, Na^+ channels and transporters in astrocytes are less prominent and contribute to various roles that contribute to homeostatic astrocytic functions and are not directly related to action potential generation. In astrocytes, sodium ions are generally transported via (Verkhratsky and Nedergaard, 2018):

- The H^+/Na^+ antiporter, a membrane protein that play a major role in pH and Na^+ homeostasis

- NKCC cotransporter that mediates electrically neutral movement of Na^+ , K^+ and 2Cl^- across the cell membrane and mainly participate in water regulation in the process of regulatory volume decrease (RVD)(explained in Chapter water homeostasis, see 1.4.1.8)
- Voltage-dependent channels that in astrocytes play an important role in some astrocytic functions, particularly ion and neurotransmitter homeostasis and reactive astrogliosis (McNeill et al., 2021)
- $\text{Na}^+/\text{Ca}^{2+}$ transporter, which makes use of an inwardly directed gradient for Na^+ ions generated by Na^+/K^+ -ATPase to remove excess Ca^{2+} from the astrocyte to prevent excitotoxicity (Verkhratsky et al., 2018)
- Na^+/K^+ -ATPase that extrudes intracellular Na^+ using ATP as an energy substrate helps to maintain resting membrane potential

An essential requirement in the K^+ spatial buffering mechanism is the low permeability of glial cells to anions such as Cl^- , as the low anion conductance ensures that net uptake of KCl will not occur upon rises of $[\text{K}^+]_e$. Several chloride channels and transporters have been described in astrocytes (Verkhratsky and Nedergaard, 2018):

- Voltage-dependent Cl^- channels (ClC-2) activated by hyperpolarisation
- Volume-regulated anion channels (VRACs) that help extrude Cl^- during the process of RVD (explained in Chapter water homeostasis, see 1.4.1.8)(Elorza-Vidal et al., 2019)
- NKCC cotransporter, mediating electrically neutral movement of Na^+ , K^+ and 2Cl^- across the cell membrane and participating in the process of RVD (explained in Chapter water homeostasis, see 1.4.1.8)
- The $\text{Cl}^-/\text{HCO}_3^-$ anion exchange system that is involved in pH regulation

Comprehensive information on astroglial function and their expression of ion channels can be found in Verkhratsky and Nedergaard (2018)(Fig.4).

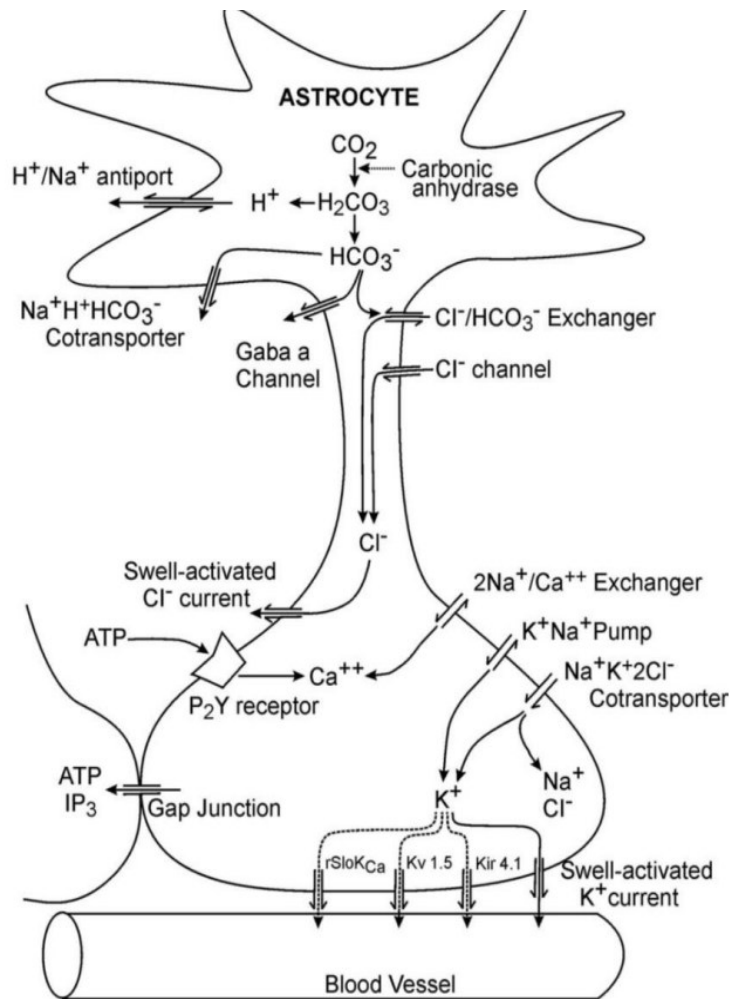


Fig. 4 Ion homeostasis in glia. Glial cells maintain the extracellular K^+ concentration via activation of Na^+/K^+ -ATPase, and $Na^+/K^+/2Cl^-$ cotransporter. Excess intracellular K^+ is shunted to the blood vessels via Kir 4.1, Kv1.5, RSloKCa channels, and swell-activated K^+ currents. Chloride ions are transported into the cell by the $Na^+/K^+/2Cl^-$ cotransporter and the Cl^-/HCO_3^- exchanger. Several Cl^- channels including inwardly and outwardly rectifying anion currents and swell-activated Cl^- channels have been described. Sodium ions channels allow Na^+ influx via the H^+/Na^+ antiport, and the $Na^+/K^+/2Cl^-$ cotransporter, while extrusion occurs via the Na^+/K^+ -ATPase and the $2Na^+/Ca^{2+}$ exchanger. The efflux of HCO_3^- occurs via activation of the GABA_A channels, the $Na^+/H^+/HCO_3^-$ cotransporter, and the Cl^-/HCO_3^- exchanger. Protons are actively extruded by the Na^+/H^+ antiport. Intracellular Ca^{2+} concentration increases via import through $2Na^+/Ca^{2+}$ exchanger or release of Ca^{2+} from intracellular stores induced by ATP binding to the P2Y receptor. **Abbreviations:** GABA, γ -Aminobutyric acid; ATP, adenosine triphosphate; IP₃, inositol trisphosphate; P2Y receptor, purinergic G protein-coupled receptor; RSloKCa, rat large-conductance Ca^{2+} -activated K^+ channel; Kv1.5, potassium voltage-gated channel, member 5; Kir4.1, inwardly rectifying potassium channel, member 4.1. From Simard and Nedergaard (2004).

1.4.1.7 Acid/base balance

Homeostatic control over cytosolic and extracellular pH is of a crucial importance for the brain function, because even mild acidification or alkalinisation significantly affects neuronal excitability and synaptic transmission. Extracellular pH (pH_e) and intracellular pH (pH_i) are maintained around 7.3-7.4 and 7.2, respectively (Casey et al., 2010). Brain cells continuously produce and release protons into the ECS, with the rate of acid production corresponding to the levels of neuronal activity and metabolism. Most of the H^+ generated during energy metabolism from the reaction of CO_2 with water (catalysed by carbonic anhydrase) is rapidly buffered by the bicarbonate buffer system and converted back by carbonic anhydrase. CO_2 is then removed by cerebral circulation and transported to the lungs to be exhaled (Deitmer et al., 2015). Secondary active transport is employed to remove excess H^+ or HCO_3^- , wherein for the efflux of H^+ or the influx of HCO_3^- . Transporters include H^+/Na^+ exchange, $\text{Na}^+/\text{HCO}_3^-$ cotransport, and Na^+ -dependent and Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchange. Additionally, there are a number of acid/base-coupled carriers, which are linked to the transport of metabolites, such as lactate and amino acids (Deitmer et al., 2015). Neuronal activity leads to acidification of the ECS and alkalization of the cytoplasm (Chesler, 2003; Theparambil et al., 2020). However, even small pH fluctuations significantly affect synaptic transmission and neuronal excitability (Chesler, 2003). Acidification also promotes cell swelling via acid-sensing ion channels (ASIC) that are upregulated and activated by low extracellular pH, and which open to inwardly conduct Na^+ , to a lesser degree also Ca^{2+} (Huang et al., 2015; Verkhratsky and Nedergaard, 2018; Cegielski et al., 2022).

1.4.1.8 Water homeostasis

The ability of astrocytes to manage the balance of water and ions is essential for maintaining a stable brain environment and ensure proper neuronal function. The regulatory volume decrease/increase (RVD/RVI) are critical cellular mechanisms that allow astrocytes to maintain volume homeostasis in response to osmotic stress (Hoffmann et al., 2009)(Fig.5).

RVD occurs when cells are exposed to conditions leading to cell swelling (hypoosmotic stress). To counteract this swelling and restore basal cell volume, astrocytes activate several mechanisms:

- The ion channels that facilitate the efflux of K^+ and Cl^- from the cell. This ion movement creates an osmotic gradient that drives water out of the cell (especially through AQP channels), reducing cell volume back to its normal state
- The release of organic osmolytes such as taurine, betaine, and myo-inositol
- The VRACs that are permeable not only to inorganic (Cl^-) but also to organic osmolytes such as taurine and excitatory amino acids (EAA)(Benfenati and Ferroni, 2010)

Activation of TRPV4 channels stimulate RVD by increasing $[Ca^{2+}]_i$, which elicit the efflux of K^+ and Cl^- via Ca^{2+} -activated K^+ and Cl^- channels (Hoffmann et al., 2009)(Fig.5). Although this mechanism has been demonstrated *in vitro* (Benfenati et al., 2011; Iuso and Krizaj, 2016; Jo et al., 2015), it has not been confirmed in acute brain slices.

RVI is the process that occurs when cells are exposed to hypertonic conditions, leading to cell shrinkage. To counteract this shrinkage and restore normal cell volume, the following mechanisms are activated:

- The ion transporters, such as the Na^+/H^+ exchanger, NKCC1, Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger. The accumulation of ions inside the cell creates an osmotic gradient that draws water back into the cell, increasing cell volume
- The increase in the organic osmolytes uptake

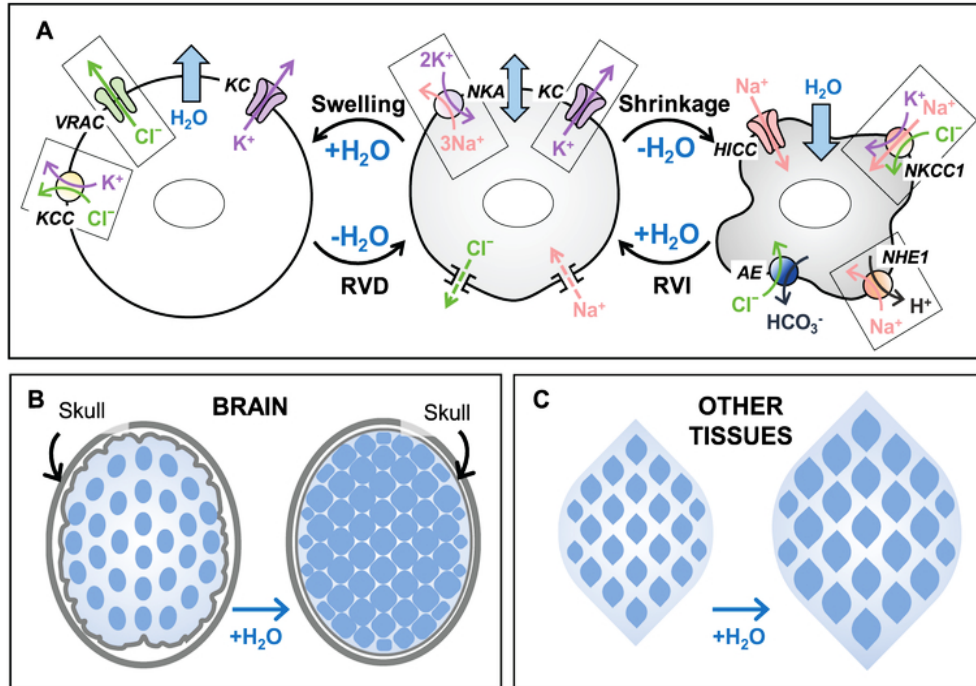


Fig. 5 Basic principles of RVD and RVI and difference in osmotic homeostasis within the brain and in other tissues. (A) Under isosmotic conditions (central picture), the work of Na⁺/K⁺-ATPase (NKA) and the activity of K⁺ channels (KC), set the transmembrane ion gradients and negative membrane potential. Hypoosmotic cell swelling (left picture) triggers the activation of the volume-regulated anion channel (VRAC) and several K⁺,Cl⁻ cotransporters (KCC) leading to regulatory volume decrease (RVD). Exposure to hyperosmotic media (right picture) stimulates the Na⁺/K⁺/2Cl⁻ cotransporter 1 (NKCC1) and the Na⁺/H⁺ exchanger 1 (NHE1). NHE works in cooperation with the Cl⁻/HCO₃⁻ anion exchangers (AE). In some cell types, cell shrinkage also opens hypertonicity induced non-selective cation channels (HICC). Their combined activity leads to regulatory volume increase (RVI). (B, C) The main differences in cell volume control between the brain and other tissues are due to the fixed volume of space in the CNS imposed by the rigid skull. From Wilson and Mongin (2018).

Astrocytes are essential for the regulation of the balance of water within the brain, ensuring that the delicate microenvironment required for neuronal activity is preserved. This regulatory function is critical not only for normal brain function, but also for response to various pathological conditions in which the balance of water is disrupted. Given its importance, the next subchapter of my thesis will be dedicated to water transport mechanisms.

1.5 Water transport mechanisms

Cell volume is transiently and locally challenged by neuronal activity, which promotes shifts in ion concentrations, thus challenging osmotically cell homeostasis (Colbourn et al., 2019). In contrast to ions or small molecules which transmembrane movements usually requiring specific channels or transporters, water can use multiple options, such as slow diffusion through the membrane, co-transport with ions and other substrates, using primary and secondary active transporters, or flow through the aquaporin channels (AQP)(Agre et al., 1993; Steffensen et al., 2015; Zeuthen, 2010).

Membrane transporters that can actively transport water include glucose transporters, namely sodium-glucose transport protein 1 (SGLT1), GLUT1, and GLUT2 (Fischbarg et al., 1990; Zeuthen et al., 2006, 2007); or chloride transporters, such as NKCC1 and chloride potassium co-transporter (KCC)(Zeuthen and MacAulay, 2012; Zhang et al., 2021). Water is also transported by monocarboxylate transporter (MCT1) or excitatory amino acid transporter (EAAT1)(Loo et al., 1996; MacAulay and Zeuthen, 2010).

To prevent excessive cell volume changes, astrocytes can activate an efficient regulatory mechanism resulting in RVD/RVI. To proteins that are tightly associated with regulation of cellular water content in astrocytes belong VRAC (Benfenati and Ferroni, 2010), TRPV4 (Jo et al., 2015) or sulfonylurea receptor 1 (SUR1)-transient receptor potential melastatin 4 (TRPM4) complex (Stokum et al., 2018). Our special focus in this dissertation was aimed on the role of the AQP4 and TRPV4 channels and their possible cooperation in water regulation.

1.5.1 Aquaporin channels

The aquaporin channels are a family of hydrophobic proteins that consists of about 300 amino acids and assemble in the the cell membrane in form of tetramers. Each monomer has six membrane spanning α -helical domains that surround a water pore and intracellularly located both the NH_2^- and COOH^- termini. Two asparagine–proline–alanine motifs form a pore that is selective to water and/or solutes. Transport through AQPs is bidirectional and driven by osmotic pressure or by the solute concentration between the intracellular and extracellular space (Verkman and Mitra, 2000).

In mammals, there are 13 known subtypes of AQPs (Li and Wang, 2017). AQPs are expressed in various tissues including the kidneys, lungs, gastrointestinal tract, and CNS. The aquaporin channels are divided into 3 subfamilies based on the channel permeability for

different molecules. For example, AQP0-6 and AQP8 are primarily water selective, whereas aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10) are also permeable to glycerol, urea, and some monocarboxylates (for example, lactic acid)(Li and Wang, 2017). Lastly, super- or unorthodox aquaporins (AQP11 and 12) are localised in the cytoplasm and have been proposed to be involved in regulation of intracellular water transport, organelle volume, and intra-vesicular homeostasis (Gonen and Walz, 2006). In addition, most AQPs are not permeable to ions, except from AQP6, AQP8, AQP11 and AQP12 (Rojek et al., 2008).

AQPs are dynamically regulated by:

- Activation of intracellular signalling pathways that induce phosphorylation/dephosphorylation;
- Changes in pH that lead to changes in the protonation state of amino acids;
- Binding or chemical reaction with metal ions;
- Protein-protein interactions;
- Internalisation;
- Binding of an inhibitor.

The aforementioned regulations affect the abundance of the AQP membrane or mediate conformational changes that modify the permeability of the channel. More long-lasting regulation is mediated by mRNA changes, protein synthesis, and degradation rate that modulate AQP abundance in the membrane (Zelenina, 2010).

1.5.1.1 Aquaporins in the CNS

The aquaporins present in the brain include: AQP1, 3, 4, 5, 6, 8, 9, and 12 (Badaut et al., 2002). However, the most represented and studied AQPs in the brain are AQP1, 4 and 9. AQP1 is expressed in epithelial cells of choroidal plexus and spinal cord neurons; AQP4 in the membrane of perivascular and perisynaptic/perinodal astrocytic processes and in the basolateral membrane of ependymal cells; and AQP9 in astrocytes, endothelial cells of subpial blood vessels and some catecholaminergic neurons (Zelenina, 2010). AQP1 is implicated in cerebrospinal fluid formation, while AQP9 in the brain energy metabolism. A special position for ischemia pathology has AQP4 that is involved in the formation and resolution. AQP4 channels at perisynaptic astrocyte processes and at contacts with the nodes

of Ranvier and nonmyelinated axons is involved in the clearance of K^+ that was released during neuronal activity and signal propagation (Nagelhus and Ottersen, 2013; Zelenina, 2010).

1.5.1.2 Structure of AQP4

From six characterised AQP4 isoforms (a-f), AQP4a (M1), AQP4c (M23), and AQP4e (Mz) are described in detail (Lisjak et al., 2017; Moe et al., 2008). M1 is a relatively long isoform with translation initiation at Met-1, while M23 is relatively short with translation initiation at Met-23. The Mz isoform has 41 extra amino acids compared to M1 and has been found in rat at very low levels (Moe et al., 2008). In the physiological state, M23 is the dominant isoform; however, during ischemia, the expression of M1 is significantly increasing (Hirt et al., 2009).

It has been discovered that AQP4 is a structural component of OAPs and is crucial for OAPs formation, as no OAPs have been observed in AQP4^{-/-} mice (Verbavatz et al., 1997) or a reduced number of OAPs has been shown in AQP4 knock-in mutant mice (Zhu et al., 2022). Moreover, M23 and M1 isoforms ratio regulates the OAPs size due to the ability of M23 isoform to stabilize OAPs and assist the formation of wider arrays (Furman et al., 2003). The biological role of OAPs remains unknown; however, it has been proposed that they could facilitate water transport, polarisation to astrocyte foot processes, and cell-cell adhesion (Lisjak et al., 2017; Smith et al., 2014; Verkman et al., 2011; Wolburg et al., 2011).

1.5.1.3 AQP4 protein-protein interactions

The localisation of AQP4 is secured by the binding of the AQP4 C-terminus to the postsynaptic density 95, disc-large, zona occludens 1 (or PDZ) domain of syntrophin, a component of the dystrophin-associated protein complex (Neely et al., 2001). Dystrophin binds actin protein with a transmembrane α -dystroglycan, and also connects to dystrobrevin, a scaffold for binding up to four syntrophin molecules (Newey et al., 2000). Different syntrophin isoforms are localised in different tissues, with the α -syntrophin (α -syn) that is expressed primarily in skeletal and cardiac muscle and in the brain (Peters et al., 1997).

AQP4 has been reported to associate with Na^+/K^+ -ATPase in the cerebellum (Illarionova et al., 2010). Na^+/K^+ -ATPase creates the Na^+ concentration gradient used by astrocyte secondary active transporters for K^+ and neurotransmitter uptake; however, it is unknown whether the association with Na^+/K^+ -ATPase can influence AQP4 water permeability or localisation (Illarionova et al., 2010). AQP4 has also been shown to associate

with the metabotropic glutamate receptor (mGluR) 5 in the cerebellum, suggesting the possible regulation of AQP4 by glutamate. In addition, it has been suggested that AQP4, Na⁺/K⁺-ATPase and mGluR5 may form a complex on astrocytic membrane, since the same region of AQP4 NH₂-terminus has been shown to associate with both these proteins (Illarionova et al., 2010).

A functional link between AQP4 and a gap junction protein connexin 43 (Cx43) was implied since AQP4-containing OAPs were detected near gap junctions (Rash et al., 2005), but there is no evidence of direct interaction or even a closer colocalization of AQP4 and Cx43 in the brain (Zelenina, 2010). Their expression is interconnected, as in mice, the deletion of AQP4 or α -syntrophin induced upregulation of Cx43 (Jullienne et al., 2018; Katoozi et al., 2017; Rao et al., 2021).

Interactions of the AQP4 channel with several other proteins have been proposed, such as glutamate transporter 1 and the μ -opioid receptor. Their impact on the function of AQP4 remains unknown. Several proteins that are possibly associated with AQP4-containing vesicles targeting have been identified, such as calmodulin and μ -subunit of clathrin assembly protein complexes 2 and 3 (Tornroth-Horsefield et al., 2022).

The localisation of Kir4.1 mirrors the AQP4 distribution on the cell membrane (Benfenati and Ferroni, 2010) and developmental studies revealed overlapping expression of Kir4.1 and AQP4 in astrocytes (Wurm et al., 2006). Nagelhus and colleagues proposed that AQP4 complements Kir4.1 channels in spatial buffering of K⁺ (Nagelhus et al., 2004). Experimental work reinforces the idea of functional coupling between AQP4 and Kir 4.1 showing that deletion of α -syn leads to delayed K⁺ clearance following high frequency activation of excitatory pathways in acute hippocampal slices (Amiry-Moghaddam et al., 2003; Anderova et al., 2014; Dmytrenko et al., 2013). Similarly, deletion of AQP4 induced delayed K⁺ clearance (Binder et al., 2006; Strohschein et al., 2011). On the contrary, Zhang and Verkman (2008) reported against functionally significant AQP4–Kir4.1 interactions, thus, the subject remains controversial (Zhang and Verkman, 2008).

AQP4 has been shown to form functional complexes with TRPV4 or SUR1-TRPM4 (Benfenati et al., 2011; Jo et al., 2015; Stokum et al., 2018). Both are cation channels permeable for monovalent cations, while TRPV4 is also permeable for Ca²⁺ (Rakers et al., 2017). The expression of TRPM4 is upregulated after CNS injuries and subsequently replaces TRPV4 in its interaction with AQP4 (Gerzanich et al., 2019; Stokum et al., 2016).

During this process, AQP4 shifts from the astrocytic endfeet to a widespread location throughout plasmatic membrane (Lafrenaye and Simard, 2019). The association of AQP4 with TRPM4 and its partner, SUR1, results in the creation of a channel complex, which mediates water transport (Stokum et al., 2018). In addition, blocked astrocyte swelling has been detected in TRPM4 knockout mice cold injury (Stokum et al., 2018). The complex of TRPV4 and AQP4 regulates water homeostasis and mediates several processes dependent on Ca^{2+} influx through TRPV4. In retinal Müller glial cells under hypoosmotic stress, water influx through AQP4 leads to the activation of TRPV4 via stretch-sensitive phospholipase A2 (PLA2) and the synthesis of eicosanoid messengers from the AA. The opening of TRPV4 then results in a subsequent increase in intracellular Ca^{2+} and cell swelling (Iuso and Krizaj, 2016; Jo et al., 2015)(Fig.6). Kitchen and colleagues showed that in hypotonicity and hypoxia models, TRPV4 channels are activated and an influx of Ca^{2+} ions into astrocytes binds to calmodulin and, through phosphorylation of AQP4, promotes its translocalization into the plasma membrane (Kitchen et al., 2020).

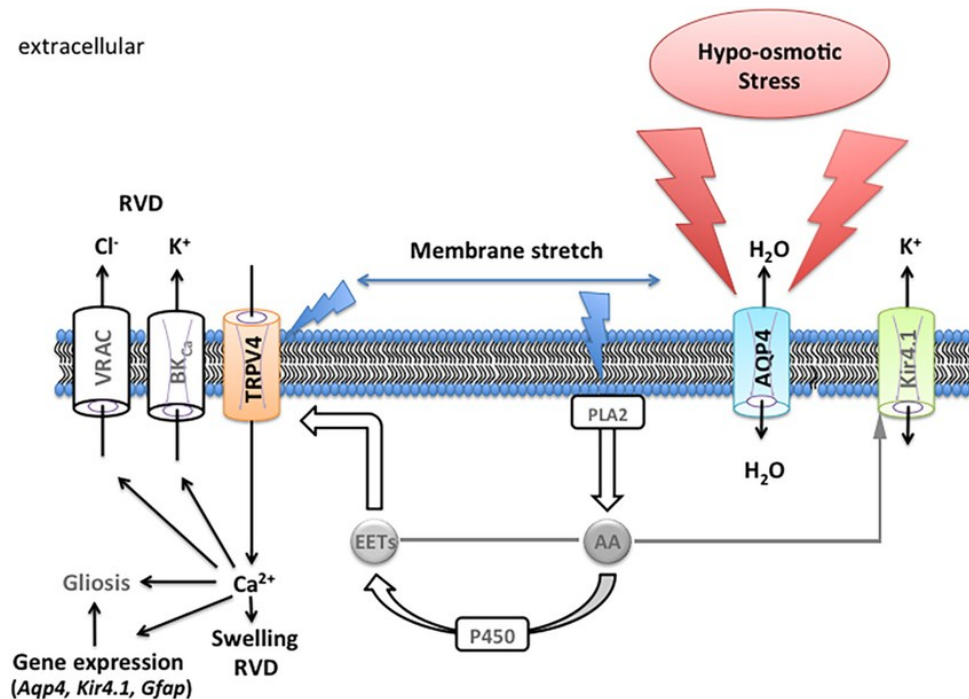


Fig. 6 Proposed schema of AQP4 interactions with TRPV4 in the Müller glia. Hypoosmotic stress resulting in cell swelling results in membrane stretching activates TRPV4 and stretch-sensitive PLA2. The product of PLA2, AA is further converted into EETs, which activate TRPV4 channels, but may also suppress Kir4.1. Ca^{2+} entering the cell induces both cell swelling and RVD and may modulate gene expression. High levels of Ca^{2+} may also stimulate the activity of BKca and VRAC

channels and facilitate RVD. **Abbreviations:** AQP4, aquaporin 4; TRPV4, transient receptor potential vanilloid-type 4; PLA2, phospholipase A2; AA, arachidonic acid; EET, epoxyeicosatrienoic acid; Kir4.1, inward rectifying potassium channels type 4.1; RVD, regulatory volume decrease; BK_{Ca}, big conductance Ca²⁺ activated K⁺ channels; VRAC, volume regulated anion channel. From Jo et al. (2015).

1.5.1.4 Role of AQP4 in brain water balance

The localisation of AQP4 at the tissue-fluid interface plays an important role in regulating water exchange across the BBB and at the brain-cerebrospinal fluid interface (Badaut et al., 2002). For clearing of the interstitial solutes, AQP4 channels are essential in the analogue of the lymphatic system, the glymphatic system in the brain (Gomolka et al., 2023; Mestre et al., 2018). The glymphatic influx occurs in the subarachnoid space, where the CSF enters the periarterial spaces. There, the astrocytes ensheath the arterioles and CSF with their endfeet with polarised localisation of AQP4 (Iliff et al., 2013a, 2013b). A directional flow of extracellular fluid through the ECS exits along the perivenous spaces (Rasmussen et al., 2022). The glymphatic system is active primarily during sleep (Hablitz et al., 2020), and contributes to the clearance of β -amyloid (Iliff et al., 2012), tau (Harrison et al., 2020) and other solutes, such as lactate (Lundgaard et al., 2017).

During physiological conditions, siphoning of K⁺ from active neurons is associated with water uptake into astrocytes. AQP4 water channels along the perivascular and subpial endfeet membranes increase the water permeability of the BBB. The presence of AQP4 facilitates the transport of water into the subarachnoidal space.

1.5.2 Transient receptor potential channels

The transient receptor potential (TRP) channel family is a large class of proteins, which is widely distributed in mammalian tissues. It consists of six subfamilies: TRPV (vanilloid), TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin) and TRPP (polycystic) (Montell, 2005). TRP channels mediate a variety of sensations, such as pain, temperature, or taste. Some TRP channels act as sensors of osmotic pressure, stretch, and vibrations. Most of the TRP channels are activated by lipid signalling. All TRP channels have a relatively non-selective permeability to cations, including sodium, calcium, and magnesium ions.

The TRPV channel subfamily contains six members (TRPV1-6). In the CNS, the most studied TRP channel is TRPV4. The TRPV4 channel is expressed in various types in the

CNS and through other mammalian tissues. In the rats and mice brains, TRPV4 can be found in peripheral nociceptive neurons, central neurons, astrocytes, microglia, oligodendrocyte precursor cells (OPCs), vascular endothelial cells and smooth muscle cells of cerebral arteries (Konno et al., 2012; Marrelli et al., 2007; White et al., 2016).

Shibasaki et al. detected that only a subset of astrocytes, about 30 %, express the TRPV4 channel (Shibasaki et al., 2014). The increase in $[Ca^{2+}]_i$ evoked by the activation of TRPV4 channels by the application of a TRPV4-specific ligand 4 α -phorbol-12,13-didecanoate in TRPV4-positive astrocytes was shown to propagate to neighbouring TRPV4-negative astrocytes through gap junctions or by ATP release into the extracellular space (Shibasaki et al., 2014). Functionally, TRPV4-positive astrocytes are suspected to play a role in the synchronisation of neuronal excitability via the release of gliotransmitters (Shibasaki et al., 2014). TRPV4 channels in neurons have been observed to potentiate the activation of the glutamate N-methyl-D-aspartate (NMDA) receptor. Experiments on hippocampal pyramidal neurons during ischemia or under hypotonic conditions have showed an increase in the NMDA-activated current, aggravating the glutamate excitotoxicity (Li et al., 2013).

The available evidence points to pro- (Beeken et al., 2022; Liu et al., 2018) and anti- (Konno et al., 2012) inflammatory effects of the activation of the TRPV4 channel in microglial cells, and the role of TRPV4 channels in microglia is inconclusive and needs to be further addressed (Nguyen et al., 2022). Interestingly, hypothermia can reduce microglia migration provoked by ischemia, suggesting that microglia movement can be modulated by temperature (Nishimoto et al., 2021). In OPCs, stimulation of TRPV4 channels by agonist GSK1016790A augmented their proliferation (Ohashi et al., 2018).

Endothelial and astrocytic TRPV4 channels are involved in the regulation of cerebral blood flow, improving hypoperfusion in the affected area (Dunn et al., 2013; Filosa et al., 2013; Kohler et al., 2006). Moderate increases in endfoot $[Ca^{2+}]_i$ dilate parenchymal arterioles, whereas high endfoot $[Ca^{2+}]_i$ constricts them (Girouard et al., 2010). In general, the activity of the TRPV4 channel is potentiated by modest elevations of $[Ca^{2+}]_i$ and inhibited by high $[Ca^{2+}]_i$ (Dunn et al., 2013).

1.5.2.1 Structure and gating of the TRPV4 channel

TRPV4 is a tetrameric channel (Shigematsu et al., 2010) consisting of 871 amino acids. Each subunit contains six transmembrane segments with a pore forming loop between

TM5 and TM6 and COOH and NH₂ intracellular termini. Moreover, the ankyrine repeat domain at the N-terminus anchors the TRPV4 channel to the cytoskeleton and provides the way to sense the stretch of the membrane (Nilius et al., 2004; Nilius and Droogmans, 2003).

TRPV4 has been shown to be activated by heat, acidic pH, citrate, mechanical stress, or oxidative stress (Garcia-Elias et al., 2014). Shibasaki et al. showed that TRPV4 channels are opened at physiological body temperature (~37°C) and mediate neuronal depolarisation, suggesting their role in regulation of neuronal activity (Shibasaki et al., 2007). However, the temperature sensitivity is dependent on the species, tissue, or cell type expressing TRPV4 channels (Toft-Bertelsen and MacAulay, 2021).

The mechanism of hypotonicity- and swelling-induced TRPV4 stimulation includes activation of the PLA2 pathway by membrane stretching. Arachidonic acid generated from membrane phospholipids is metabolised to epoxyeicosatrienoic acid (EET), which activates TRPV4 channels (Cao et al., 2018; Nilius et al., 2004; Plant and Strotmann, 2007; Watanabe et al., 2003). Phosphorylation by protein kinase C is another way TRPV4 channels can be activated. G protein-coupled receptor stimulation activates phospholipase C (PLC)-diacylglycerol (DAG)-protein kinase C (PKC) signalling (Liu et al., 2003). Both ways are based on site specific phosphorylation of TRPV4 serine and threonine residues (Fan et al., 2009)(Fig.7).

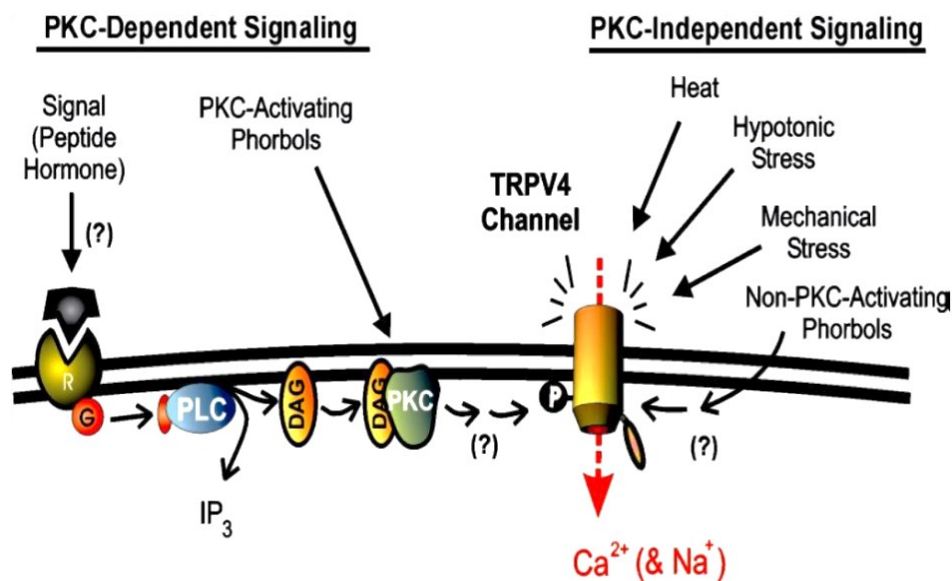


Fig. 7 Model showing potential converging pathways regulating TRPV4 channels. The TRPV4 channel appears to be regulated by at least two pathways, one protein kinase C (PKC)-dependent,

the other PKC-independent. The range of stimuli for the two pathways is indicated (black arrows). TRPV4 channels are permeable to calcium and monovalent cations, such as sodium. **Abbreviations:** R, receptor; G, G protein; PLC, phospholipase C; DAG, diacylglycerol; IP3, inositol trisphosphate; P, potential phosphorylation site; ?, unknown potential signalling pathways controlling TRPV4 activation. From O'Neil and Brown (2003).

1.5.2.2 Protein-protein interactions of TRPV4

In neurons, TRPV4 channel activation promotes the activity of NMDA receptor (Jie et al., 2015; Li et al., 2013). However, in astrocytes, TRPV4 channels also interact with mGluR and play a role in neurovascular coupling. Activation of mGluRs leads to $[Ca^{2+}]_i$ increase that triggers the synthesis of AA metabolites, such as EET. In turn, EET stimulates the opening of TRPV4 channels (Dunn et al., 2013).

TRPV4 channel activation evokes inositol-3-phosphate receptor(IP3R)-mediated Ca^{2+} oscillations by triggering of Ca^{2+} -induced Ca^{2+} release from the ER in astrocytes, neurons, and endothelial cells (Barrett et al., 2018; Dunn et al., 2013; Heathcote et al., 2019). Further, Ca^{2+} influx through TRPV4 channels activates ryanodine receptors (RyRs) on the ER, which can also induce Ca^{2+} release events (Garcia et al., 2019). RyR2 has been shown to modulate neuronal development and apoptosis through changes in the Ca^{2+} concentration, suggesting the role of TRPV4 channels in the regulation of apoptosis (Hajnoczky et al., 2000). Transient increases in $[Ca^{2+}]_i$ drive the release of chemical transmitters such as glutamate or ATP that modulate synaptic transmission and neuronal excitability (Parpura et al., 1994; Pryazhnikov and Khiroug, 2008). IP3R-dependent Ca^{2+} signalling is also critical for neurovascular coupling, the process by which local cerebral blood flow is modulated in response to neuronal activity (Otsu et al., 2015).

TRPV4 channels were shown to functionally couple with Ca^{2+} -activated K^+ channels (K_{Ca})(Earley et al., 2005; Feetham et al., 2015). K_{Ca} channels are divided into three subtypes with: big conductance (BK_{Ca}), intermediate conductance (IK_{Ca}), and small conductance (SK_{Ca}). BK_{Ca} channels are activated by Ca^{2+} released from the ER and have a great impact on the regulation of vascular tone (Earley et al., 2005; Garcia et al., 2019).

K-ATP channels sensitive to cellular ATP/adenosine diphosphate (ADP) levels activate when the concentration of ATP is low and hyperpolarise the cell (allowing K^+ efflux), thus, reducing cellular excitability (Tinker et al., 2018). In astrocytes, acute drop in cerebral

perfusion (ischemia/hypoxia) induces Ca^{2+} responses mediated by opening of TRPV4 channels. The influx of Ca^{2+} through the TRPV4 channels can trigger the release of ATP from astrocytes. TRPV4 channel activation, therefore, oppose the activity of K-ATP channel (Turovsky et al., 2020).

In Chapter 1.5.1.3, the co-operation of AQP4 and TRPV4 in astrocytes has been described, indicating the role of TRPV4 channels in volume regulation (Benfenati et al., 2011).

1.5.2.3 Role of TRPV4 in water homeostasis

As a result of cell swelling and concomitant stretch of the plasma membrane, the mechanosensitive TRPV4 channels open (Ryskamp et al., 2015). In cultured astrocytes and Müller glia in retina, TRPV4 channels were shown to functionally co-operate with AQP4 in the process of RVD (Benfenati et al., 2011; Jo et al., 2015), which prevents the detrimental swelling in response to hypoosmotic stress. Increased cytosolic Ca^{2+} concentration (due to activation of TRPV4 channels) activate K_{Ca} channels, chloride channels open in parallel, and subsequent efflux of K^{+} and Cl^{-} drives the osmotic transport of water (Iuso and Krizaj, 2016; Okada et al., 2001).

TRPV4-induced phosphorylation of AQP4 via calmodulin or protein kinase A-dependent mechanism, promotes AQP4 translocation into the plasma membrane (Kitchen et al., 2020). Inhibition of calmodulin in a rat spinal cord injury with trifluoperazine suppressed AQP4 in the blood-spinal cord barrier and decreased the extent of edema (Kitchen et al., 2020).

During ischemia, TRPV4 channels activation was shown to amplify tissue damage and BBB disruption (Jie et al., 2015), while in vascular smooth muscle cells it may promote dilation of cerebral vessels via opening of BK_{Ca} channels (Szarka et al., 2018). These changes lead to a more pronounced vasogenic edema.

1.6 Pathological cell swelling and edema development

Depletion of ATP and oxygen during ischemia leads to a collapse of the active transport system and dysregulation of ion homeostasis. Cell ion and volume homeostasis is further challenged due to the BBB disruption caused by MMP activity. Ischemia-induced edema comprises specific stages described as cytotoxic edema, ionic edema, and vasogenic edema (Gu et al., 2022; Stokum et al., 2016)(Fig. 8).

Cytotoxic edema is the initial step in the pathological process of development of ischemia-evoked cerebral edema. The loss of active transport mechanisms due to the lack of energy leads to dysregulation of ionic transmembrane gradients that cause cell depolarisation and cell swelling (Halstead and Geocadin, 2019; Stokum et al., 2016)(Fig.8).

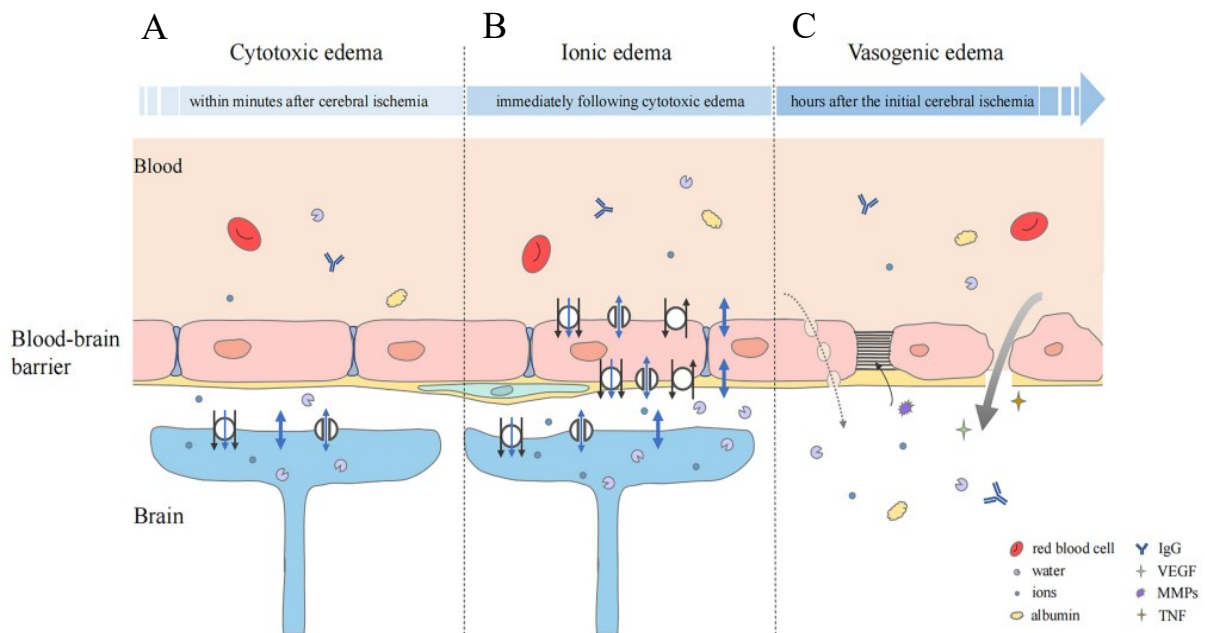


Fig. 8 Progression of brain ischemia-evoked edema. A) Cytotoxic edema is particularly prominent in astrocytes (blue endfeet). Ischemia induces the influx of ions (black arrow) by changing the osmotic gradients. The water (blue single-/double-headed arrows) follows the ions into the astrocytes in three ways: simple diffusion, transport through aquaporin channels, and water co-transport with ions. B) In ionic edema, the influx of ions (black arrow) and water (blue single-/double-headed arrows) is further potentiated by the activity of channels and transporters in the endothelial cells of blood vessels. C) Vasogenic edema is characterised by a disruption of the blood-brain barrier. The transport of ions, water, and serum proteins (such as albumin or IgG) may occur directly (thick gray arrow) or via pinocytotic vesicles (thin gray arrow). Multiple factors, including VEGF, MMPs, and pro-inflammatory cytokines (such as TNF) mediate neuroinflammation and tight junction degradation. **Abbreviations:** IgG, immunoglobulin type G; VEGF, vascular endothelial growth factor; MMPs, matrix metalloproteinases; TNF, tumour necrosis factor. From Gu et al. (2022).

Cytotoxic edema further progresses to ionic edema characterised by the early stage of endothelial dysfunction. Due to the ion concentration gradient formed by cytotoxic edema, Na^+ , Cl^- , and water are transported across endothelial cells into the brain parenchyma (Gu

et al., 2022). Water can move through simple diffusion (MacAulay, 2021), by a co-transport with ions or molecules, such as lactate or GABA (Hamann et al., 2010; MacAulay and Zeuthen, 2010), and through aquaporin channels (Stokum et al., 2016).

The stage of vasogenic edema is characterised by the disruption of the BBB and plasma proteins extravasation into the interstitial space. The process is associated with the entry of water into the brain parenchyma, either through a reverse pinocytosis or paracellular pathway, when tight junctions are weakened (Gu et al., 2022). The expression of vascular endothelial growth factor (VEGF) is triggered, resulting in a decrease in the amount of tight junction protein and increased vascular permeability (Hu et al., 2022).

The clearance routes of extracellular edema are still not sufficiently understood. Studies indicate that redundant water is removed from the parenchyma along the perivascular spaces, whereupon it is removed from subarachnoid space by mechanisms of CSF absorption (Stokum et al., 2016).

1.6.1 Volume changes in neurons and astrocytes during ischemia

Cytotoxic edema during ischemia is particularly developed in astrocytes (Liang et al., 2007; Stokum et al., 2015). While astrocytic RVD (described in Chapter 1.4.1.8) mitigates initial cell swelling, overwhelming stress during ischemia can impair RVD mechanisms and results in the development of excessive cytotoxic edema and exacerbation of brain damage. During ischemic injury, RVD mechanism may be detrimental, as it potentiates neurotoxicity due to an increased extracellular concentration of glutamate released through VRACs. Other neurotransmitters and osmolytes can also be released during RVD and affect synaptic transmission and overall brain excitability (Mongin, 2016). Moreover, astrocytic glutamate uptake during ischemia can be reversed further enhancing excitotoxic damage (Gouix et al., 2009).

Cerebral ischemia increases the abundance of the SUR1-TRPM4 and of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in the endfeet of perivascular astrocytes raising the endfoot concentration of Ca^{2+} . This increase in Ca^{2+} stimulates calmodulin-dependent translocation of AQP4 to the plasma membrane and water influx (Stokum et al., 2023).

The mechanisms of neuronal swelling are far less investigated than those of astrocytes. Strikingly, neurons do not express AQP4 *in vivo* (Andrew et al., 2007; Papadopoulos and Verkman, 2013); however, they seem to express AQP9 and AQP1 (Badaut et al., 2004;

Fukuda et al., 2012), which fulfil different roles than volume regulation (Oshio et al., 2006). During hypotonic challenge in living brain slices, astrocytes swelled readily, but neurons did not show any change in cell volume (Andrew et al., 2007). In contrast, several studies show that neurons swell extensively, for example, during focal cerebral ischemia (Risher et al., 2012, 2009). It seems that the neuronal swelling depends on severity of the insult/osmotic challenge as neurons have been reported to withstand greater osmotic stresses than glial cells due to the lack of functional aquaporin channels (Steffensen et al., 2015). These data have been challenged by Murphy et al., whose experiments detected neuronal swelling even during osmotic stresses (Murphy et al., 2017).

In neurons, we can see that swelling is localised mostly to the dendrites, which are endowed with a high density of postsynaptic ionotropic receptors. This phenomenon is known as dendritic beading or the formation of synaptic varicosities, which is particularly pronounced under pathological conditions (Greenwood and Connolly, 2007). During more severe pathological conditions, including cortical spreading depression, energy failure, and excitotoxicity, neuronal swelling is extensive (Rungta et al., 2015; Steffensen et al., 2015; Zhou et al., 2010). Neurons transport water molecules across their cell membrane, usually by co-transport mechanisms. For instance, in many cell types, water may be co-transported with K^+ and Cl^- via the NKCC1 and KCC2 co-transporters (Steffensen et al., 2015; Takezawa et al., 2023), with glutamate (via EEAT/GLAST, GLT-1), GABA (via GAT), or via lactate co-transporters (via MCT2)(MacAulay, 2021; MacAulay et al., 2004). Neurons start to swell within seconds during spreading depolarisation in the *in vivo* model of stroke (Kirov et al., 2020; Murphy et al., 2008; Risher et al., 2010; Steffensen et al., 2015). In acute brain slices, neurons swell, when ischemia is modelled by solutions with either elevated $[K^+]_e$ or lowered/omitted oxygen and glucose. In the recovery solution, astroglial volume recovered quickly, while the neurons remained swollen (Andrew et al., 2007). Unlike neurons, astrocytes better recover from brief ischemic insult in cortical slices, possibly thanks to the expression of aquaporin channels facilitating water efflux (Risher et al., 2009).

1.6.2 Ischemia

Ischemia is the restriction of blood supply that results in tissue damage. It is accompanied with hypoxia (the deprivation of adequate oxygen supply), or, in most serious cases, anoxia (complete deprivation of oxygen supply). Ischemia triggers a process known as the ischemic cascade, a series of biochemical reactions that damage nervous tissue.

Without restoration of the blood supply, ischemia can lead to cell death and irreversible damage to brain tissue.

Following focal cerebral ischemia, both neurons and glia in the infarct core are damaged. The core is surrounded by a so-called penumbra, a border zone, where the metabolic activity is partially preserved, but residing cells are electrically unexcitable (Astrup et al., 1981; Kaufmann et al., 1999). Without treatment, cells within penumbra would deteriorate due to the presence of depolarisation events, such as, peri-infarct depolarizations and spreading depression (Dreier, 2011; Fabricius et al., 2006). The process of peri-infarct depolarisation is amplified by calcium transients from activation of TRPV4 channels (Rakers et al., 2017).

1.6.2.1 Ischemic cascade

The lack of oxygen during ischemia leads to cessation of oxidative phosphorylation and disruption of ATP production. ATP supply depends on continuous blood perfusion and falls to zero within minutes of the onset of anoxia. Cells switch to anaerobic glycolysis and produce lactic acid. The amount of ATP is slowly depleted and ATP-related transport fails (such as Na^+/K^+ -ATPase)(Ferdinand and Roffe, 2016). Consequently, a rise in the $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$ and $[\text{K}^+]_e$ occurs (Hansen, 1985; Hossmann et al., 1977; Perez-Pinzon et al., 1995), and since cells can no longer maintain effective osmotic homeostasis via active pumping, water enters into the cells, causing swelling (Elkin et al., 2010). The increase in $[\text{Na}^+]_i$ is aggravated by acidosis and increased activity of Na^+/H^+ exchangers (Kahlert and Reiser, 2004).

Astrocytes may retain their membrane potentials for a longer time than neurons as they produce ATP also by anaerobic glycolysis and from stored glycogen (Bonvento and Bolanos, 2021). Furthermore, they benefit from the presence of gap junctions that help astrocytes work as one unified ion sink (Wilson and Mongin, 2018).

The detrimental effect of the accumulation of Ca^{2+} , which cannot be transported either to the ECS nor into internal storages (owing to the lack of energy for Ca^{2+} pumps), induces the release of Ca^{2+} from the intracellular endoplasmic reticulum. Excess Ca^{2+} triggers the release of glutamate from neurons that activates NMDA receptors permeable for Ca^{2+} . In addition, the loss of the Na^+ gradient that fuels the glutamate uptake system, together with the activity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, facilitates a further increase in $[\text{Ca}^{2+}]_i$ (Lai et al., 2014). Accumulation of Ca^{2+} promotes the production of free radicals and activation of

calcium-dependent enzymes (e.g., calpain, endonucleases, ATPases and phospholipases). The degradation of phospholipids increases membrane permeability, allowing the influx of ions and toxic chemical substances into the cell (Andrabi et al., 2020). Ischemic disturbances within mitochondria promote the opening of the permeability transition pore, a nonspecific channel that increases the permeability of the inner mitochondrial membrane and induces apoptosis by releasing proteins cytochrome c, endonuclease G, and apoptosis-inducing factor into the cytosol (Andrabi et al., 2020; Suhaili et al., 2017; Szydlowska and Tymianski, 2010).

Moreover, glutamate initiates Ca^{2+} -dependent synthesis of NO utilizing neuronal nitric-oxide synthase (NOS) (Araki et al., 2020). A large amount of NO has both a detrimental and protective role. Excess NO modulates the proteins of several signalling pathways through S-nitrosylation and induces tissue damage and cell apoptosis. However, its vasodilatory effect might also help increase the post-ischemic blood flow (Iadecola et al., 1994; Schulz, 2004; Wang et al., 2022).

Apart from constitutively expressed proteins, non-selective cation channels upregulated by ischemia or oxidative and nitrosative stress provide new ways for Ca^{2+} influx. There channels include TRPM4-SUR1 and a nonselective cation channel $\text{NC}_{\text{Ca-ATP}}$ (regulated by SUR1), which is triggered by ATP depletion (Simard et al., 2009; Stokum et al., 2023).

As anaerobic metabolism and lactic acid generation decrease the pH_e below 6.8, the compensatory ion and water fluxes through the Na^+/H^+ exchanger and the $\text{Na}^+/\text{HCO}_3^-$ co-transporter in astrocytes aggravate cytotoxic edema (Ehresman et al., 2021; Kempinski et al., 1988; Staub et al., 1990).

In summary, an ischemic injury begins with severe hypoperfusion and progresses to excitotoxicity and oxidative damage, which in turn promote microvascular injury, blood-brain barrier dysfunction, and initiate post-ischemic inflammation. All these events exacerbate the initial injury and can result in permanent cerebral damage (Fig. 9).

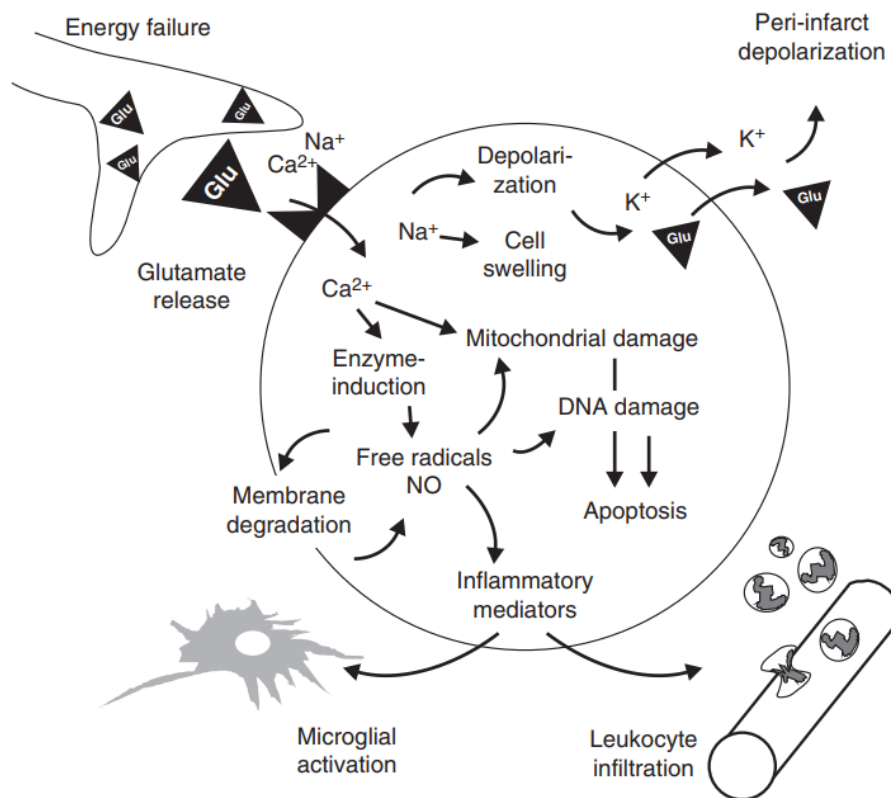


Fig. 9 The ischemic cascade and mediators of ischemic injury. Energy failure following ischemia leads to glutamate excitotoxicity. The accumulation of Na^+ in the cytosol induces cell swelling and cell depolarisation, which induces release of neurotransmitter glutamate. Waves of peri-infarct depolarisation further compromise the balance of the ischemic neurons. Increase in the intracellular concentration of Ca^{2+} triggers the activation of downstream mediators of ischemic damage including free radicals and nitric oxide production, phospholipases, inducing mitochondrial, membrane and DNA damage. Concomitantly, apoptotic pathways are initiated. The release of inflammatory mediators then amplifies tissue damage. From Endres et al. (2008).

1.6.2.2 Tissue changes following ischemia

Although reperfusion of ischemic brain tissue is critical for restoring its physiological functions, it can paradoxically result in secondary damage, called ischemia/reperfusion injury, which is mediated, for example, by reactive oxygen species/ reactive nitrogen species (ROS/RNS) released by inflammatory cells (Bugger and Pfeil, 2020; Sun et al., 2018). The increase in free radicals induces the synthesis of transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), hypoxia inducible factor 1 (HIF-1), interferon regulator factor 1 (IRF1), and signal transducer and activator of transcription 3 (STAT3), which trigger the transcription of several pro-inflammatory genes (Crack and Wong, 2008). This results in upregulation of cytokines and consequently in increased expression of adhesion molecules on the endothelial cell surface, which mediate

the adhesion of leukocytes to the endothelia (Lakhan et al., 2009). Consequently, neutrophils and later monocytes/macrophages, as well as several subtypes of T cells, invade the site of ischemia and induce further neuronal death (Chen et al., 2021).

Furthermore, excess production of ROS/RNS, among other factors, promotes the generation of MMPs, especially MMP-9 (Zinnhardt et al., 2015). MMP activation in early ischemic brain damage intensifies the inflammatory response and leads to BBB disruption (Candelario-Jalil et al., 2022, 2009). In contrast, MMP-9 plays a major role in the migration of neural progenitor cells into the peri-infarct area, the process important in the lesion repair and neurovascular remodelling (Kang et al., 2008; Yang et al., 2011).

Cytokine, debris, or molecules (also termed damage-associated molecular pattern) released from dead cells trigger microglia activation in the ischemic brain (Gulke et al., 2018). The dual role of microglia has been proposed during the different phases of ischemia (Lalancette-Hebert et al., 2012; Qin et al., 2019; Sun et al., 2015). Microglia with a pro-inflammatory profile were termed as M1-type activated microglia, while an anti-inflammatory profile of microglia was termed M2-type activated microglia (Martinez and Gordon, 2014). M1-type activated microglia contribute to an increase in the inflammation and cytotoxicity levels through the release of the cytokines, interleukins, chemokines, MMPs, and ROS/RNS (Martinez and Gordon, 2014). In contrast, M2-type activated microglia promote the recovery of injured tissue and decrease inflammatory levels (Martinez and Gordon, 2014). The evidence supporting their protective function shows that microglia promote neurogenesis, suppress inflammation during stroke, and have neuroprotective properties (Chen and Trapp, 2016; Denes et al., 2007; Neumann et al., 2006; Taylor et al., 2017). Microglia also prevent the secretion of astrocyte-mediated inflammatory factors during ischemia and promote plasticity (Alia et al., 2017; Jin et al., 2017). However, recent studies employing single-cell RNA sequencing have identified more distinct subgroups of microglia, indicating that the situation is considerably more complex than previously understood (Hammond et al., 2019; Shi et al., 2020).

Activated astrocytes are usually classified into A1- and A2-type; however, similar to microglia, their population is, in fact, more heterogeneous with many intermediate phenotypes (Escartin et al., 2021; Guo et al., 2021; Liddel and Barres, 2017). A1-type astrocytes release pro-inflammatory cytokines with inhibitory effects on oligodendrogenesis and OPC differentiation (Miyamoto et al., 2020; Traiffort et al., 2020). In contrast, A2-type

astrocytes have neuroprotective functions releasing anti-inflammatory cytokines and trophic factors, such as brain-derived neurotrophic factor (BDNF) or vascular endothelial growth factor (VEGF)(Dvorianchikova et al., 2009; Escartin et al., 2021; Liddelow and Barres, 2017).

Following ischemic insult in rats, astrocyte reactivity is initiated from 4 h to 1 day, reaches maximum at 4 days, and persists until 28 days (Nowicka et al., 2008). Active astrocytes (reactive astrocytes; RA) change their morphology, phenotype, and expression pattern (Li et al., 2022). Around the lesion core, hypertrophic processes of RAs form a capsule layer to cover the infarction area (Huang et al., 2014). RAs secrete a large amount of chondroitin sulfate proteoglycan and other ECM components, which together with other cellular components (fibroblasts, pericytes, RAs, microglia and monocyte-derived macrophages) form the glial scar (Dias et al., 2021). The formation of the glial scar can have both protective and detrimental consequences. The glial scar limits further inflammation, thereby inhibits enlargement of the ischemic penumbra (Yang, 2020). Some studies highlighted the importance of the glial scar in axon regeneration (Anderson et al., 2016; Liu et al., 2014) and post-stroke angiogenesis (Williamson et al., 2021). However, it is widely believed that the glial scar creates a major physical and chemical barrier against regeneration of neurons as it creates an inhibitory environment for axonal growth (Li et al., 2022). Despite the presence of glial scar delayed atrophy, cytotoxic edema, and neuron loss is present in regions adjacent to the infarct core. These findings suggest that the glial scar is not able to completely and impermeably segregate areas of necrosis from surviving brain tissue (Zbesko et al., 2018).

In addition to astrocytes and microglia other glial cell types are affected by ischemia. Pericytes are a component of the neurovascular unit and BBB with contractile function (Gurler et al., 2022). Persistent contraction of pericytes during ischemia leads to occlusion of microvasculature, while pericyte death impairs the integrity of BBB (Gurler et al., 2022). Surviving pericytes activate and secrete MMP-9, ROS and growth factors important in angiogenesis such as platelet-derived growth factor- β (PDGF- β), VEGF and transforming growth factor- β (TGF- β). Pericytes can also contribute to recovery after ischemia by differentiating into neural, glial, and vascular cells (Gurler et al., 2022). To cells highly sensitive to oxidative stress and excitotoxicity to belong also oligodendrocytes and OPCs (Dewar et al., 2003; Tanaka et al., 2001). The trophic factors released by M2-type microglia and A2-type astrocytes increase oligodendrogenesis and OPC differentiation and help in

myelin repair (Huang et al., 2023; Traiffort et al., 2020; Zhang et al., 2024). OPCs, also known as NG2-glia, were significantly decreased in the infarct core but elevated in the penumbra (Tanaka et al., 2001). NG2-glia contribute to the formation of glial scars (Valny et al., 2017) and under ischemic conditions, NG2-glia differentiate rather into reactive astrocytes than into oligodendrocytes (Honsa et al., 2016).

Apart of the cellular effects of ischemia, the ECM also undergoes significant changes, often in response to the tissue attempt to repair itself. Increased/decreased production of ECM and proteases in neurons and glial cells underlies the process of the ischemia-evoked ECM remodelling. Partial and localised loss of the PNNs was detected early after middle cerebral artery occlusion (MCAO) (Dzyubenko et al., 2023, 2018; Hartig et al., 2017; Karetko-Sysa et al., 2011). The effect lasted for around 2 weeks after MCAO, as indicated by the fluorescence intensity quantification of *Wisteria floribunda* agglutinin (WFA) staining (Hobohm et al., 2005). The decomposition of PNNs in the vicinity of the lesion leads to an increase in neuronal plasticity (Reichelt et al., 2019). Another feature after injury is the accumulation of ECM components at lesion sites. ECM molecules secreted by RAs form a component of the glial scar and inhibit neural outgrowth from the perilesional area to the ischemic core (Jones et al., 2003; Li et al., 2023; Siebert et al., 2014).

1.6.3 Models of cytotoxic edema

The research of cytotoxic edema is complicated due to the multitude of simultaneous changes that occur in brain after the injury. The experimental models, allowing the examination of cytotoxic edema development/resolution under controlled conditions, were implemented for better understanding. However, there are general issues, independent from the choice of model, potentially conflicting the reality of human stroke in the experimental setting. These include genetic/epigenetic differences from humans, differences in brain anatomy, differences in vascular anatomy, or differences in the immune system (Sommer, 2017).

In vitro models allow for the investigation of specific basic biochemical and molecular mechanisms underlying ischemia. There are two principal ways to induce ischemia-like events: either the deprivation of oxygen and glucose or the chemical or enzymatic blockade of the cellular metabolism (Holloway and Gavins, 2016). Compared to *in vivo* models, there is a need for a longer period of energy deficiency to induce neuronal death. The simulation of ischemia/perfusion can be achieved by a return to standard perfusion or culture conditions.

In vitro models allow for an isolated analysis of glutamate excitotoxicity by exposing the culture or slices to glutamate or other specific agonists of excitatory receptors (Sommer, 2017; Vargova et al., 2001).

In vivo animal models of cerebral ischemia are characterised as a global, focal, and multifocal restriction of cerebral blood flow. Focal ischemia results from artery blockage and is localised into a specific area that is supplied by the certain artery. Global ischemia occurs when blood flow to the whole brain is drastically reduced; commonly it is induced by cardiac arrest, severe hypotension, or carbon monoxide poisoning. In multifocal ischemia, there is an uneven distribution of reduced cerebral blood flow.

Depending on the duration (and reversibility) of the ischemia, animal models may induce permanent or transient ischemia, where the latter is followed by blood reperfusion (Baranovicova et al., 2023).

In vivo, focal ischemia is usually modelled by induction of MCAO, transient or permanent. Several different techniques may be utilised (Sommer, 2017), such as:

- The intraluminal suture MCAO model, which does not require craniectomy. To induce vessel occlusion, a monofilament is introduced into the internal carotid artery
- Direct occlusion of a cerebral vessel done by clipping, ligation or hooks (with the possibility of reperfusion). Cauterisation followed by transection on the other hand, results in permanent ischemia. All these direct approaches have in common that a craniectomy with an incision of the dura mater is necessary
- The embolic stroke models: thromboembolic clot model and non-thromboembolic, microsphere, or macrosphere-induced stroke model, with transient cases allowing clot dissolution for reperfusion
- Endothelin-1 model. Application onto an exposed vessel or directly on the brain surface or stereotactically injected into the brain parenchyma leads to vasoconstriction inducing downstream ischemia
- The photothrombotic stroke model. For stroke induction, a photosensitive dye is systemically applied followed by illumination of the brain through the intact skull with light of a specific wavelength. The illumination activates the dye leading to the formation of singlet oxygen and superoxide, which in turn results in endothelial

injury, platelet activation, and aggregation. The procedure can be transient if it is followed by the administration of a clot-dissolving agent

The *in vivo* model that is used to induce permanent severe global ischemia is the terminal ischemia/anoxia method, a controlled experimental procedure simulating extreme conditions of oxygen and blood deprivation to brain tissue, mimicking the final stages of ischemia. This can be achieved by cutting the systemic oxygen supply by stopping ventilation or blood flow, such as inducing cardiac arrest or by Common Carotid Artery Ligation (CCAL). The terminal nature of the experiment means that the animals do not recover and it is often used to study the most severe outcomes of ischemic stroke (Sommer, 2017). Transient global ischemia models include Common Carotid Artery Occlusion (CCAO), a temporary clamping of both common carotid arteries, and Two-Vessel Occlusion (2VO), a simultaneous occlusion of the bilateral common carotid arteries, often combined with hypotension.

To investigate the impact of ischemia on the development of cytotoxic edema, we employed three established methodologies: oxygen-glucose deprivation (OGD) in acute brain slices; and terminal ischemia/anoxia and permanent MCAO (pMCAO) *in vivo*, both are a model of global and focal permanent ischemia, respectively. These techniques are well-established and provide complementary insights into the mechanisms underlying cytotoxic edema.

2 AIMS AND HYPOTHESES

The objectives of this thesis were established to investigate the roles of AQP4 and TRPV4 channels, as well as their functional interaction, in the development of cytotoxic edema. To assess the involvement of these channels, we measured alterations in brain diffusion parameters associated with cell swelling in different models of cell swelling/ischemia. These measurements were further supplemented by observations of early chronic tissue changes induced by ischemic injury.

We hypothesised that:

1. The absence of AQP4 and TRPV4 channels will affect the development of cytotoxic edema and that the effect of their loss depends on the severity of the insult.
2. The absence of AQP4 complex affects K^+ homeostasis during oxygen-glucose deprivation (model of ischemia) and terminal ischemia/anoxia, as AQP4 is believed to functionally cooperate with Kir channels.
3. AQP4 and TRPV4 channels functionally cooperate in the regulation of cell volume in the brain tissue.
4. Ischemia evokes alterations in ECM content and protective PNNs that can affect the damage or regeneration abilities, and these alterations differ between control mice and animals lacking AQP4, TRPV4 or both proteins.

To confirm our hypotheses, following aims were proposed:

1. To evaluate the effect of deletion of AQP4 or TRPV4 on the acute cell swelling in the *in vivo* model of terminal ischemia/anoxia (see Chapter 4.1.1).
2. To detect the changes in the extracellular K^+ concentration *in vivo* after terminal ischemia/anoxia in AQP4 and TRPV4 deficient mice and controls (see Chapter 4.1.2).
3. To determine the changes in the ECS diffusion parameters *in situ* in models of acute cell swelling (hypoosmotic stress and hyperkalemia) in order to elucidate the role of AQP4, TRPV4 and AQP4/TRPV4 complex in volume regulation (see Chapter 4.2.1 to 4.2.3).
4. To detect the changes in the ECS diffusion parameters *in situ* during oxygen-glucose deprivation (model of ischemia) in AQP4, TRPV4 and AQP4/TRPV4 deficient mice and controls (see Chapter 4.2.4).

5. To evaluate the changes in the extracellular K^+ concentration *in situ* during oxygen-glucose deprivation in AQP4, TRPV4 and AQP4/TRPV4 deficient mice and controls (see Chapter 4.2.4.1).
6. To correlate *in situ* and *in vivo* results in AQP4, TRPV4 and AQP4/TRPV4 deficient mice and controls, we examined the subacute and early chronic changes implementing MRI measurements of the lesion volume at day 1 and day 7 following pMCAO (see Chapter 4.3.1 and 4.3.2).
7. To evaluate the ECM alterations following pMCAO, we conducted a PNN analysis (see Chapter 4.3.4).

3 MATERIALS AND METHODS

3.1 Theoretical background

3.1.1 Ion-selective microelectrodes

The ion-selective microelectrodes (ISMs) are implemented in the RTI measurements to measure the activity of ions in the fluid and convert them into electrical potential, which can be detected with a voltmeter. The ISMs tip is filled with a liquid electrochemical membrane (selectively permeable ion-exchanger) that separates the ISM internal solution from the external experimental solution. Diffusion of the permeant ion takes place through the membrane. This flux gives rise to a net movement of electrical charge, and electric potential difference is generated across the membrane. The Nernst equation describes the size of this membrane potential as:

$$E = E_0 + slog(a_i) \quad (1),$$

where E_0 is the reference potential in millivolts, s is the Nernst response (“slope”) [$s = (2.303 RT/zF)$, where R is the gas constant ($8.314 \text{ JK}^{-1}\text{mol}^{-1}$), T is the absolute temperature in kelvins K, F is the Faraday electrochemical equivalent ($9.6487 \times 10^4 \text{ Cmol}^{-1}$) and z is the charge of the ion] and a_i is the activity of the reference ion.

Coexisting ions interfere with the ion of interest; thus, for the measurements the Nernst equation is modified to Nicolsky-Eisenman equation:

$$E = E_0 + slog \left[a_i + \sum_j K_{ij}^{pot} (a_j)^{\frac{z_i}{z_j}} \right] \quad (2),$$

where E is a measured potential, E_0 is a constant potential difference that includes various interfacial potentials between the glass wall and electrolyte; s is Nernst response (‘slope’, explained above), a_j/a_i is the activity of j^{th}/i^{th} ion (where i is the measured ion and j is the interfering ion), respectively. K_{ij}^{pot} is the potentiometric selectivity coefficient.

The ISM senses also biopotentials from the whole surrounding environment; thus, second (reference) barrel of the ISM assembly measures these stable biopotentials (action or synaptic potentials, for example), which are later subtracted from the signal (Nicholson, 1993).

Lux and Neher invented the method in 1973 using a K^+ -ISM. Later, in 1981, Nicholson and Phillips defined the real-time iontophoretic method using TMA^+ -ISM (Neher and Lux, 1973; Nicholson and Phillips, 1981).

3.1.1.1 Calculation of the ECS diffusion parameters

Within the ECS, the main mechanism of molecular transport is diffusion. The molecules in a free water medium follow Brownian motion, which is described by Fick's laws. This movement is a result of molecule diffusion in random directions and collisions with molecules of the solvent. A substance naturally following a random path from the regions with its higher concentration to the regions with its lower concentration.

The Fick's laws describe diffusion in the free medium such as water. However, diffusion through the brain is hindered by many obstacles that slow down the velocity of diffusion and prolong the path taken by the substance. The mentioned diffusion equations are obtained from Nicholson and Phillips (Nicholson and Phillips, 1981) and are based on the Fick's first law, which defines the microscopic Brownian motion of ions on macroscopic level.

The microscopic flow of diffusing molecules (J) in the direction of the concentration gradient is presented as a vector for which the following equation applies:

$$J = -D\nabla C_0 \quad (3),$$

where D is the diffusion coefficient of the solute in water and C_0 is its extracellular concentration, while here is used ∇C_0 as a concentration gradient.

Brain tissue is more complex and the ECS geometrical structure and the present molecules limit the free diffusion, and the diffusing substance behaves differently than in the free medium. The diffusion in the brain, therefore, may be described by the introduction of the diffusion parameters. The extracellular volume fraction α is defined as:

$$\alpha = \frac{V_0}{V} \quad (4),$$

where V represents the volume of the tissue and V_0 is the volume of the ECS. In aqueous solution or dilute gel α is supposed to be equal to 1.

Tortuosity defines the hindered diffusion of the substance in the ECS, and it is defined as:

$$\lambda^2 = \frac{D}{D^*} \quad (5),$$

where D is a diffusion coefficient measured in a free medium ($\text{cm}^2.\text{s}^{-1}$) and D^* is an effective diffusion coefficient measured in tissue.

Lastly, uptake (k') represents the loss or clearance of the substance from the ECS into cells, across the BBB, or by degradation.

From Equation (3), the equation for isotropic homogeneous porous medium, introducing the ECS diffusion parameters, is defined as:

$$J = -\alpha D^* \nabla C_0 \quad (6),$$

where α is a volume fraction, D^* is an effective diffusion coefficient and ∇C_0 is the concentration gradient of a substance in the ECS.

Due to the distribution of experimental molecules in a representative elementary volume of brain tissue, a form of ‘volume averaging’ is applied. In experimental work, where diffusion measurements are made over distances of the order of 100 μm the local structural properties are averaged, and the following macroscopic equation applies:

$$\frac{\partial C}{\partial t} = D^* \nabla^2 C + \frac{Q}{\alpha} - \mathbf{v} \nabla C - \frac{f(C)}{\alpha} \quad (7),$$

where C is the actual concentration of a probe in the interstitial space, and is a function both of time t and position. The position is represented by the vector $\mathbf{v} = (x, y, z)$ or radial coordinate r in a spherically symmetric system. The symbol ∇ and ∇^2 symbolize the first and second spatial derivatives in the appropriate coordinate system. α is volume fraction, D^* is an effective diffusion coefficient, and C is the concentration of the substance. Q is the volume average of the source density in the medium (the number of molecules/ions per unit of volume). The source term Q is divided by the volume fraction α , reflecting the fact that molecules released into the ECS are restricted to a smaller volume than if they had access to the entire brain tissue. The third term represents the contribution of bulk flow. Flow is defined by its velocity vector $\mathbf{v}$ and it forms a scalar product with the concentration gradient ∇C . Bulk flow have little influence on short-term and near-distance diffusion and may be, therefore, neglected. The final term, $f(C)$, represents loss or clearance of the probe.

The source term can be further defined as:

$$Q = \frac{In}{F} \quad (8),$$

where I is the current, n is the transport number of the ISM and F is Faraday’s electrochemical equivalent.

When the RTI method is used, a micropipette releases TMA^+ , or another suitable probe ion, by iontophoresis with constant source strength Q into either brain tissue or an agarose gel. An ISM located about 100 μm from the release point measures the increasing concentration of the probe ion during the iontophoretic pulse as a function of time according

to equation (9), which is the solution to equation (7) in a spherically symmetric coordinate system:

$$C(t) = \frac{Q\lambda^2}{8\pi D\alpha r} \left[\exp\left(r\lambda\sqrt{\frac{k'}{D}}\right) \operatorname{erfc}\left(\frac{r\lambda}{2\sqrt{Dt}} + \sqrt{k't}\right) + \exp\left(-r\lambda\sqrt{\frac{k'}{D}}\right) \operatorname{erfc}\left(\frac{r\lambda}{2\sqrt{Dt}} - \sqrt{k't}\right) \right] \quad (9),$$

where Q is the source strength (the amount of substance delivered per unit time into unit of tissue volume). Other parameters are tortuosity λ , volume fraction α , uptake k' and diffusion coefficient D . The function erfc is a complementary error function.

Concentration C is recorded at distance r from the origin ($r = 0$) where the source Q is located. The loss of probe ions from the ECS is represented by a first-order kinetic constant, k' ; this may include entry into cells or loss across the blood-brain barrier. When the loss of probe ion is negligible ($k' = 0$), equation (9) reduces to:

$$C(t) = \frac{Q\lambda^2}{4\pi D\alpha r} \operatorname{erfc}\left(\frac{r\lambda}{2\sqrt{Dt}}\right) \quad (10),$$

3.1.2 Basic principles of magnetic resonance imaging

MRI is an imaging technique that allows us to visualise internal structures in living animals. MRI makes use of the quantum-mechanical properties of nuclei with odd nucleus numbers, which have their own magnetic moment and, thus, may interact with an external magnetic field. For imaging, it is practical to select nuclei that are abundant in the measured tissues to detect a sufficient response to excitation. Such nuclei are ^1H , ^{13}C , ^{19}F , ^{31}P . The hydrogen nucleus is the most suitable and is widely used in biology and medicine imaging. It has great sensitivity and is present in most important structures, including water that has two hydrogen nuclei.

The magnetic moments of individual nuclei are randomly orientated in space. When the sample is placed into the external magnetic field, the average magnetic moment of a high percentage of protons becomes aligned with the direction of this field. The protons align in two possible orientations: parallel (low energy state) and antiparallel (high energy state) to the applied magnetic field. A slightly greater number of protons exist in the parallel direction, resulting in a measurable net magnetic moment (B_0). In addition to different energy states of the parallel and antiparallel spin, the magnetic moments of individual nuclei experience a periodic tilted motion called precession. All nuclei in a single magnetic field precess at the same angular frequency, which is known as the Larmor frequency and is given by the equation:

$$\omega_0 = \gamma B_0 \quad (11),$$

ω_0 is frequency of rotation (the number of rotations per second); γ is the gyromagnetic ratio (Larmor constant), which describes the ratio of mechanic and magnetic properties of the nucleus; B_0 is the strength of the magnetic field. The constant γ is the gyromagnetic ratio that represents the precession frequency of the nuclei in a field intensity of 1 T.

During the resting conditions, moments of the precession movement cancel out; however, when the tissue is exposed to the radiofrequency (RF) waves the magnetic dipole moments (MDMs) of individual nuclei begin to precess in phase, leading to formation of a macroscopic magnetic moment. The RF pulse is tuned to the resonance (Larmor) frequency and shifts protons from the low-energy state (parallel) to higher energy states. Depending on the amount of applied energy, it may result in a switch from the longitudinal (z axis) macroscopic magnetic moment to the transverse magnetic moment (y axis) or to reverse of the longitudinal magnetic moment against the direction of the external magnetic field. The electromagnetic pulse that promotes this process is referred to as a 90-degree (Fig. 10) or 180-degree pulse, respectively. When the radiofrequency field is turned off, the MRI sensors are able to detect energy released as the protons that are realigning with the magnetic field, in a process called relaxation. Different tissues relax at different rates when the transmitted RF pulse is switched off. The time taken for the nuclei to fully relax is measured in two ways. The first is the time taken for the magnetic moment to return to its resting state (longitudinal orientation), and the second is the time needed for the precession to return to its resting state (dephasing of the spin). The first is called T1 relaxation, the second T2 relaxation (Fig. 10).

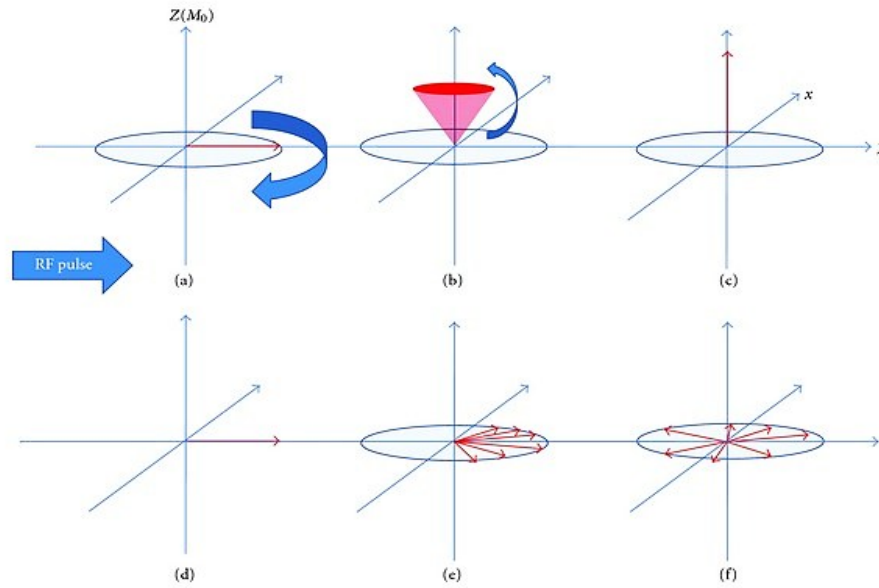


Fig. 10 A scheme of T1 and T2 relaxation. Magnetic resonance imaging utilises the behaviour of protons, within varying magnetic fields, to produce signals that are convertible into images. The net magnetisation of hydrogen nuclei in the brain is imagined as a vector (in the z and x-y planes) representing the strength and direction of its magnetic field as it spins around its axis (its magnetic dipole moment, MDM). The MDMs of protons align with the external magnetic field of the scanner (referred to here as M_0 shown along the z axis). When a short radiofrequency (RF) pulse is applied, it shifts the MDMs of protons from alignment in the z direction into the x-y plane (a, d). During the relaxation phase, the vector amplitude on the z-axis will gradually increase (b, e), while the vector amplitude in the x-y plane slowly decreases (c, f). T1 relaxation is the process by which the net magnetisation returns to its initial maximum value (M_0)(longitudinal orientation, z axis), a parallel to B_0 . T2 relaxation is the process by which the transverse components of magnetisation (x-y planes) decay or dephase. From Silva et al. (2012)

To acquire images with better contrast, pulse sequences are usually employed. A fundamental pulse sequence is the spin-echo sequence, which begins with a 90-degree pulse to excite a measurable response. Magnetic field inhomogeneities degrade the signal-to-noise ratio of MR measurements, and the signal decays with a higher rate of T2, corresponding to the T2* time constant. After a time (t) from the first pulse, a 180-degree pulse is applied and the transverse vectors are flipped 180°. The time at which this echo is produced is the TE (time to echo). It is produced at exactly $2t$, t being the time at which the 180° RF pulse is applied (i.e. the 180° RF pulse is applied at TE/2). At TE the vectors return to be in phase, the rapidly dephasing protons catch up with slowly dephasing protons and an echo is produced.

3.1.2.1 Diffusion-weighted imaging

Diffusion-weighted magnetic resonance imaging (DW-MRI), in which water diffusion characteristics are determined through the generation of water apparent diffusion coefficient (ADC_w) maps, utilises standard spin echo sequence. The tissue with higher water experience greater signal loss when magnetic field gradients are used in experimental sequence.

A single $T2^*$ weighted image after a 90° pulse, known as the $b=0$ image, is obtained, serving as a baseline. In the sequence, two diffusion gradients are added before and after the 180° RF pulse. The first diffusion gradient dephases the spins. The second, symmetrical, diffusion gradient rephases and returns a signal only from the stationary spins that have remained within the area. Spins that have moved out of the area are not rephased, fall out of phase, and lose signal. The diffusion gradient is applied in multiple directions. The minimum number of directions is 3 running perpendicular to each other (e.g. x-, y-, and z-axes). Immediately following the second diffusion gradient, an image acquisition module is played out. The intensity of each image element (voxel) reflects the best estimate of the rate of water diffusion at that location.

The b -value is an important parameter determining the sensitivity of the resulting images to diffusion of water molecules. Higher b -values correspond to stronger diffusion weighting and more sensitive images and vice versa. A larger b -value is achieved with increasing the gradient amplitude and duration and by widening the interval between gradient pulses. The pulse sequence described above is called the Stejskal-Tanner scheme (Stejskal and Tanner, 1965). The images can be arithmetically combined to generate diffusion-weighted maps and ADC maps.

The ADC value is obtained from the equation:

$$ADC = -\frac{1}{b} \ln \left(\frac{S_{DWI}}{S_{b=0}} \right) \quad (12),$$

where ADC is an apparent diffusion coefficient, b is b value, S_{DWI} is the signal intensity of isotropic diffusion weighted imaging and $S_{b=0}$ is signal intensity of $b=0$.

3.2 Experimental setup and protocols

3.2.1 Animals

Transgenic mice of postnatal age P80–120, both male and female, were used in the experiments. The exact number of animals used in each experiment is indicated in the results. As previously described by Nolte et al., mice astrocytes were fluorescently labelled and expressed EGFP under the control of the human GFAP promoter (line designation TgN(GFAP-EGFP), FVB background)(Nolte et al., 2001). This procedure was performed to allow parallel experiments in which visualisation of astrocytes was necessary for morphometric analysis. The mice were either cross-bred with the Trpv4-deficient strain (on C57BL/6N background) with excised exon 12 encoding transmembrane pore domains 5 and 6 (Liedtke and Friedman, 2003), or with Aqp4-deficient mice (genetic background B6 mixed with Balb/c). The embryos of the Aqp4-deficient mice were obtained from Riken BRC (acc.no. CDB0758K-1; <http://www.cdb.riken.jp/arg/mutant%20mice%20list.html>) and breeding lines were established through embryo transfer in The Czech Centre for Phenogenomics (Vestec, Czech Republic). Homozygous Trpv4⁻, and Aqp4-deficient lines were established together with animal lines deficient in both channels, homozygous Aqp4/Trpv4-positive line was used as control (Ctrl). All procedures involving laboratory animals were performed in accordance with the Council Directive 2010/63EU of the European Parliament and the Council of 22 September 2010, on the protection of animals used for scientific purposes and animal care guidelines approved by the Institute of Experimental Medicine, Academy of Sciences of the Czech Republic (Animal Care Committee on April 7, 2011; approval number 49/2019). All efforts were made to minimise both the suffering and the number of animals used.

3.2.2 Experimental solutions

The composition of the isolation solution, artificial cerebrospinal fluid (aCSF) and solutions modelling hypoosmotic stress (aCSFH-50 and aCSFH-100), hyperkalemia (aCSFK⁺), and oxygen-glucose deprivation (OGD) are listed in Tab. 1. All solutions, except OGD, were equilibrated with 95% O₂ and 5% CO₂ (Carbogen, Siad, Branany, Czech Republic) to achieve a pH of 7.4. The OGD solution was saturated with 5% O₂, 5% CO₂, and 90% N₂. The osmolality was measured using a vapour pressure osmometer (Vapro 5520, Wescor, Logan, USA).

Tab. 1 Contents of experimental solutions.

Compounds	aCSF [mM]	Isolation solution [mM]	aCSF _{H-50} [mM]	aCSF _{H-100} [mM]	aCSF _{K⁺} [mM]	OGD [mM]
NaCl	122	-	98	67	75	122
NMDG	-	110	-	-	-	-
KCl	3	2.5	3	3	50	3
NaHCO ₃	28	24.5	28	28	28	28
Na ₂ HPO ₄	1.25	1.25	1.25	1.25	1.25	1.25
Glucose	10	20	10	10	10	-
CaCl ₂	1.5	0.5	1.5	1.5	1.5	1.5
MgCl ₂	1.3	7	1.3	1.3	1.3	1.3
HEPES	-	-	-	-	-	-
Osmolality (mOsmol/kg)	~300	~300	~250	~200	~300	~300

Abbreviations: aCSF, artificial cerebro-spinal fluid; aCSFH-50, 250mOsmol hypotonic solution; aCSFH-100, 200mOsmol hypotonic solution; aCSFK⁺, 50mM K⁺ solution; NMDG, N-methyl-D-glucamine; OGD, oxygen-glucose deprivation.

3.2.3 Models of cytotoxic edema and ischemia

3.2.3.1 Terminal ischemia/anoxia in vivo

Cardiac arrest was induced by intraperitoneal injection (i.p.) of 0.5 ml of saturated MgCl₂. An electrocardiograph recording confirmed that heart arrest occurred.

3.2.3.2 Acute cell swelling in situ

Mice were deeply anesthetised with sodium pentobarbital (100 mg/kg, i.p.; Sigma 208 Aldrich, Germany) and perfused transcardially with ice-cold (~4°C) isolation solution. The brains were dissected, placed into the ice-cold (~4°C) isolation solution, and cut into 400 µm thick coronal brain slices using a HM 650 V microtome with a vibrating blade (MICROM Int. GmbH, Waldorf, Germany). For recovery, the slices were kept for 1 hour in a continuously bubbled (with 95% O₂ and 5% CO₂) aCSF solution at room temperature. Individual brain slices (from region 1.06 to 2.7 caudal from Bregma) were then placed under the upright Zeiss microscope into the experimental chamber (Luigs and Neumann, Germany) perfused with aCSF enriched with 0.1 mM TMA⁺ flowing at a rate of 4 ml/min, which was continuously bubbled with 95% O₂ and 5% CO₂.

The ECS diffusion parameters were measured in acute brain slices, which were exposed to four different models of brain pathological conditions, resulting in osmotic disbalance and cell swelling – hypoosmotic aCSF of 250 or 200 mOsm/l (aCSFH-50 or aCSFH-100, respectively); aCSF solution with 50mM K⁺ (aCSFK⁺) and solution for oxygen-glucose deprivation (see Tab. 1). The data were averaged from the experiments on 48 Ctrl, 39 AQP4^{-/-}/TRPV4^{-/-}, 43 AQP4^{-/-} and 46 TRPV4^{-/-} mice, totally.

Previous studies (Salman et al., 2017; Shibasaki et al., 2015) demonstrated that TRPV4 activity is inhibited at room temperature. To activate the TRPV4 channels and maintain viable slices we raised the temperature of the bath solution to 33°C, which represents a compromise value, that activates the TRPV4 channels but does not harm the slices. After stabilisation of the ECS diffusion parameter values, we began the 20-min application of aCSFH-50, aCSFH-100, OGD, or aCSFK⁺ solutions followed by a 20-min washout with aCSF.

3.2.3.3 Permanent middle cerebral artery occlusion

Mice were anesthetized using a vaporiser (Tec-3, Cyprane Ltd., Keighley, UK) with 2% isoflurane (Abbot, IL, USA). A cut between the orbit and the external auditory meatus was performed and 1–2 mm hole was drilled through the frontal bone. The exposed middle cerebral artery (MCA) was occluded by electrocoagulation with bipolar tweezers (SMT, Czech Republic). To ensure permanent vessel occlusion, the MCA was later transected. During surgery, body temperature was maintained at 37 ± 1 °C using a heating pad (SurgiSuite, Kent Scientific, USA).

3.2.4 Preparation of ion-selective microelectrodes

Ion-selective electrodes were fabricated from borosilicate capillary glass (Ruckl and sons, Otovice) 2.5 mm in diameter and 11 cm long. The tubes were halved, and one long and one short tube were combined (fixed with a polyethylene ring). The glass melts at relatively low temperatures, so the electrode pair may be twisted around each other by 360°, while heated over a burner. Next, two double-barreled microelectrodes were obtained by pulling the tube assembly on a vertical puller (Narishige, Japan). The glass surface is highly hydrophilic and, therefore, repels organic liquids, such as ion-exchanger. Therefore, it has to be modified by applying a thin layer of the silane to the tip of inner surface of the ion-selective channel in a process of silanization. We silanized the ISMs by exposing them to vapours of the silicone compound (e.g., hexamethyldisilazane) in a closed chamber at high

temperature, while the reference channel was prefilled with distilled water (protecting the tip from silanization). The electrodes were inserted over a continuously heated (45-50°C) hexamethyldisilazane for 20 minutes and then baked for 20 minutes in the oven (200°C).

3.2.4.1 ISMs for TMA⁺ measurements

A tip of the ISM was broken against a glass tube under the microscope (at 2-3 μm) and the reference barrel was backfilled with 150 mM NaCl solution. The ISM was dipped into ion exchanger (IE 190, World Precision Instruments, RRID:SCR_008593) for 10-30 seconds and the process was completed by back-filling of the ion-selective barrel with 100 mM TMA⁺-chloride solution. Potential air bubbles were removed by electrocauter. TMA⁺ ions, in low (micromolar) concentrations, are not toxic and do not cross the membrane. Therefore, TMA⁺ is a good candidate for monitoring dynamic changes in the ECS (Lehmenkuhler et al., 1993; Nicholson and Phillips, 1981).

The iontophoretic electrodes were made from theta-capillary double barrel borosilicate glass (EC1 30-0118, model TGC200-15, Harvard apparatus). The capillary was pulled on a vertical puller (Narishige, Japan) and bent to circa 40° by heating the tip. Next, the tip was broken at 2-3 μm against a glass under the microscope and both barrels were back-filled with 100 mM TMA⁺-chloride solution.

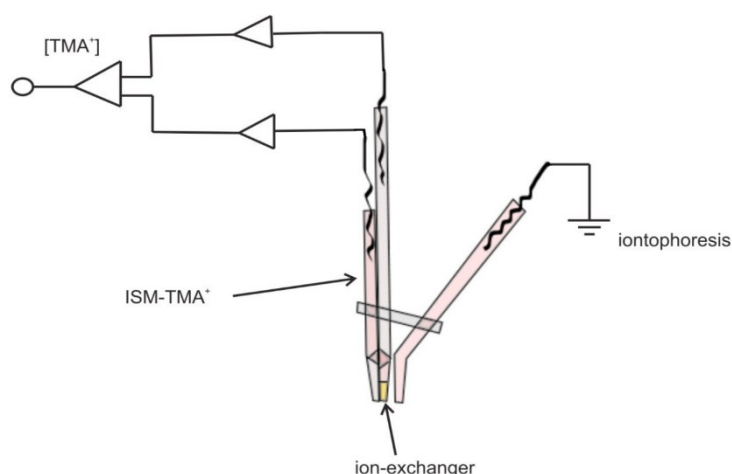


Fig. 11 Scheme of the ion-selective microelectrode. The electrode used for RTI method consists of the double-barreled ISM (on the left) and the iontophoretic electrode (on the right). The ISM is made of two barrels – reference, filled with 150 mM NaCl (grey) and ion-selective one with ion-exchanger at the tip (yellow) back-filled with a solution of 100 mM TMA⁺ chloride (pink). The iontophoretic electrode contains the solution of 100 mM TMA⁺ chloride (pink). The electrodes are connected to the differential amplifier and iontophoresis via silver chlorinated wires. **Abbreviations:** ISM, ion-selective microelectrode; RTI, real-time iontophoretic method; TMA⁺, tetramethylammonium ion. Source: author.

3.2.4.2 ISMs for detection of potassium concentration

ISM for measurements of potassium concentration were fabricated as described in Chapter 3.2.4. The tip of the ion-selective barrel was filled with the liquid ion-exchanger IE 190 (WPI, Inc., Sarasota, USA, RRID:SCR_008593) and back-filled with 100 mM KCl, whereas the reference barrel contained 150 mM NaCl.

3.2.4.3 Calibration of the ion-selective microelectrodes

The ISMs (for TMA⁺ and K⁺ concentration measurements) were calibrated in 5 solutions with different concentration of the measured ion with the respective background of NaCl to keep the ionic strength of the solution constant. The data were fitted to the Nikolsky equation to determine the electrode slope and interference utilising the VOLTORO software (kindly provided by C. Nicholson, New York University School of Medicine, USA, unpublished data).

The electrodes for the RTI measurements were calibrated in 0.1% agar (agarose gel with 1 mM TMA⁺ in physiological saline containing 150 mM NaCl and 3 mM KCl) to acquire the values of tip separation (distance in μm) and electrode transport number by fitting the diffusion curve. Transport number of the electrode should be in the range 0.3-0.5. The occurrence of a smaller transport number could mean a blockage of a channel, while a higher number indicates a significant bulk flow.

3.2.5 Experimental protocols for ISMs experiments

The reference and ion-selective channels were connected by silver chlorinated wires (Ag-AgCl) with a low-noise differential amplifier (Ujec, 1988) and the whole apparatus was grounded.

3.2.5.1 Acquisition of the ECS diffusion parameters in vivo and in situ

The iontophoretic electrode was connected to a generator of rectangular electric current (Single Channel Iontophoresis Generator ION - 100; Dagan Corporation, Minneapolis, Minnesota, USA). The iontophoresis parameters involved a +20 nA bias current that was continuously applied to maintain a constant transport number, with a +200 nA current step of 24 s duration to generate the diffusion curve. A digital oscilloscope (model 310, Nicolet Instrument Corporation) was employed to capture the diffusion curve and the curve was fitted utilising the VOLTORO program. The values of α , λ and k' were calculated by the program. The examples of the diffusion curves recorded in the agar and brain tissue are shown in (Fig. 12).

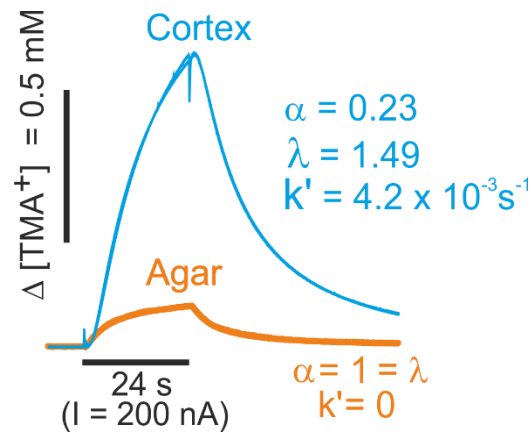


Fig. 12 Representative TMA⁺ diffusion curves obtained in agar gel or neocortical tissue.

Representative TMA⁺ diffusion curves with corresponding values of the ECS volume fraction (α) and tortuosity (λ) recorded in agar gel (orange) and cortical tissue (blue) mice. Each experimental diffusion curve is superimposed on a theoretical one, which was analysed for the calculation of the ECS diffusion parameters. The amplitude of the diffusion curve is indirectly proportional to the α value, and the shape of the curve reflects the tortuosity value. Source: author.

In experiments *in vivo*, following a series of control measurements during normoxia, the ECS diffusion parameters were recorded at 4-min intervals up to 32 min after the induction of terminal ischemia by cardiac arrest.

In experiments *in situ*, diffusion curves were captured and analysed every 5 min in the cortex of acute brain slices at a depth of 200 μ m. The slices were perfused by either aCSF or four different solutions modelling cytotoxic cell swelling (see Tab. 1). To verify that all slices are viable and non-ischemic, the diffusion experiments *in situ* were first performed at room temperature (24°C), and the obtained values were compared to our database of the extracellular diffusion parameter values from the previous studies during normoxia and anoxia; if the value of α was lower than 0.10 and, concomitantly, λ was higher than 1.80, the slice was considered to be anoxic. Before the application of the experimental solutions, we raised the temperature of the bath solution to 33°C order to activate the TRPV4 channels and maintain viable slices.

3.2.5.2 Measurements of the extracellular potassium concentration during terminal ischemia/anoxia *in vivo* and during OGD *in situ*

The potassium electrode response signal was registered using the multifunction DAQ device USB-6001 (National Instruments, Austin, USA) and the NI LabVIEW Signal Express

2015 software. The sampling frequency was 200 Hz. The voltage was captured every 1 min. Based on the electrode characteristics, the measured voltage was converted to $[K^+]_e$ values implementing modified Nernst equation. Location of the K^+ and the experimental protocol *in vivo* and *in situ* corresponded with those implemented for the ECS diffusion parameter measurements as described above.

3.2.6 Magnetic resonance imaging after pMCAO

Magnetic resonance imaging after pMCAO was implemented to determine the extent of subacute and early chronic post-ischemic damage and to correlate the RTI measurements *in situ* with *in vivo* experiments. The pMCAO surgery and MRI measurements were performed by Denisa Kirdajova and Sara Camacho Garcia.

3.2.6.1 Magnetic resonance imaging

The volume of the ischemic lesion 1 day (D1) and 7 days (D7) after pMCAO was quantified by T2-weighted MRI. Additionally, DW-MRI was performed to determine the ADC_w at D1. Daniel Jirak and Eyar Shany performed the MRI and processed and analysed the data. Ivan Vorisek performed and analysed the DW-MRI.

MRI experiments were performed at a 4.7 T MR scanner (Bruker BioSpec, Ettlingen, Germany) equipped with a head homemade surface coil and with a 200 mT/m gradient system (190 μ s rise time). During MRI experiments, the animals were anaesthetised with isoflurane (1.5% in a mixture of 40% O₂ and 60% N₂O) and placed in a heated mouse holder. Nine T2-weighted sagittal images (TE of 20 ms, TR of 2.4 s, 4 acquisitions, field of view - FOV 1.92 x 1.92 cm², matrix size 256 x 128, slice thickness of 0.8 mm, interslice distance 1.2 mm) were used to position the coronal slices for T2-weighted and DW-MRI measurements.

The **T2-weighted MRI** was performed employing 2D Rapid Acquisition with a Relaxation Enhancement (RARE) and a multi-spin echo sequence, allowing the acquisition of T2-weighted coronal and axial images. The basic sequence parameters were: Repetition Time (TR)=3300 ms, effective echo time (TE_{eff})=36 ms, number of acquisitions (NA)=1 (axial plane) and 4 (coronal plane), acquisition time=1 min 19 s (axial plane) and 5 min 16 s (coronal plane), slice thickness=0.6 mm, turbo factor=8 and spatial resolution 137 × 137 mm², excitation and refocusing pulses: Hermite with 2700 Hz bandwidth.

DW-MRI measurements were performed as previously described (Sykova et al. 2005) using an experimental magnetic resonance spectrometer BIOSPEC 4.7 T system (Bruker, Ettlingen, Germany) equipped with a 200 mT/m gradient system (190 μ s rise time) and a homemade head surface coil. A coronal slice was acquired using the following parameters: time interval between gradient pulses Δ = 30 ms, b factors = 136, 329, 675, 1035, 1481 and 1825 s/mm², TE = 46 ms, TR = 200 ms, field of view 1.92 x 1.92 cm², matrix size = 256 x 128, one 0.8 mm thick coronal slice. DW-MRI images were measured using the stimulated echo sequence and the diffusion gradient direction pointed along the rostrocaudal direction.

3.2.7 Immunohistochemistry

Controls and experimental mice after pMCAO were anesthetised with sodium pentobarbital (100 mg/kg, i.p.), and transcardially perfused with 20 ml of physiological solution containing heparin (2500 IU/100 mL; Zentiva, Prague, Czech Republic) followed by perfusion with 20 ml of 4% paraformaldehyde. The brains were dissected, post-fixed in 4% paraformaldehyde overnight, and placed stepwise in solutions with gradually increasing sucrose concentrations (10%, 20%, 30%) for cryoprotection. Coronal slices (30 μ m) were prepared using Hyrax C50 cryostat (Zeiss, Gottingen, Germany).

Upon staining, the slices were incubated in a blocking solution containing 5% ChemiBLOCKER (Merck, Darmstadt, Germany) and 0.5% Triton X-100 (Merck, Darmstadt, Germany) in phosphate buffer saline (PBS) for 1 hour. Slices with primary antibody against the Ca²⁺-binding adapter molecule (Iba1; diluted 1:500, Ab178846, Abcam, Cambridge, UK) diluted in a blocking solution were incubated overnight at 4 °C, followed by a 2-hour incubation with species-specific secondary antibodies diluted in a blocking solution at room temperature. The corresponding secondary antibody (goat anti-rabbit IgG conjugated with Alexa-Fluor 594, Thermo Fisher Scientific, Waltham, MA, USA) was diluted at 1:200.

The primary GFAP antibody (diluted 1:800, conjugated to Cy3, C-9205, Sigma Aldrich, Merck, Darmstadt, Germany) is conjugated with Cy3 dye, thus, no secondary antibody was required.

For the WFA antibody staining, slices were preincubated in PBS containing 1% H₂O₂ for 10 minutes. Blocking was carried out in the PBS containing 0.3% Tritone and 3% goat serum for 2 hours. Incubation with primary antibody (diluted 1:400, L1516, Sigma Aldrich, Merck, Darmstadt, Germany) was carried out overnight at 4°C. Slices were further incubated

with secondary antibody Streptavidin 488 (diluted 1:400, S32354, Thermo Fisher Scientific, Waltham, MA, USA) for 2 hours at 4°C.

After immunostaining, the slices were mounted onto microscope slides using Aqua-Poly/Mount (Polysciences Inc, Eppelheim, Germany).

The images were obtained using a confocal laser scanning microscope (Zeiss LSM5DUO, Carl Zeiss, Mainz, Germany) with a 20x oil objective. The laser intensity, gain, offset, and pinhole settings were kept constant for all the images. Semi-automatic quantitative analysis of WFA staining was performed utilizing automated software Polygon AI (RRID:SCR_022148, Rewire AI, Portland, OR, USA, <https://rewireneuro.com/polygonai>), which was developed based on the previous ImageJ (RRID:SCR_003070) macro plug-in PIPSQUEAK (Slaker et al., 2016). The brightness intensity of at least 15 PNNs/animal was analysed, while each PNN was assigned to one of three categories of staining intensity: high (staining intensity higher than 75th percentile of control data), medium, low (staining intensity below 25th percentile of control data). The contingency tables with data to control animals were created to display the distribution of the PNNs staining intensity.

3.2.8 Statistical analysis

3.2.8.1 Measurements of the ECS diffusion parameters and potassium concentration

Data are expressed as mean $\pm$ SEM, N represents the number of animals in the group, and n represents the number of applications in individual slices. Statistical analysis was performed using the R-project statistical package (R Core Team, R Foundation for statistical Computing, Vienna, Austria, <http://www.R-project.org>, RRID:SCR_001905) using N for *in vivo* measurements and n for *in situ* measurements. If not stated otherwise, all datasets conform to the assumption of ANOVA model and Tukey's test, i.e. normal distribution and variance homogeneity verified by a D'Agostino-Pearson omnibus K2 test.

For *in vivo* measurements, time course analysis of ECS parameters was performed by a two-tailed Student's t-test followed by Benjamini-Hochberg post-hoc test. Statistical analysis of the differences between the experimental groups was performed using one-way and two-way ANOVA analysis, followed by the Tukey HSD test. The P and F values of the ANOVA tests as well as an adjusted p value acquired by post-hoc test, are provided. Differences were considered significant if the value of P or p < 0.05.

For *in situ* experiments, the statistical analyses of the differences in ECS diffusion parameters among the experimental groups, and of the ECS diffusion parameter change within the experimental groups during applications, were performed using two-way ANOVA with Tukey's post-test. Differences were considered significant when the value of $p < 0.05$, very significant when $p < 0.01$, and extremely significant when $p < 0.001$.

3.2.8.2 MRI experiments

The image analysis of MRI experiments was performed using ImageJ software (version 1.48v, National Institutes of Health, USA). The volume of the lesion was measured in 23 mice by three experienced investigators. During ImageJ analysis, the edges of increased intensity areas were manually delineated for the axial and coronal T2-weighted images. The average pixel area of the two planes for each animal was calculated to determine the lesion volume.

Maps of the ADC_w were calculated using a custom-made software by a linear least square algorithm. Data are presented as mean $\pm$ SEM. Statistical analyses of group differences were performed using a one-way ANOVA and Bonferroni's post hoc test. The differences between the groups were considered statistically significant when $p < 0.05$, very significant when $p < 0.01$, and extremely significant when $p < 0.001$. N represents the number of mice used.

3.2.8.3 PNNs immunostaining

Data are presented as a count of PNNs assigned to each group or as a percentage distribution. Statistical analyses of group differences were performed using a R-project statistical package (R Core Team, R Foundation for statistical Computing, Vienna, Austria, <http://www.R-project.org>, RRID:SCR_001905) utilizing Fisher's exact test with Bonferroni correction for multiple tests. Differences between groups were considered statistically significant when $p < 0.017$ (due to Bonferroni correction). N represents the number of mice used, while n represents the number of PNNs.

4 RESULTS

4.1 The effect of terminal ischemia/anoxia *in vivo*

We performed *in vivo* experiments to measure the diffusion parameters after the induction of terminal ischemia/anoxia to determine the role of AQP4 and TRPV4 channels in acute cell swelling in severe pathological states *in vivo*. The total number of animals used for this method was 18; 6 mice in each experimental group. The ECG monitored cardiac arrest, which occurred in all animal groups within 4-5 min after i.p. administration of MgCl₂ ($F_{2,12} = 0.26$, $p = 0.773$).

4.1.1 ECS diffusion parameters during terminal ischemia/anoxia in AQP4 and TRPV4 knockout mice

We did not find any significant differences in the resting values of α , λ or k' between TRPV4^{-/-} and Ctrl mice, while the α value in the AQP4^{-/-} mice was significantly higher than in the controls ($F_{2,30} = 8.92$, $p < 0.001$)(Fig.13). Induction of ischemia/anoxia resulted in a decrease in α and an increase in λ in all animal groups ($F_{1,30} = 1238$, $p < 0.001$; $F_{1,30} = 1290$, $p < 0.001$, respectively). The final values were similar in all experimental groups (Fig. 13 and Tab. 2).

To evaluate the change rate and the time from ischemia induction to the fastest parameter change (inflection point), a four-parametric logistic (sigmoid) function was fitted on the time course of α and λ changes (Fig. 13d). The analysis revealed that the rate of α and λ change was significantly slower ($F_{2,15} = 46.2$, $p < 0.001$; $F_{2,15} = 36.7$, $p < 0.001$, respectively) and the time taken to the fastest parameter change was significantly longer ($F_{2,15} = 5.21$, $p < 0.03$; $F_{2,15} = 8.84$, $p < 0.02$, respectively) in AQP4^{-/-} and TRPV4^{-/-} mice when compared to the Ctrl animals (Fig. 13 and Tab. 2).

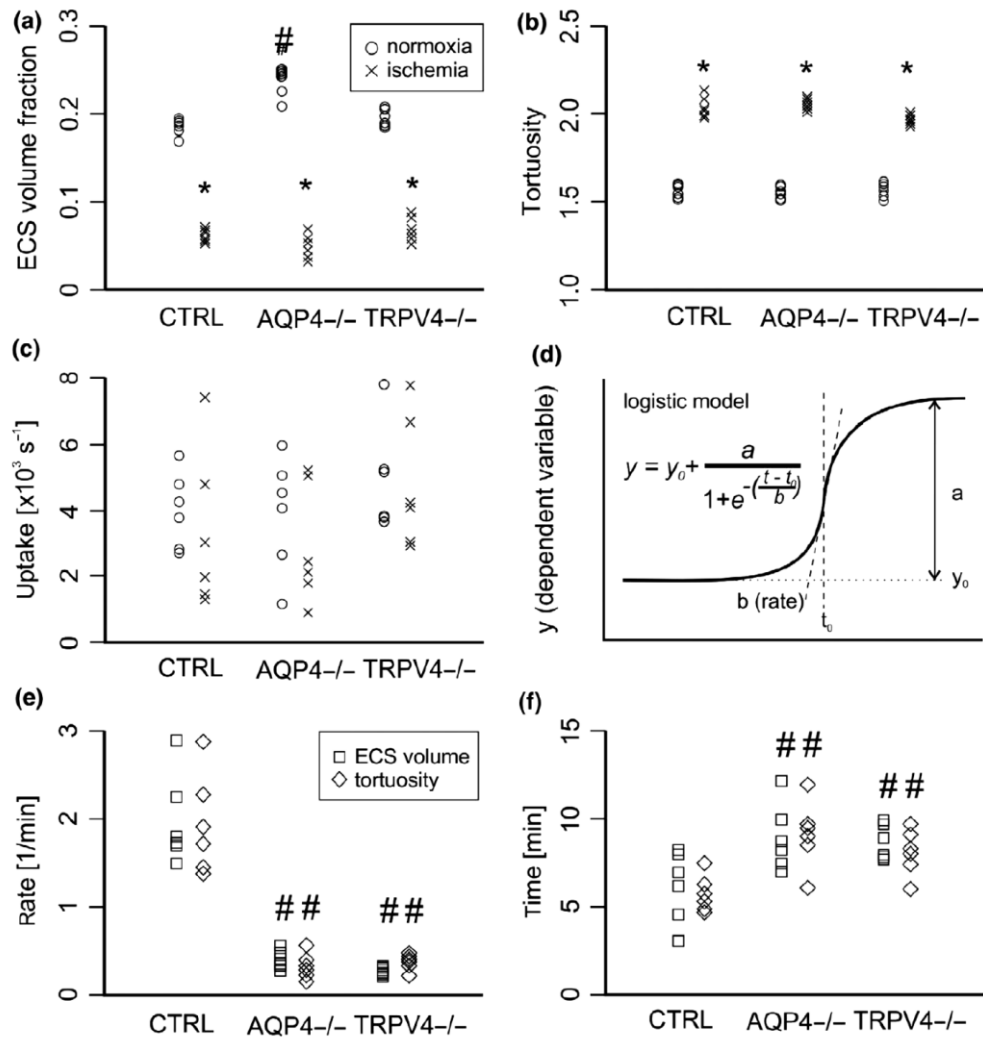


Fig. 13 ECS diffusion parameters *in vivo* before and after induction of ischemia. Values of the ECS volume fraction (a); tortuosity (b), non-specific uptake (c), sigmoid function (d) used for evaluation of the time course of changes. The change rate (e) and the time to the fastest changes (f) of α and λ . The rate of α and λ change was significantly slower and the time taken to the fastest parameter change was significantly longer in AQP4^{-/-} and TRPV4^{-/-} mice when compared to the Ctrl animals (CTRL)(e, f). Note the increased initial values of α in AQP4^{-/-} mice when compared to CTRL (a). Data are expressed as mean $\pm$ SEM. Statistically significant differences (*, $p < 0.001$) in parameters before and after induction of ischemia are marked by asterisks. Statistically significant differences (#, $p < 0.001$) among transgenic mice are indicated by a hashtag. **Abbreviations:** CTRL, control mice; AQP4^{-/-}, AQP4-deficient mice; TRPV4^{-/-}, TRPV4-deficient mice.

Tab. 2 The time and rate to the fastest change of ECS volume fraction and tortuosity after ischemia induction.

		Ctrl		AQP4 ^{-/-}		TRPV4 ^{-/-}	
		values	N	values	N	values	N
Rate (min⁻¹)	α	2.00 ± 0.25	6	0.34 ± 0.03*	6	0.23 ± 0.02*	6
	λ	1.95 ± 0.26	6	0.30 ± 0.06*	6	0.37 ± 0.04*	6
Time (min)	α	5.52 ± 0.88	6	8.49 ± 0.83*	6	8.34 ± 0.40*	6
	λ	4.91 ± 0.46	6	8.60 ± 0.83*	6	7.59 ± 0.57*	6

Data are expressed as mean ± SEM. Asterisks indicate statistically significant differences (*, $p < 0.001$) when compared to the control mice. **Abbreviations:** Ctrl, control mice; AQP4^{-/-}, AQP4-deficient mice; TRPV4^{-/-}, TRPV4-deficient mice; α , ECS volume fraction; λ , tortuosity; N, number of animals.

4.1.2 Extracellular potassium concentration during terminal ischemia/anoxia

We did not detect any significant differences in the basal values of $[K^+]_e$ among control, AQP4^{-/-} or TRPV4^{-/-} mice (3.3 ± 0.2 mM, 3.3 ± 0.2 mM, 3.6 ± 0.2 mM, respectively; $F_{2,12} = 0.42$, $p = 0.664$). After 32 min from the induction of terminal ischemia/anoxia the $[K^+]_e$ reached similar values in control, AQP4^{-/-} or TRPV4^{-/-} mice (72.6 ± 3.3 mM, 65.8 ± 2.3 mM, 69.5 ± 5.5 mM, respectively; $F_{2,12} = 0.69$, $p = 0.69$). The sigmoid function analysis of the time course of $[K^+]_e$ changes showed a slower change rate ($F_{2,12} = 178$; $p < 0.001$ for both control-AQP4^{-/-} and control-TRPV4^{-/-} comparisons) and a longer time to the fastest parameter change ($F_{2,12} = 37.0$; $p < 0.001$ for both control-AQP4^{-/-} and control-TRPV4^{-/-} comparisons) in AQP4^{-/-} and TRPV4^{-/-} mice when compared to control animals (Fig. 14).

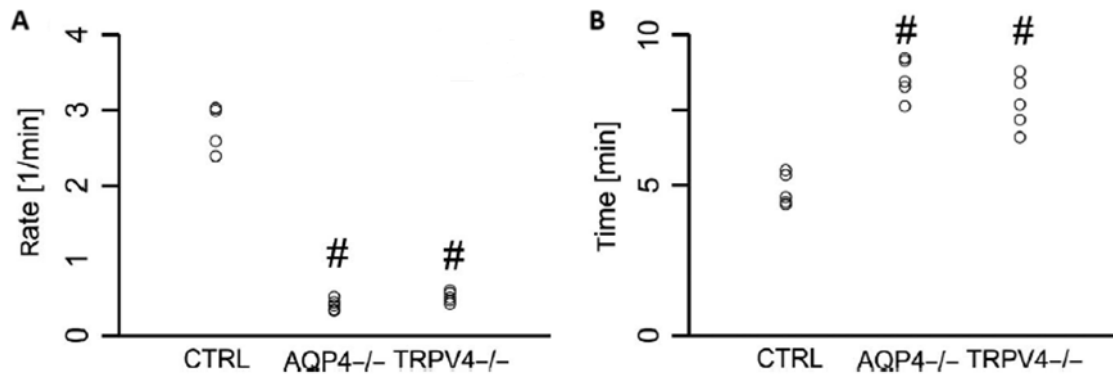


Fig. 14 Extracellular potassium concentration *in vivo* before and after induction of ischemia. The rate of change (A) and time to the fastest changes (B) in $[K^+]_e$ during the development of cerebral edema. The rate of $[K^+]_e$ change was significantly slower and the time taken to the fastest parameter change was significantly longer in AQP4^{-/-} and TRPV4^{-/-} mice when compared to the Ctrl animals. Data are expressed as mean $\pm$ SEM. Statistically significant differences (#, $p < 0.001$) among transgenic mice are indicated by a hashtag. **Abbreviations:** CTRL, control mice; AQP4^{-/-}, AQP4-deficient mice; TRPV4^{-/-}, TRPV4-deficient mice.

4.2 The effect of AQP4 and TRPV4 deletion on the diffusion parameters of the ECS in models of cytotoxic edema/ischemia *in situ*

The RTI method was employed to obtain the ECS diffusion parameters: α , λ , and k' in acute brain slices to study the tissue changes evoked by acute cell swelling/ischemia in control animals and mice lacking AQP4 and/or TRPV4 channels. The measurements were performed in the cortex and resting values were recorded to verify the viability of the tissue slice. The diffusion curves were then obtained every 5 min during a 20-min application of solutions modelling cytotoxic edema (hypoosmotic stress, hyperkalemia, OGD), as well as during the subsequent 20-min washout. The data were collected from 48 Ctrl mice, 46 AQP4^{-/-} mice, 43 TRPV4^{-/-} mice and 39 AQP4^{-/-}/TRPV4^{-/-} mice.

4.2.1 ECS diffusion parameters under resting conditions

Initially, resting values of α , λ , and k' were obtained from the cortex bathed in 22-24°C (room temperature, RT) aCSF. When the resting values confirmed the viability of the slice (see Section 3.2.5.1), the aCSF was heated to 33°C to activate the TRPV4 channels.

We showed a significantly higher resting value of α in AQP4^{-/-} mice compared to Ctrl animals, which persisted on a lower level of significance when the temperature was increased. On the other hand, the resting values of α from the TRPV4^{-/-} and AQP4^{-/-}/TRPV4^{-/-} mice did not differ significantly from the Ctrl animals (Fig. 15 and Tab. 3). We did not detect any significant alterations among the experimental groups in the resting values of λ or k' (Tab. 3).

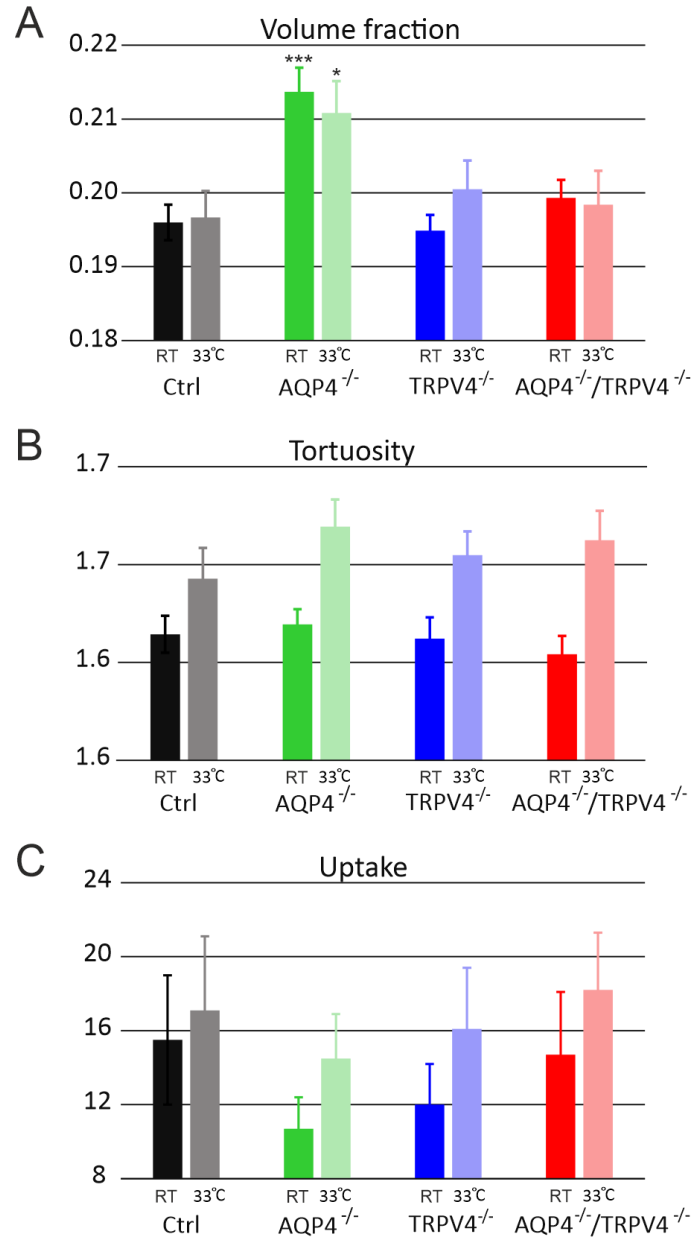


Fig. 15 ECS diffusion parameters *in situ* under resting conditions. (A) Resting values of the ECS volume fraction at room temperature (RT, 22-24°C) and at 33°C in all experimental groups. Note that the resting value at room temperature in AQP4^{-/-} mice is significantly increased compared to Ctrl animals and this significance is preserved after the temperature elevation to 33°C. (B) Resting values of tortuosity at RT and 33°C in all experimental groups. (C) Resting values of uptake at RT and 33°C

in all experimental groups. Data are expressed as mean $\pm$ SEM. The asterisks indicate significant differences between Ctrl and AQP4^{-/-} mice (*, p<0.05, ***, p<0.001). **Abbreviations:** Ctrl, control mice; AQP4^{-/-}, AQP4-deficient mice; TRPV4^{-/-}, TRPV4-deficient mice; AQP4^{-/-}/TRPV4^{-/-}, AQP4- and TRPV4-deficient mice; RT, room temperature (22-24°C).

Tab. 3 ECS diffusion parameters *in situ* under the resting conditions.

		Ctrl		AQP4 ^{-/-}		TRPV4 ^{-/-}		AQP4 ^{-/-} /TRPV4 ^{-/-}	
		values	N/n	values	N/n	values	N/n	values	N/n
α	RT	0.196 $\pm$ 0.002	33/48	0.214 $\pm$ 0.003	26/46	0.195 $\pm$ 0.002	25/43	0.200 $\pm$ 0.002	24/39
	33°C	0.201 $\pm$ 0.003	33/48	0.217 $\pm$ 0.004	26/46	0.202 $\pm$ 0.003	25/43	0.203 $\pm$ 0.004	24/39
λ	RT	1.614 $\pm$ 0.009	33/48	1.619 $\pm$ 0.008	26/46	1.612 $\pm$ 0.011	25/42	1.604 $\pm$ 0.009	24/39
	33°C	1.644 $\pm$ 0.013	33/48	1.665 $\pm$ 0.012	26/46	1.641 $\pm$ 0.011	25/42	1.657 $\pm$ 0.014	24/39
k' [10 ⁻³ s ⁻¹]	RT	15.5 $\pm$ 3.5	33/48	10.7 $\pm$ 1.7	26/46	12.0 $\pm$ 2.2	25/43	14.7 $\pm$ 3.4	24/39
	33°C	17.1 $\pm$ 4.0	33/48	14.5 $\pm$ 2.4	26/46	16.1 $\pm$ 3.3	25/43	18.2 $\pm$ 3.1	24/39

Data are expressed as mean $\pm$ SEM. **Abbreviations:** Ctrl, control mice; AQP4^{-/-}, AQP4-deficient mice; TRPV4^{-/-}, TRPV4-deficient mice; AQP4^{-/-}/TRPV4^{-/-}, AQP4- and TRPV4-deficient mice; RT, room temperature (22-24°C); α , ECS volume fraction; k' , uptake; λ , tortuosity; N, number of animals; n, number of experiments.

4.2.2 Hypoosmotic stress

Hypoosmotic stress was implemented as a model of cytotoxic edema. Two different solutions modelling either mild (aCSFH-50) or severe (aCSFH-100) hypoosmotic stress were used (Tab. 1). In all experimental groups, α was significantly reduced by the application

of both aCSFH-50 and aCSFH-100 ($p < 0.001$ in all groups) with no significant difference between the Ctrl mice and other experimental groups. Hypoosmotic conditions did not induce any significant changes among groups in either λ or k' (Fig. 16 and Tab. 4). During the washout period after mild hypoosmotic stress, the α gradually returned to the pre-application resting values in all experimental groups. To a lesser extent, a similar trend was observed following severe hypoosmotic stress (Fig. 16 and Tab. 4).

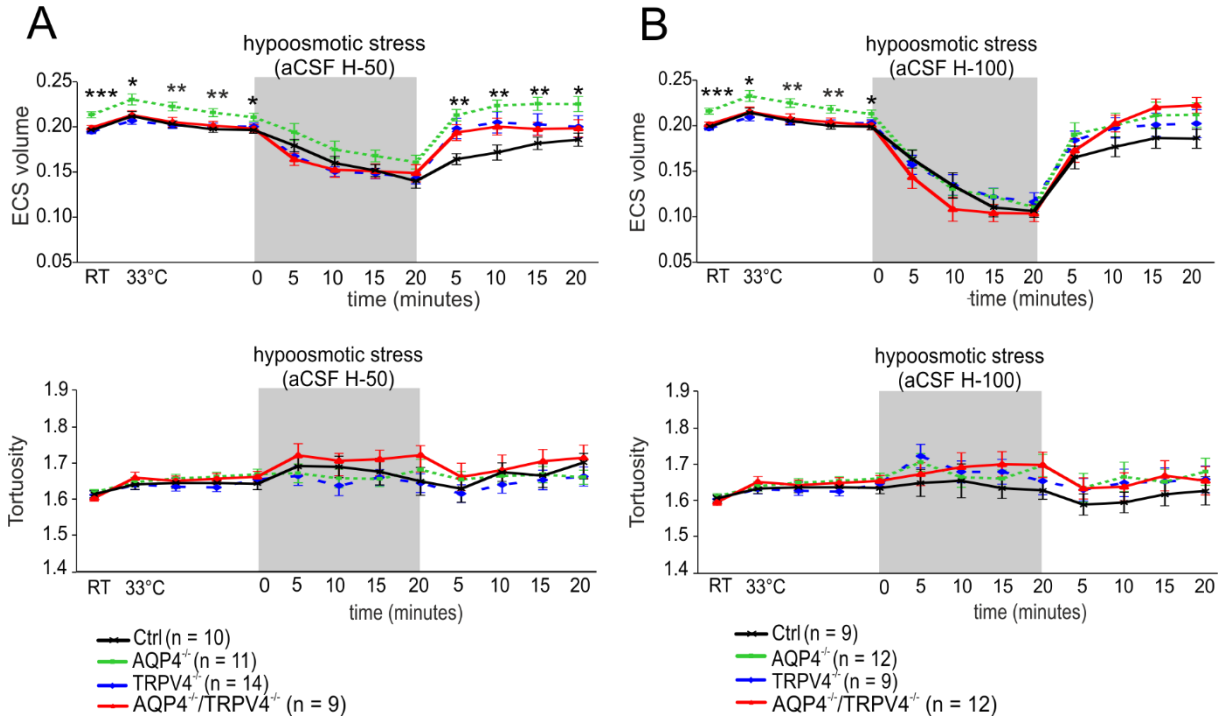


Fig. 16 The effect of hypoosmotic stress on ECS diffusion parameters *in situ*. (A) Averaged data of the ECS volume fraction (up) and tortuosity (down) measured at room temperature (RT) and at 5-minute intervals after the temperature increase to 33°C, during (grey area) and after the application of a mild hypoosmotic solution (aCSF H-50) in Ctrl, AQP4^{-/-}, TRPV4^{-/-} and AQP4^{-/-}/TRPV4^{-/-} mice. (B) Averaged data of ECS volume fraction (up) and tortuosity (down) measured at RT and at 5-minute intervals after the temperature increase to 33°C, during (grey area) and after application of aCSF H-100 in Ctrl, AQP4^{-/-}, TRPV4^{-/-} and AQP4^{-/-}/TRPV4^{-/-} mice. We did not observe any difference in response to mild or severe hypoosmotic stress between Ctrl mice and the knockout groups. Data are expressed as mean $\pm$ SEM. Asterisks indicate significant differences between Ctrl and AQP4^{-/-} mice (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$). **Abbreviations:** Ctrl, control mice; AQP4^{-/-}, AQP4-deficient mice; TRPV4^{-/-}, TRPV4-deficient mice; AQP4^{-/-}/TRPV4^{-/-}, AQP4- and TRPV4-deficient mice; RT, room temperature; aCSF, artificial cerebrospinal fluid; H-50, 250mOsm hypoosmotic solution; H-100, 200mOsm hypoosmotic solution; ECS, extracellular space; n, number of experiments.

Tab. 4 ECS diffusion parameters *in situ* during cell swelling-inducing conditions (hypoosmotic stress, hyperkalemia, OGD).

			Ctrl		AQP4 ^{-/-}		TRPV4 ^{-/-}		AQP4 ^{-/-} /TRPV4 ^{-/-}	
			values	N/n	values	N/n	values	N/n	values	N/n
α		RT	0.196 ± 0.002	33/48	0.214 ± 0.003	26/46	0.195 ± 0.002	25/43	0.200 ± 0.002	24/39
		33°C	0.201 ± 0.003	33/48	0.217 ± 0.004	26/46	0.202 ± 0.003	25/43	0.203 ± 0.004	24/39
	<i>H-50</i>	20 th min	0.140 ± 0.008	6/10	0.161 ± 0.008	6/11	0.143 ± 0.006	6/14	0.149 ± 0.010	8/9
	<i>H-100</i>	20 th min	0.103 ± 0.007	8/9	0.108 ± 0.007	10/12	0.113 ± 0.010	7/9	0.101 ± 0.009	10/12
	<i>OGD</i>	20 th min	0.107 ± 0.004	11/14	0.109 ± 0.008	10/12	0.141 ± 0.009	7/9	0.207 ± 0.009	8/8
	<i>K⁺</i>	20 th min	0.123 ± 0.006	9/13	0.144 ± 0.008	7/11	0.129 ± 0.008	7/11	0.118 ± 0.007	9/10

λ		RT	1.614 ± 0.009	33/48	1.619 ± 0.008	26/46	1.612 ± 0.011	25/42	1.604 ± 0.009	24/39
		33°C	1.644 ± 0.013	33/48	1.665 ± 0.012	26/46	1.641 ± 0.011	25/42	1.657 ± 0.014	24/39
	<i>H-50</i>	20 th min	1.650 ± 0.038	6/10	1.681 ± 0.029	6/11	1.645 ± 0.026	6/14	1.722 ± 0.036	8/9
	<i>H-100</i>	20 th min	1.636 ± 0.024	8/9	1.705 ± 0.030	10/12	1.662 ± 0.037	7/9	1.706 ± 0.035	10/12
	<i>OGD</i>	20 th min	1.651 ± 0.034	11/14	1.621 ± 0.034	10/12	1.647 ± 0.039	7/9	1.646 ± 0.047	8/8
	<i>K⁺</i>	20 th min	1.710 ± 0.044	9/13	1.731 ± 0.036	7/11	1.770 ± 0.041	7/11	1.704 ± 0.048	9/10

k' [10 ⁻³ s ⁻¹]		RT	15.5 ± 3.5	33/48	10.7 ± 1.7	26/46	12.0 ± 2.2	25/43	14.7 ± 3.4	24/39
		33°C	17.1 ± 4.0	33/48	14.5 ± 2.4	26/46	16.1 ± 3.3	25/43	18.2 ± 3.1	24/39
	<i>H-50</i>	20 th min	11.8 ± 2.5	6/10	12.9 ± 2.3	6/11	16.8 ± 2.5	6/14	21.7 ± 2.6	8/9
	<i>H-100</i>	20 th min	15.3 ± 6.5	8/9	8.3 ± 1.2	10/12	10.7 ± 2.3	7/9	12.6 ± 2.6	10/12
	<i>OGD</i>	20 th min	7.94 ± 2.4	11/14	5.1 ± 0.9	10/12	15.0 ± 5.6	7/9	8.63 ± 2.5	8/8
	<i>K⁺</i>	20 th min	13.3 ± 4.2	9/13	11.2 ± 2.0	7/11	9.1 ± 1.7	7/11	9.66 ± 3.1	9/10

Data are expressed as mean $\pm$ SEM. **Abbreviations:** Ctrl, control mice; AQP4^{-/-}, AQP4-deficient mice; TRPV4^{-/-}, TRPV4-deficient mice; AQP4^{-/-}/TRPV4^{-/-}, AQP4- and TRPV4-deficient mice; RT, room temperature; H-50, 250mOsm hypoosmotic solution; H-100, 200mOsm hypoosmotic solution; OGD, oxygen-glucose deprivation; K⁺, artificial cerebrospinal fluid with elevated K⁺ concentration; α , ECS volume fraction; k' , uptake; λ , tortuosity; N, number of animals; n, number of experiments.

4.2.3 The effect of hyperkalemia

Extracellular hyperkalemia is a prominent feature of acute ischemia. The application of a 50 mM potassium solution (aCSFK⁺, Tab. 1) in the acute brain slices was implemented to model these conditions. Similar to hypoosmotic stress, α values significantly decreased during the application of aCSFK⁺ solution in all experimental groups ($p < 0.001$), with no differences observed between the Ctrl and single or double-knockout mice. Hyperkalemic environment did not induce any significant changes in λ or k' in any of the experimental groups (Fig. 17 and Tab. 4). During washout, α gradually returned to its initial values in the Ctrl, TRPV4^{-/-}, and AQP4^{-/-}/TRPV4^{-/-} mice, while an overshoot above the initial values was detected in AQP4^{-/-} mice ($p < 0.001$)(Fig. 17).

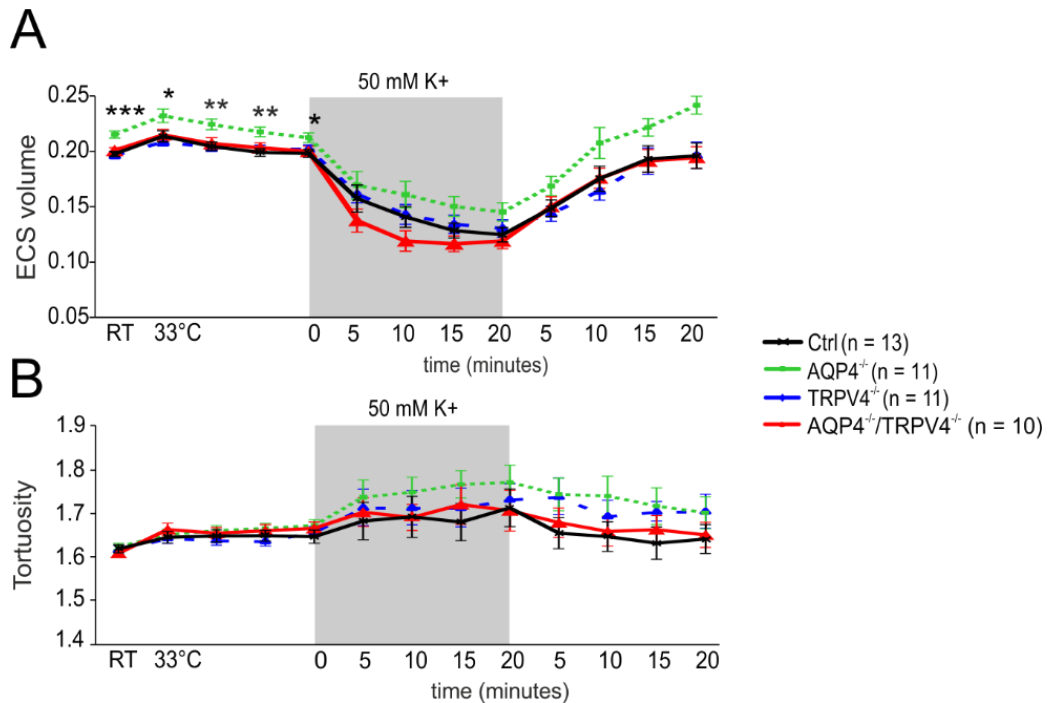


Fig. 17 The effect of hyperkalemia on the ECS diffusion parameters *in situ*. (A) Averaged data of the ECS volume fraction recorded in Ctrl, AQP4^{-/-}, TRPV4^{-/-} and AQP4^{-/-}/TRPV4^{-/-} mice at room temperature (RT) and at 5-minute intervals after the temperature increase to 33°C, during application

of aCSFK⁺ (grey area) and subsequent washout. **(B)** Averaged data of tortuosity recorded in Ctrl, AQP4^{-/-}, TRPV4^{-/-} and AQP4^{-/-}/TRPV4^{-/-} mice at RT and at 5-minute intervals after the temperature increase to 33°C, during application of aCSFK⁺ (grey area) and subsequent washout. The higher ECS volume fraction in AQP4^{-/-} mice is depicted. Data are expressed as mean ± SEM. The asterisks indicate significant differences between Ctrl and AQP4^{-/-} animals (*, p<0.05, **, p<0.01, ***, p<0.001). **Abbreviations:** Ctrl, control mice; AQP4^{-/-}, AQP4-deficient mice; TRPV4^{-/-}, TRPV4-deficient mice; AQP4^{-/-}/TRPV4^{-/-}, AQP4- and TRPV4-deficient mice; aCSFK⁺, 50 mM K⁺ solution; n, number of experiments; ECS, extracellular space; RT, room temperature (22-24°C).

4.2.4 Oxygen-glucose deprivation

Oxygen-glucose deprivation *in situ* is the most commonly used model of stroke due to its similarity to models of cerebral ischemia *in vivo* (Sommer, 2017). During the application of OGD solution, we detected a significant decrease in α ($p < 0.001$) in the Ctrl mice, in comparison with its resting values but no changes in λ or k' (Fig. 18 and Tab. 4). The OGD-evoked decrease in α in AQP4^{-/-} mice was comparable to that in Ctrl animals; however, the decrease in TRPV4^{-/-} mice was significantly smaller than that in Ctrl animals. The α values in the Ctrl mice as well as in both single knockout strains remained decreased even during/after a 20 min washout (Fig. 18). Surprisingly, in AQP4^{-/-}/TRPV4^{-/-} the α , λ , and k' remained unaffected by OGD application throughout the entire time course of the experiment ($p > 0.99$, $p > 0.98$, and $p > 0.86$, respectively; evaluated in the 20th minute of application (Fig. 18 and Tab. 4).

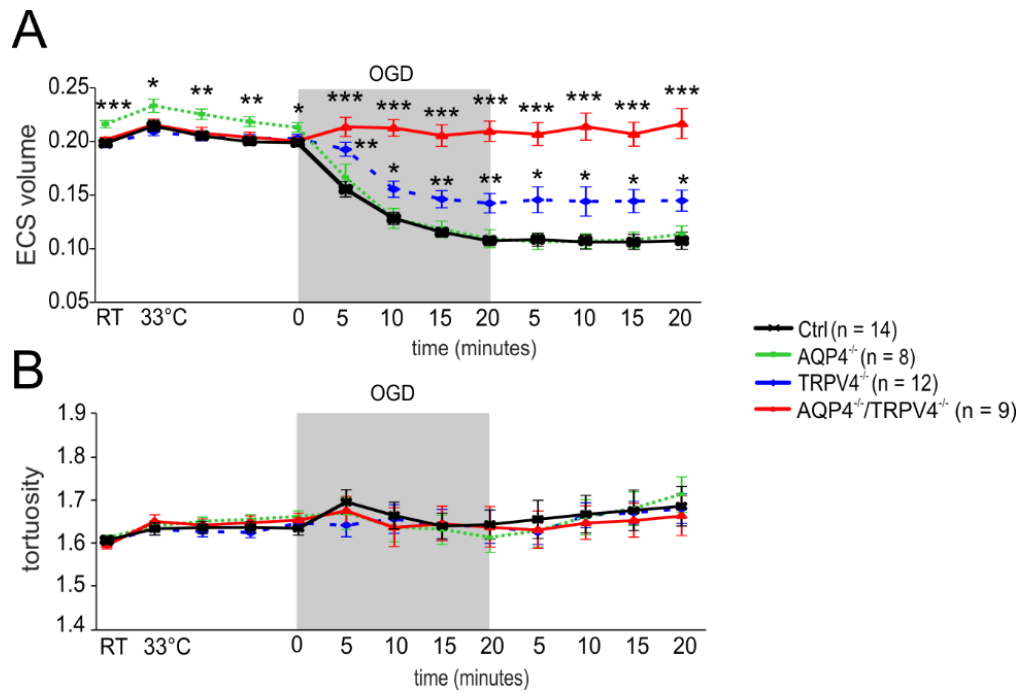


Fig. 18 The effect of oxygen-glucose deprivation (OGD) on the ECS diffusion parameters *in situ*. (A) Averaged data of the ECS volume fraction recorded in Ctrl, AQP4^{-/-}, TRPV4^{-/-} and AQP4^{-/-}/TRPV4^{-/-} mice at room temperature (RT) and at 5-minute intervals after the temperature increase to 33°C, during the application of OGD (grey area) and subsequent washout. (B) Averaged tortuosity data recorded in Ctrl, AQP4^{-/-}, TRPV4^{-/-} and AQP4^{-/-}/TRPV4^{-/-} mice at RT and at 5-minute intervals after the temperature increase to 33°C, during the application of OGD (grey area) and subsequent washout. Data are expressed as mean $\pm$ SEM. The asterisks indicate significant differences between AQP4^{-/-}/TRPV4^{-/-} or TRPV4^{-/-} and Ctrl mice (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$). **Abbreviations:** Ctrl, control mice; AQP4^{-/-}, AQP4-deficient mice; TRPV4^{-/-}, TRPV4-deficient mice; AQP4^{-/-}/TRPV4^{-/-}, AQP4- and TRPV4-deficient mice; OGD, oxygen-glucose deprivation; n, number of experiments; ECS, extracellular space; RT, room temperature (22-24°C).

4.2.4.1 Extracellular potassium concentration during OGD

Utilising the ISMs, we estimated the changes in extracellular K⁺ concentrations evoked by OGD. The experiments were performed on 15 Ctrl, 10 AQP4^{-/-}, 9 TRPV4^{-/-} and 13 AQP4^{-/-}/TRPV4^{-/-} mice.

The value of [K⁺]_e at room temperature was similar in all experimental groups (4.4 ± 0.4 mM in Ctrl, 3.7 ± 0.4 mM in AQP4^{-/-}, 4.5 ± 0.2 mM in TRPV4^{-/-} and 4.2 ± 0.3 mM in AQP4^{-/-}/TRPV4^{-/-} mice). These differences persisted during washout (Fig. 19). There were no significant differences in [K⁺]_e during OGD between AQP4^{-/-} or TRPV4^{-/-}, and Ctrl animals; the maximum values reached 8.4 ± 0.5 mM in Ctrl mice, 7.3 ± 1.0 mM in AQP4^{-/-} mice and 8.1 ± 0.6 mM in TRPV4^{-/-} mice (Fig. 19). In contrast, significant differences in

$[K^+]_e$ between the Ctrl and AQP4^{-/-}/TRPV4^{-/-} mice appeared already at the elevation of bath temperature to 33°C (6.3 ± 0.4 mM in the Ctrl and 4.9 ± 0.3 mM in AQP4^{-/-}/TRPV4^{-/-}), and were even more pronounced during OGD application with a significantly lower rise in $[K^+]_e$ (5.9 ± 0.5 mM) in AQP4^{-/-}/TRPV4^{-/-} animals.

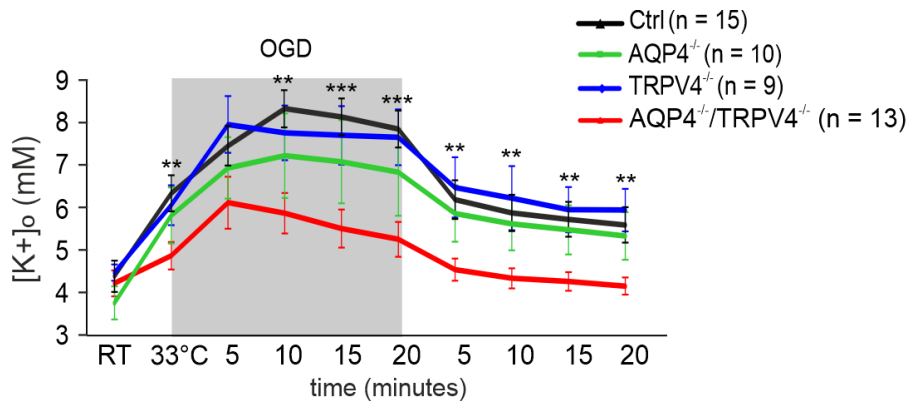


Fig. 19 The effect of oxygen-glucose deprivation (OGD) on the extracellular potassium concentration $[K^+]_e$ *in situ*. The average values of $[K^+]_e$ (recorded originally in 1-minute intervals) are depicted in 5-minute intervals at room temperature (RT), 33°C, during the application of OGD (grey area) and subsequent washout in Ctrl, AQP4^{-/-}, TRPV4^{-/-} and AQP4^{-/-}/TRPV4^{-/-} mice. Data are expressed as mean ± SEM. The asterisks indicate significant differences between AQP4^{-/-}/TRPV4^{-/-} and Ctrl mice (**, $p < 0.01$, ***, $p < 0.001$). **Abbreviations:** Ctrl, control mice; AQP4^{-/-}, AQP4-deficient mice; TRPV4^{-/-}, TRPV4-deficient mice; AQP4^{-/-}/TRPV4^{-/-}, AQP4- and TRPV4-deficient mice; RT, room temperature; OGD, oxygen-glucose deprivation; n, number of experiments.

4.3 Cortical damage and brain diffusion properties following middle cerebral artery occlusion

To assess how the RTI data correspond with the changes on the macroscopic level, we employed MRI methods (T2-weighted and diffusion-weighted MRI) to assess the lesion volume and ADC_w value.

4.3.1 The impact of AQP4 and TRPV4 deletion on the volume of the ischemic lesion

The volume of the ischemic lesion in 6 Ctrl, 5 AQP4^{-/-}, 6 TRPV4^{-/-} and 5 AQP4^{-/-}/TRPV4^{-/-} mice was quantified employing T2-weighted MRI at 1 day (D1) and 7 days (D7) following pMCAO (Fig. 20,21) to assess the role of AQP4 and TRPV4 channels in post-ischemic development of the lesion. Our experiments discovered that the AQP4^{-/-} as well as the TRPV4^{-/-} mice developed about a twice larger lesion volume D1 after pMCAO when

compared to the Ctrl animals. The decrease in the lesion volume between D1 and D7 was comparable in both single knockouts and did not significantly differ from that in Ctrl mice (Fig. 20,21 and Tab. 5). However, the volume of the lesion at D7 was still significantly enlarged in both the TRPV4^{-/-} as well as AQP4^{-/-} mice as compared to the Ctrl animals. Interestingly, quantification of a lesion volume in the AQP4^{-/-}/TRPV4^{-/-} mice brought quite the opposite results, revealing a notably smaller lesion volume at D1 when compared to both single knockouts and Ctrl animals (Fig. 20,21 and Tab. 5). Compared to the Ctrl mice, the values of the lesion volume in the AQP4^{-/-}/TRPV4^{-/-} at D7 were not significantly different (Fig. 20,21 and Tab. 5); however, they were still significantly smaller when compared to both single knockouts.

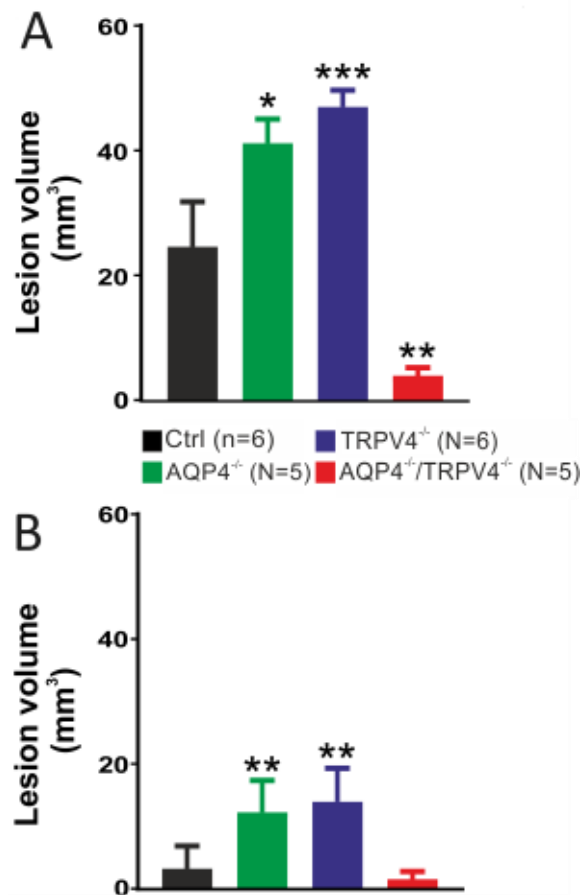


Fig. 20 Quantification of the ischemic volume lesion using T2-weighted magnetic resonance imaging of the mouse brain 1 day (D1) and 7 days (D7) after pMCAO. (A) Bar graph showing comparison of the size of the ischemic lesion in Ctrl, AQP4^{-/-}, TRPV4^{-/-} and AQP4^{-/-}/TRPV4^{-/-} mice at D1 after pMCAO. Note the increased volume of lesion in the single knockout mice as compared to Ctrl animals. Interestingly, AQP4^{-/-}/TRPV4^{-/-} mice had a smaller lesion volume than Ctrl mice and both single knockouts. (B) Bar graph showing the comparison of ischemic lesion size in Ctrl, AQP4^{-/-}, TRPV4^{-/-} and AQP4^{-/-}/TRPV4^{-/-} mice at D7 after pMCAO. The volume of the lesion decreased between D1 and D7 in all experimental groups. The increased lesion volume in the single knockout

versus Ctrl mice persisted on D7, while AQP4^{-/-}/TRPV4^{-/-} mice had smaller lesion volume than both single knockouts. Data are expressed as mean ± SEM. The asterisks indicate significant differences between AQP4^{-/-}/TRPV4^{-/-} and Ctrl mice (*, p<0.05, **, p<0.01, ***, p<0.001). **Abbreviations:** Ctrl, control mice; AQP4^{-/-}, AQP4-deficient mice; TRPV4^{-/-}, TRPV4-deficient mice; AQP4^{-/-}/TRPV4^{-/-}, AQP4- and TRPV4-deficient mice; pMCAO, permanent middle cerebral artery occlusion; n, number of animals.

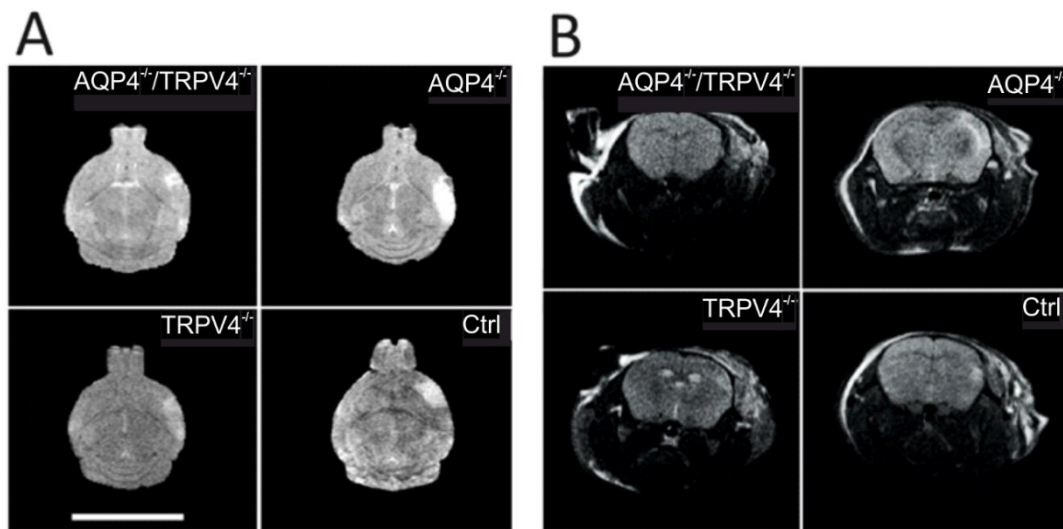


Fig. 21 Representative T2-weighted images of mouse brains 1 day (D1) and 7 days (D7) after pMCAO. (A) Representative T2-weighted images of the brains of Ctrl, AQP4^{-/-}, TRPV4^{-/-} and AQP4^{-/-}/TRPV4^{-/-} mice at D1 after pMCAO **(B)** Representative T2-weighted images of the brains of Ctrl, AQP4^{-/-}, TRPV4^{-/-} and AQP4^{-/-}/TRPV4^{-/-} mice at D7 after pMCAO. The lesion is visible as a light hyperintense area in the left hemisphere (on the right part of the brain in the picture). **Abbreviations:** Ctrl, control mice; AQP4^{-/-}, AQP4-deficient mice; TRPV4^{-/-}, TRPV4-deficient mice; AQP4^{-/-}/TRPV4^{-/-}, AQP4- and TRPV4-deficient mice; pMCAO, permanent middle cerebral artery occlusion.

Tab. 5 Average size of the ischemic lesions

Experimental group	Average size of the lesion (mm ³)			
	D1	D7	% of decrease	N
Ctrl	24.8 ± 7.3	3.3 ± 1.5	87.52 ± 2.31	6
AQP4 ^{-/-}	41.5 ± 3.9	12.4 ± 2.3	70.61 ± 4.57	5
TRPV4 ^{-/-}	47.4 ± 2.7	14.1 ± 2.4	71.11 ± 13.47	6
AQP4 ^{-/-} /TRPV4 ^{-/-}	4.1 ± 1.3	1.6 ± 0.6	54.05 ± 14.79	5

Data are presented expressed as mean ± SEM, **Abbreviations:** Ctrl, control mice; AQP4^{-/-}, AQP4-deficient; TRPV4^{-/-}, TRPV4-deficient; AQP4^{-/-}/TRPV4^{-/-}, AqQP4- and TRPV4-deficient; D1, 1 day after ischemia; D7, 7 days after ischemia; N, number of animals.

4.3.2 The impact of AQP4/TRPV4 complex deletion on apparent diffusion coefficient of water

T2-weighted MRI revealed a reduced impact of pMCAO in the AQP4^{-/-}/TRPV4^{-/-} mice at D1. Therefore, we performed DW-MRI in 6 AQP4^{-/-}/TRPV4^{-/-} and 7 Ctrl mice to confirm the attenuating effect of simultaneous deletion of AQP4 and TRPV4 on the outcome of pMCAO in the early phase. Indeed, the ADC_w values measured in AQP4^{-/-}/TRPV4^{-/-} mice on the ipsilateral ischemic side ($609 \pm 16 \mu\text{m}^2 \text{s}^{-1}$) did not significantly differ from the contralateral non-injured side ($601 \pm 13 \mu\text{m}^2 \text{s}^{-1}$) and were comparable to the values in the contralateral hemisphere in the Ctrl mice ($665 \pm 18 \mu\text{m}^2 \text{s}^{-1}$) (Fig. 22,23 and Tab. 6). In Ctrl animals, the ADC_w on the ipsilateral side was significantly lower than on the contralateral side ($481 \pm 17 \mu\text{m}^2 \text{s}^{-1}$ and $665 \pm 18 \mu\text{m}^2 \text{s}^{-1}$, respectively; $p < 0.001$) indicating more pronounced cell swelling and brain edema (Fig. 22,23 and Tab. 6).

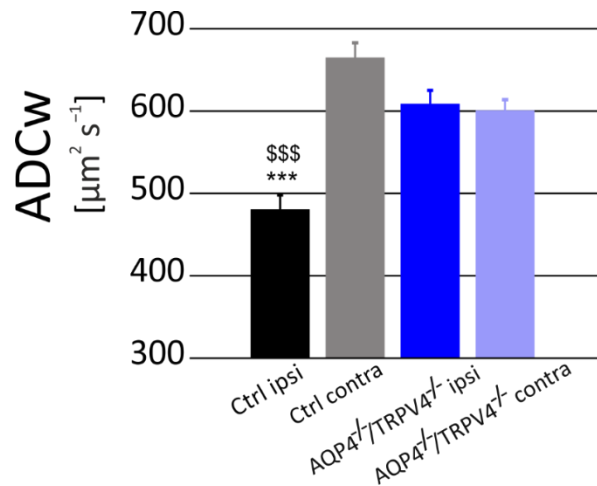


Fig. 22 Quantification of ADC_w using diffusion-weighted magnetic resonance imaging 1 day (D1) after pMCAO. Bar graph comparing ADC_w in Ctrl and AQP4^{-/-}/TRPV4^{-/-} mice at D1 after pMCAO. Note the decreased ADC_w in the Ctrl mice ipsilateral hemisphere, which is the result of more pronounced cell swelling that is not present in AQP4^{-/-}/TRPV4^{-/-} mice at D1 after pMCAO. Data are expressed as mean $\pm$ SEM. Asterisks indicate significant differences between AQP4^{-/-}/TRPV4^{-/-} and Ctrl mice (***, $p < 0.001$) and dollars indicate significant differences between ipsilateral and contralateral hemisphere (***, $p < 0.001$). **Abbreviations:** Ctrl, control mice; AQP4^{-/-}/TRPV4^{-/-}, AQP4- and TRPV4-deficient mice; ADC_w, apparent diffusion coefficient of water; ipsi, ipsilateral hemisphere; contra, contralateral hemisphere; pMCAO, permanent middle cerebral artery occlusion.

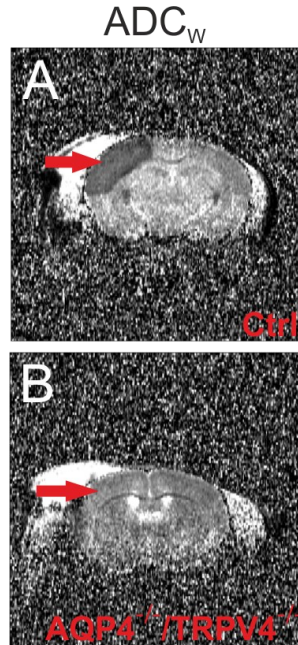


Fig. 23 Representative diffusion-weighted images 1 day (D1) after pMCAO. Note the decreased ADC_w (a darker area, red arrow) in Ctrl mice (A) at D1 following pMCAO. Such decrease is not present in $AQP4^{-/-}/TRPV4^{-/-}$ mice (B). **Abbreviations:** Ctrl, control mice; $AQP4^{-/-}/TRPV4^{-/-}$, AQP4- and TRPV4-deficient mice; ADC_w , apparent diffusion coefficient of water; pMCAO, permanent middle cerebral artery occlusion.

Tab. 6 Average ADC_w at D1 after pMCAO

	Experimental group			
	Ctrl	Ctrl	$AQP4^{-/-}/TRPV4^{-/-}$	$AQP4^{-/-}/TRPV4^{-/-}$
hemisphere	ipsilateral	contralateral	ipsilateral	contralateral
$ADC_w [\mu m^2 s^{-1}]$	481±17	665±18	609±16	601±13
N	7	7	6	6

Data are expressed as mean $\pm$ SEM. **Abbreviations:** Ctrl, control mice; $AQP4^{-/-}/TRPV4^{-/-}$, AQP4- and TRPV4-deficient; D1, 1 day after ischemia; ADC_w , apparent diffusion coefficient of water; pMCAO, permanent middle cerebral artery occlusion; N, number of animals.

4.3.3 Visualisation of astroglial and microglial reactivity following pMCAO

To assess the extent of glial cell activation, the immunohistochemical staining was implemented at D7 after pMCAO in 3 Ctrl, 3 $AQP4^{-/-}$, 3 $TRPV4^{-/-}$ and 3 $AQP4^{-/-}/TRPV4^{-/-}$ mice (Fig. 24). Antibodies against GFAP and Iba1 were employed to visualise reactive

astrocytes and microglia, respectively. At D7, microglial reactivity of similar extent was detected in all experimental strains, while astroglial reactivity was less pronounced in AQP4^{-/-}/TRPV4^{-/-} mice as compared to Ctrl mice and both single knockouts.

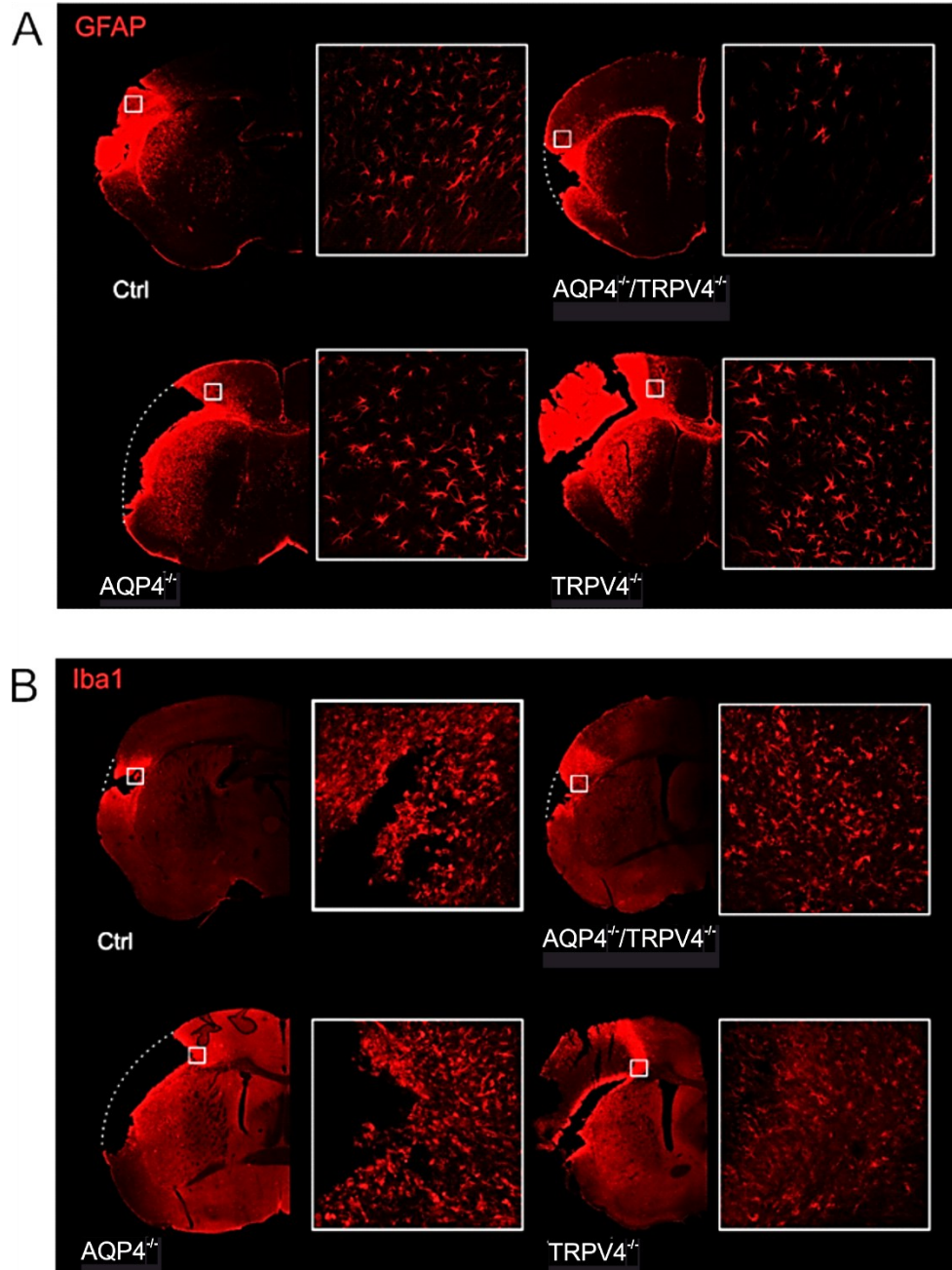


Fig. 24 Immunohistochemical staining for GFAP and Iba1 7 days (D7) after pMCAO. (A) Decreased GFAP positivity indicated a lesser astrogliosis in AQP4^{-/-}/TRPV4^{-/-} mice. **(B)** Staining with antibodies against Iba1 showed similar microglial activity at D7 in all experimental groups. The white dotted lines show the original size of the tissue slices in places where due to the extent of the ischemic damage, parts of the slices fell out. Consistent with the MRI results, we observed comparable damage in the AQP4^{-/-}/TRPV4^{-/-} as in the Ctrl mice, and significantly larger damage in the AQP4^{-/-} or TRPV4^{-/-} mice. **Abbreviations:** Ctrl, control mice; AQP4^{-/-}; AQP4-deficient mice,

TRPV4^{-/-}; TRPV4-deficient mice; AQP4^{-/-}/TRPV4^{-/-}, AQP4- and TRPV4-deficient mice; pMCAO, permanent middle cerebral artery occlusion; GFAP, glial fibrillary acidic protein; Iba1, ionized Ca²⁺-binding adapter molecule.

4.3.4 The effect of pMCAO on the perineuronal nets

The expression of the PNNs, with a possible protective role against oxidative stress (Cabungcal et al., 2013; Morawski et al., 2004), was evaluated by immunohistochemical staining for WFA at D7 after pMCAO in 6 mice in each experimental group (Ctrl, AQP4^{-/-}, TRPV4^{-/-} and AQP4^{-/-}/TRPV4^{-/-})(Fig. 25 and Tab. 7). The staining intensity of individual PNNs was analysed and each net was labelled either as of high, medium, or low intensity (see Chapter 3.2.7).

In the non-ischemic mice, the PNNs were present in the cortex with comparable distribution in all studied genotypes. At D7, the ipsilateral hemisphere, relative to the pMCAO injury, was characterised by a decreased WFA positivity in the control and AQP4^{-/-}/TRPV4^{-/-} mice as compared to the non-ischemic and contralateral cortex. In AQP4^{-/-} and TRPV4^{-/-} we did not detect a similar decrease in WFA staining intensity after pMCAO and the frequency distribution of high, medium and low intensity PNNs was comparable to the non-ischemic mice (Fig. 25 and Tab. 7).

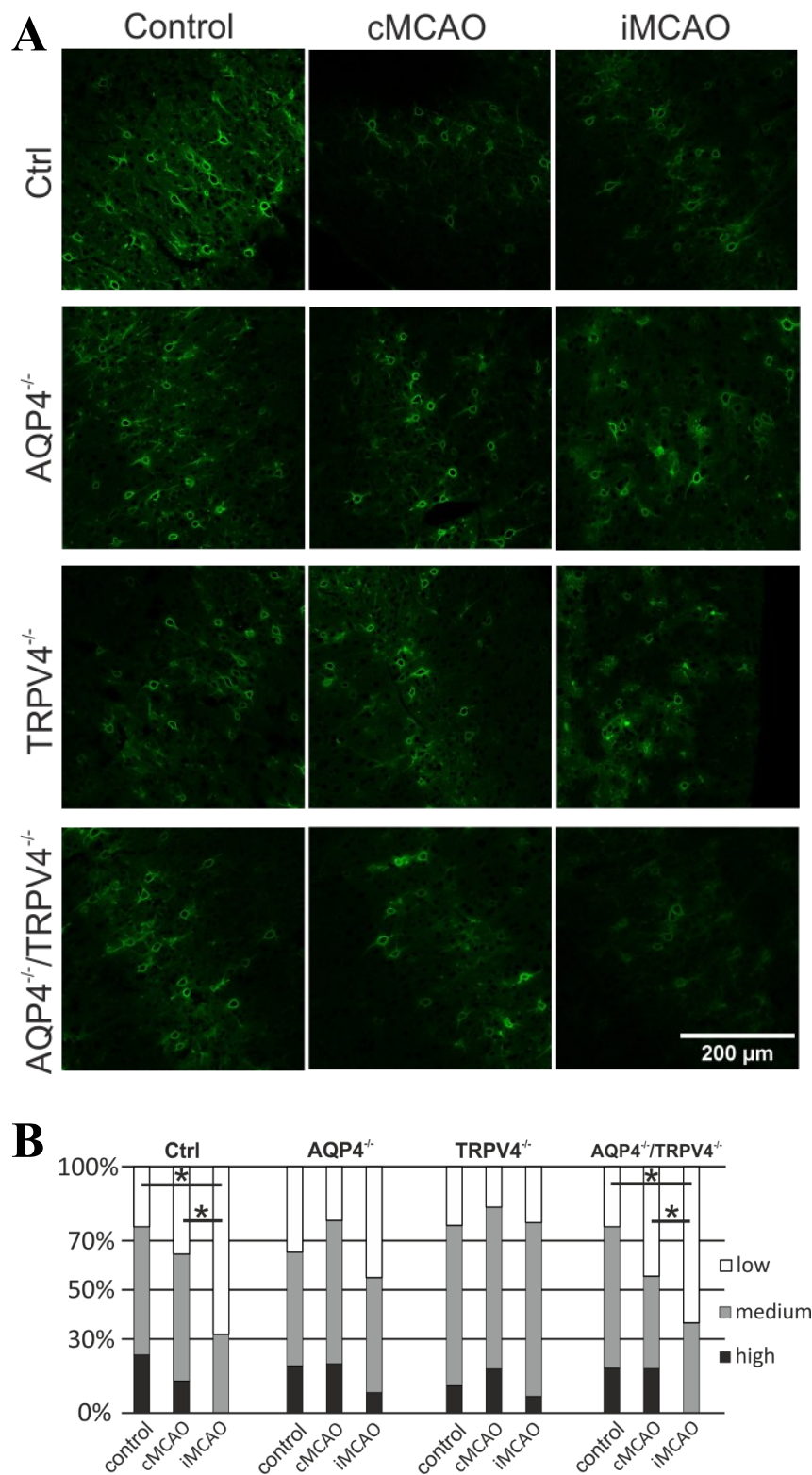


Fig. 25 *Wisteria floribunda agglutinin* (WFA) immunohistochemical staining 7 days (D7) after pMCAO. (A) A decrease in WFA positivity was detected in the Ctrl and AQP4^{-/-}/TRPV4^{-/-} mice ipsilateral cortex affected by pMCAO. In AQP4^{-/-} and TRPV4^{-/-} we did not observe any detectable decomposition of PNNs after pMCAO. (B) Frequency distribution of PNNs, subdivided into three

categories according to WFA staining intensities in the non-ischemic cortex and at D7 after pMCAO (in ipsilateral and contralateral cortex, relative to the pMCAO injury) in all experimental strains. Decreased WFA positivity was detected in the Ctrl and AQP4^{-/-}/TRPV4^{-/-} mice ipsilateral cortex affected by pMCAO. In AQP4^{-/-} and TRPV4^{-/-} we did not detect a similar decrease in WFA staining intensity after pMCAO. Asterisks represent significant differences. **Abbreviations:** Ctrl, wild type control mice; AQP4^{-/-}; AQP4-deficient mice, TRPV4^{-/-}; TRPV4-deficient mice; AQP4^{-/-}/TRPV4^{-/-}, AQP4- and TRPV4-deficient mice; control, non-ischemic cortex; cMCAO, contralateral hemisphere to the ischemic injury; iMCAO, ipsilateral hemisphere to the ischemic injury. The scale bar is depicted in the image.

Tab. 7 Distribution of PNN staining intensity at D7 following pMCAO

	Count of PNNs with high/medium/low intensity			N
	Control	cMCAO	iMCAO	
Ctrl	31/68/32	14/55/38	0/39/83	3/3
AQP4^{-/-}	32/77/58	11/32/12	5/28/27	3/3
TRPV4^{-/-}	14/82/30	12/44/11	3/31/10	3/3
AQP4^{-/-}/TRPV4^{-/-}	15/47/20	13/27/32	0/26/45	3/3

Abbreviations: Control, non-ischemic cortex; cMCAO, contralateral hemisphere to the ischemic injury; iMCAO, ipsilateral hemisphere to the ischemic injury; Ctrl, wild type control mice; AQP4^{-/-}, AQP4-deficient; TRPV4^{-/-}, TRPV4-deficient; AQP4^{-/-}/TRPV4^{-/-}, AQP4- and TRPV4-deficient; D7, 7 days after ischemia; pMCAO, permanent middle cerebral artery occlusion; PNN, perineuronal net; N, number of animals.

5 DISCUSSION

The activity of various membrane proteins facilitates the transport of ions and water over the membrane, affecting the osmolarity in the tissue. This process necessitates the regulation of cell volume to maintain proper cell function. Physiological and pathophysiological conditions challenge ion homeostasis, thereby activating the regulatory processes. Because brain tissue resides in a finite space, regulating cell volume is crucial to prevent extensive tissue damage. Severe insults to brain tissue are often accompanied by edema, which exacerbates the outcomes of the injury. The aim of new therapeutic approaches is to identify specific channels involved in the mechanisms of cell swelling mechanisms that can be targeted and regulated externally so that we could potentially alleviate the development and resolution of cytotoxic edema.

It has been previously demonstrated in the astrocyte cultures and retina that cooperation between AQP4 and TRPV4 can play a pivotal role in the regulation of the cell volume (Benfenati et al., 2011; Jo et al., 2015). The aim of my dissertation thesis was to specify the role of AQP4 and TRPV4 channels in the process of cytotoxic edema development/volume regulation in the acute, subacute, and early chronic phase following ischemia induction. For this purpose, we employed the RTI method, which detects changes in the ECS diffusion parameters including the alternations in the extracellular volume that reciprocally reflect cell volume changes. The RTI measurements are affected by swelling of all types of brain cells and show the impact of the AQP4 and/or TRPV4 deficit on the tissue edema development in wider consequences. Initially, we evaluated the role of AQP4 and TRPV4 *in vivo* in the severe model of ischemia – terminal ischemia/anoxia. Further, we assessed the differences among the AQP4, TRPV4 and AQP4/TRPV4 channel deficient mice in experimental models of cytotoxic edema: hypotonic stress, hyperkalemia, and OGD *in situ*. The RTI measurements were complemented with MRI experiments, which analysed subacute and early chronic changes following pMCAO. To detect possible protective properties of the tissue, the PNNs expression and micro/astro-glial activation were examined 7 days after pMCAO,

In the first study, we have shown that an AQP4 or TRPV4 deficit slowed down an α decrease resulting from the extracellular volume shrinkage during acute terminal ischemia *in vivo*. *In situ*, deletion of the TRPV4 channel led to a smaller ECS volume decrease during OGD application when compared to the control animals. Interestingly, in tissue with concomitant deletion of AQP4 and TRPV4, we did not detect any decrease in the ECS

volume during OGD. In any of the selected cytotoxic edema models *in situ* (OGD, hyperkalemia, mild or severe hypotonic stress), we did not observe any differences in cell swelling between the control and AQP4 deficient tissue. In contrast with acute *in vivo* and *in situ* experiments, we demonstrated that a deficit in a single AQP4 or TRPV4 protein aggravated tissue damage during a subacute phase of ischemia *in vivo*, modelled by pMCAO. However, a simultaneous deficit of both channels significantly alleviated tissue damage following pMCAO, as indicated by smaller lesion volumes, higher ADC_w, and less astrogliosis in AQP4^{-/-}/TRPV4^{-/-} mice compared to control and single knockout mice.

5.1 RTI measurements revealed increased initial ECS volume fraction in AQP4^{-/-} mice

Data obtained from cortical slices of AQP4^{-/-} mice under resting conditions revealed an approximately 14% enlargement in α compared to the control mice, with no change in λ . Moreover, *in vivo* results under normoxic condition in AQP4^{-/-} showed enlarged α by about 28% compared to the control mice (with no change in λ). Increased basal α values in AQP4^{-/-} mice were described previously by a study which employed the same technique (Yao et al., 2008). However, the α enlargement *in situ* was slightly lower than that reported *in vivo* (Yao et al., 2008) and in our previous articles concerning the deficit of the AQP4 anchoring protein α -syntrophin (Anderova et al., 2014; Dmytrenko et al., 2013). In our *in situ* measurements, an increase in temperature to 33°C before applications was accompanied by a significant α decrease, which may be associated with a higher level of neuronal activity resulting in cell swelling and compensatory shrinkage of the ECS volume (Svoboda and Sykova, 1991). In addition, it has recently been demonstrated that temperature alterations may affect the expression and end-feet localization of AQP4 channels in astrocytic membranes (Salman et al., 2017), which may also affect α values.

5.2 AQP4 or TRPV4 deficit slows down α decrease and an increase in extracellular potassium during severe ischemia *in vivo*

Despite the higher initial values of α in normoxic AQP4^{-/-} animals, the final values of α , λ and $[K^+]_e$, observed after terminal ischemia/anoxia, were similar between AQP4^{-/-}, TRPV4^{-/-} mice, and control animals. This is in agreement with previous studies of our department, which showed that the terminal values of the ECS diffusion parameters, approximately $\alpha \sim 0.05$ and $\lambda \sim 2.00$, remain consistent across various studies and comparisons, such as in young vs. adult animals, gray vs. white matter, and rats vs. mice

(Sykova and Nicholson, 2008; Vorisek and Sykova, 1997a). However, the time course of changes in the AQP4 and TRPV4 deficient mice was significantly slower than in controls, suggesting that both AQP4 and TRPV4 channels play a role in the formation of acute ischemia-induced edema *in vivo*. We have also detected a slower increase in $[K^+]_e$ in AQP4^{-/-} and TRPV4^{-/-} mice than in the control animals, that can influence cell swelling mechanisms associated with $[K^+]_e$ elevation.

The literature offers conflicting findings on whether the deletion of AQP4 and TRPV4 has a protective or detrimental effect on the development of brain edema. Some studies demonstrated aggravation of tissue damage in AQP4 knockout mice during ischemia/reperfusion injury (Shi et al., 2012a; Zeng et al., 2012) and in TRPV4 knockout mice (Pivonkova et al., 2018). In contrast, other studies showed reduced lesion size and brain edema in TRPV4^{-/-} mice (Hoshi et al., 2018; Jie et al., 2015; Rakers et al., 2017; Tanaka et al., 2020) and reduced brain edema and infarct volume in AQP4 deficient mice (Hirt et al., 2017; Katada et al., 2014; Manley et al., 2000; Yao et al., 2015). Such discrepancies are likely caused by the distinct mechanisms of tissue swelling in individual models of ischemia. Moreover, it is difficult to compare the results of these teams with our data during terminal ischemia *in vivo*, as they employed models of transient or focal ischemia.

The time course of changes in the ECS diffusion parameter and $[K^+]_e$ following cardiac arrest has been previously studied in relation with development and aging. The studies found that younger animals with higher ECS volume exhibited a slower time course of changes in both diffusion parameters and $[K^+]_e$ and suggest that reduced extracellular space increases the susceptibility of the ageing brain to anoxia/ischemia, likely due to faster extracellular acidosis and the accumulation of K^+ and toxic substances (Sykova et al., 1998; Vorisek and Sykova, 1997b). The higher initial volume in AQP4^{-/-} mice may contribute to the reduced rate of cell swelling observed in AQP4^{-/-} mice but does not account for the delayed swelling seen in TRPV4^{-/-} mice.

The obtained results indicate that the AQP4 and TRPV4 channels may play a crucial role in the velocity of acute edema development in severe pathological states *in vivo*. As the final extent of damage was similar in all strains, the main difference between control and in AQP4 and TRPV4 knockout animals was observed in the acute phase after the induction of ischemia. These findings suggest that inhibition of AQP4 and TRPV4 during acute cell swelling may extend that the therapeutic window for standard therapeutic approaches and interventions, including clot removal.

5.3 The deletion of AQP4 or TRPV4 does not affect changes in the ECS diffusion parameters evoked by hypoosmotic stress or hyperkalemia

Exposition of tissue *in situ* to solutions of varying osmolality enables us to study the cell volume changes, even though such fast osmotic shifts are rare under natural conditions (Toft-Bertelsen et al., 2018). We did not observe any differences in the ECS volume change after a 20-minute application of mild or severe hypotonic stress (aCSFH-50 or aCSFH-100, respectively) either in AQP4^{-/-}, TRPV4^{-/-} or AQP4^{-/-}TRPV4^{-/-} mice compared to control animals.

The localisation of AQP4 loses its polarisation to the astrocytic endfeet following ischemia induction. This depends on AQP4 position mainly on its binding to α -syntrophin/dystroglycan complex that reduces the lateral movement of AQP4 channels (Noell et al., 2011). Our previous studies of the ECS volume fraction and astrocyte volume changes in α -syn^{-/-} mice (Anderova et al., 2014; Dmytrenko et al., 2013) showed that the application of a severe hypotonic solution (aCSFH-100), but not a mild hypotonic solution (aCSFH-50) evoked a smaller volume response in α -syn^{-/-} mice when compared to control animals. In contrast to current results, these differences in α between α -syn^{-/-} mice and control mice were not detectable before the 20th minute of application, while the volume had already differed at the 10th minute (Anderova et al., 2014). It seems that longer hypoosmotic stimulation is required to reveal the distinction between control and AQP4^{-/-} mice. Moreover, the different temperature conditions used in the current (close to physiological temperature) and previous (room temperature) studies could also explain the discrepancy in the results as lower temperature led to an increase in surface localisation of AQP4 in human astrocytes (Salman et al., 2017). The discrepancy between the results from α -syn^{-/-} and AQP4^{-/-} mice may be related to the different animal strain used in the current study and the fact that in α -syn^{-/-} mice the AQP4 channels are still present, only their localisation is impaired.

The study on astrocyte cultures found hypotonic stress-evoked differences between astrocytes prepared from control and TRPV4^{-/-} tissue (Pivonkova et al., 2018). These findings contribute to the hypothesis that the involvement of TRPV4 and AQP4 channels in cell swelling vary when comparing *in situ* and *in vitro* models, as the cultures reflected only reflect the response of astrocytes and not of the whole tissue. In contrast, Hermanova et al. showed that astrocyte exposure to hypoosmotic stress or hyperkalemia did not reveal any differences in astrocyte swelling in the absence of AQP4, TRPV4, or both channels concomitantly (Hermanova et al., 2024).

Surprisingly, another study showed a reduced swelling in AQP4^{-/-} astrocytes compared to AQP4^{+/+} ones after exposure to mild hypoosmotic stress (20% reduction in osmolarity) but not after severe hypoosmotic stress (30% reduction in osmolarity) (Thrane et al., 2011). However, the experiments were done in slices from 10- to 20-days old mouse pups, which is approximately the age when the homeostatic functions of glia are maturing and thus these astrocytes may react differently.

In astrocytes from AQP4 knock-out (Solenov et al., 2004) or knock-down mice (Nicchia et al., 2003), the water permeability was reduced by only 50%. It is caused by the fact that many membrane transporters allow water through together with their substrates and participate to a large extent in cell swelling/volume regulation during physiological or milder pathological stimuli (Kahle et al., 2015; MacAulay et al., 2004; Steffensen et al., 2015; Takezawa et al., 2023). For example, glucose transporters - SGLT1, GLUT1, and GLUT2 (Fischbarg et al., 1990; Zeuthen et al., 2006, 2007) or NKCC1, KCC ion cotransporters (Hamann et al., 2010). Water is also transported by the monocarboxylate (lactate) transporter MCT1 or the excitatory amino acid (glutamate) transporter EAAT1 (Loo et al., 1996; MacAulay and Zeuthen, 2010). Notably, under a given osmotic gradient, cells generally swelled to the same extent regardless of the osmotic water permeability of the cell membrane. However, this swelling occurred at a slower rate in cells with reduced permeability, such as in AQP4-deficient astrocytes (Solenov et al., 2004). Additionally, TRPV4 and AQP4 were proposed to be essential for RVD in cell cultures; however, the results from TRPV4 and AQP4-lacking cell cultures are inconsistent (Benfenati et al., 2011; Jo et al., 2015; Pivonkova et al., 2018). Jo and colleagues showed that TRPV4 together with the AQP4 channels triggered RVD only in high hypotonic but not in mild hypotonic conditions (Jo et al., 2015). Despite this, we did not observe RVD in any experimental group in our study. Several studies indicated that RVD, as described in cell cultures, was mostly absent *in situ* or *in vivo* (Pivonkova et al., 2018; Risher et al., 2009).

Due to the observed co-localization of AQP4 with Kir4.1 on the membrane, a functional coupling between these proteins has been proposed (Amiry-Moghaddam et al., 2003; Connors et al., 2004; Guadagno and Moukhles, 2004; Nagelhus et al., 2004, 1999). However, we did not detect any difference in α changes after a 20-minute application of 50 mM K⁺ solution, either in the AQP4^{-/-}, TRPV4^{-/-} or AQP4^{-/-}/TRPV4^{-/-} mice as compared to control animals. Previous research on α -syn^{-/-} mice showed reduced swelling of astrocytes following the application of a 50 mM K⁺ solution, but not following the application of milder

10 mM K⁺ solution (Anderova et al., 2014), suggesting a potential role of AQP4 in K⁺ homeostasis, especially under pathological conditions. The proposed functional coupling is supported by the presence of reciprocal regulation of the gene expression of *Trpv4*, *Aqp4*, and *Kir4.1*. In the mouse retina, deficiency of both AQP4 and TRPV4 led to the reduced expression of Kir4.1 (Jo et al., 2015). A similar effect in the brain could explain the observed lower rise in [K⁺]_e following OGD in this study. Moreover, facilitated spatial buffering associated with enhanced glial coupling in AQP4^{-/-} mice (Katoozi et al., 2017; Strohschein et al., 2011) may also contribute to this phenomenon. Interestingly, AQP4^{-/-} slices had been reported to have reduced K⁺ uptake near basal [K⁺]_e, while stronger stimulation elicited similar levels of potassium uptake in AQP4^{-/-} and control mice, suggesting that Kir4.1 and AQP4 may be more involved in K⁺ and water homeostasis around resting [K⁺]_e rather than at higher concentrations (Haj-Yasein et al., 2011). The functional interplay between AQP4 and Kir4.1 is still controversial (Zhang and Verkman, 2008).

5.4 Simultaneous deletion of AQP4 and TRPV4 blocks the changes of the ECS diffusion parameters during OGD

OGD treatment became a widely used *in situ* model of ischemia, as several studies reported that the perfusion of the slices with low oxygen and glucose-free solution fairly accurately the ischemic conditions that occur *in vivo* (Holloway and Gavins, 2016; Richard et al., 2010). However, in contrast to *in vivo* conditions, *in situ* perfusion with bath solution allows the removal of the toxic metabolites, which could alleviate tissue damage.

In acute terminal ischemia *in vivo*, we observed that the lack of AQP4 or TRPV4 slows down the development of cytotoxic edema (Chmelova et al., 2019). In contrast, experiments *in situ* showed that only TRPV4 deletion (but not AQP4 deletion) attenuated the ECS volume decrease induced by OGD model of ischemia. After OGD, we did not observe any recovery after α within 20 minutes of washout in AQP4, TRPV4 or control mice. Similar findings were shown in the study by Pivonkova et al., which employed 3D morphometry to quantify changes in astrocyte volume changes. These results indicated that TRPV4 is involved in OGD-induced cell swelling, but not necessarily through the activation of AQP4 channels (Pivonkova et al., 2018). A comparable distinction was noted between TRPV4^{-/-} and AQP4^{-/-} animals in a rodent stroke model: while TRPV4 deletion significantly reduced peri-infarct depolarizations, AQP4 deletion failed to produce a similar effect (Rakers et al., 2017). Since TRPV4 channels are expressed in various cell types, including neurons, this indicates a greater contribution of neuronal swelling compared to glial swelling. Furthermore, it was

previously shown that TRPV4 activation following stroke enhances NMDA receptor function, which may facilitate glutamate excitotoxicity (Li et al., 2013).

The study of Pivonkova and colleagues using astrocyte volume quantification detected a reduced OGD-evoked astrocyte volume increase in TRPV4 deficient mice (Pivonkova et al., 2018). Furthermore, Hermanova et al. detected that deletion of AQP4 in the astrocyte cultures reduced cell swelling during OGD, while simultaneous deletion of both AQP4 and TRPV4 led to a disturbance of astrocyte volume recovery during subsequent washout (Hermanova et al., 2024). Moreover, they identified two distinct subpopulations of astrocytes with low and high volumetric responses (LRA and HRA), while the responses of HRA were more affected by the deletion of AQP4, TRPV4, or both channels (Hermanova et al., 2024). Furthermore, the analysis of gene expression revealed reduced expression of KCC1, chloride channel 2 (ClC2), and receptor GABA_B and NMDA in TRPV4^{-/-} mice and of the serine/cysteine peptidase inhibitor Serpina3n in the AQP4^{-/-} mice (Hermanova et al., 2024).

In contrast to *in vivo* studies conducted during ischemia (Chmelova et al., 2019; Hirt et al., 2017; Yao et al., 2015), we did not observe any effect of AQP4 deletion during OGD *in situ*. This could be explained by a previously seen protective effect of hypothermic (32-33°C) preconditioning that was described *in situ* (Jiang et al., 2018; Kreisman et al., 2020). Moreover, when studying α -syn^{-/-} mice, we demonstrated that severe pathological conditions are needed to reveal the role of AQP4 in cell swelling (Anderova et al., 2014; Dmytrenko et al., 2013). Therefore, it is possible that a small oxygen content and removal of toxic metabolites by perfusion make the effect of AQP4 channel deletion below the discriminative ability of the RTI method.

Unlike in single transgenic strains, the deletion of both AQP4 and TRPV4 completely blocked the α decrease during the application of OGD, which is in full agreement with *in vivo* experiments after pMCAO, where the lesion area in an early post-ischemic tissue in the double knockouts was markedly reduced 1 day after ischemia. In retinal Müller cells, the protective effect of TRPV4^{-/-} deletion has been linked to a lower expression of AQP4 (Jo et al., 2015). Therefore, the deletion of both channels in our experiments could have a more distinctive protective effect than the deletion of TRPV4 and a downregulation of AQP4 expression.

It is important to remember that changes in the ECS volume fraction reflect volume changes in several cellular components of the tissue, such as neurons or NG2-glia, which

swell and regulate their volume through different mechanisms than astrocytes. (Andrew et al., 2007; Budde and Frank, 2010; Caspi et al., 2009; Murphy et al., 2017). Therefore, changes in the ECS volume fraction do not always correspond with volume changes of astrocytes, as we have demonstrated in several papers (Anderova et al., 2014; Tureckova et al., 2022). Moreover, AQP4 channels are expressed in the brain almost exclusively in astrocytes (Saadoun et al., 2005), whereas TRPV4 channels are expressed in various cell types, where they contribute to distinct functions and processes. For example, in neurons, TRPV4 channels are involved in modulation of neuronal excitability (Shibasaki et al., 2015), while in astrocytes, they are primarily localised at the interface between brain and extracerebral liquid spaces. This supports the hypothesis that TRPV4 channels participate in osmosensation and play a crucial role in brain volume homeostasis (Benfenati et al., 2007, 2011).

The previously mentioned findings contribute to the hypothesis that the mechanisms of cell swelling, and volume regulation evoked by distinct models of cytotoxic edema may vary. The involvement of the AQP4/TRPV4 complex should be thus carefully evaluated, especially when comparing *in vivo*, *in situ* and *in vitro* models. The results may differ with the temperature used and it has been proved that general anaesthesia by ketamine injection or isoflurane inhalation reduces the ECS volume in a dose-dependent manner (Gakuba et al., 2018; Zhao et al., 2020).

Since extracellular diffusion reveals the structure of nervous tissue fairly accurately (Nicholson and Sykova, 1998), changes of the ECS volume and tortuosity may provide us with more comprehensive information about the general impact of the AQP4 and/or TRPV4 deficit on the development of cytotoxic edema. Our results indicate that AQP4 and TRPV4 channels may play a crucial role in the size of acute edema development/recovery in severe pathological states.

5.4.1 The simultaneous deletion of AQP4 and TRPV4 reduces extracellular potassium increase during OGD

While in the AQP4^{-/-} or TRPV4^{-/-} mice, the levels of $[K^+]_e$ did not differ from those in the Ctrl mice, the peak values during OGD in the AQP4^{-/-}/TRPV4^{-/-} mice were significantly lower. This finding suggests that in AQP4^{-/-}/TRPV4^{-/-} mice altered neuronal activation associated with TRPV4 deletion could play a role, as well as impaired function of K^+ channels due to AQP4 deletion. Related to that statement, it was observed that deletion of AQP4 (which is believed to co-localize with Kir4.1 channels) or its anchoring protein α -syn

slowed potassium kinetics (Amiry-Moghaddam et al., 2003; Binder et al., 2006; Dmytrenko et al., 2013). In addition, Shibasaki and colleagues showed that the influx of cations through TRPV4 in neurons may control neuronal excitability by regulating the resting membrane potential, thereby influencing the movement and distribution of ions (Shibasaki et al., 2007, 2015). However, we did not observe a significant difference in an increase in $[K^+]_e$ during OGD between the AQP4^{-/-} or TRPV4^{-/-} and Ctrl mice. It is plausible that concomitant deletion of both the AQP4 and TRPV4 channels was necessary to increase this difference to a significant level.

Since α -syntrophin serves as an anchoring protein for both AQP4 and Kir4.1 (Connors et al., 2004), the detected alterations in $[K^+]_e$ observed in α -syn^{-/-} mice may not necessarily be present in AQP4^{-/-} animals.. Electron microscopy and co-immunoprecipitation analyses showed the co-localisation of AQP4 and Kir4.1 in the retina (Connors et al., 2004; Guadagno and Moukhles, 2004; Nagelhus et al., 2004). Their functional relationship is far from being understood, as there are controversial results regarding compromised K^+ clearance in α -syn or AQP4 knockout animals (Amiry-Moghaddam et al., 2003; Binder et al., 2006; Connors et al., 2004) and enhanced glial gap junctional coupling in AQP4^{-/-} mice (Strohschein et al., 2011).

The increase in $[K^+]_e$ during OGD treatment in the control animals reached a maximum of about 8 mM, which is rather low compared to ischemia *in vivo* with a typical rise in $[K^+]_e$ exceeding 70 mM (Chmelova et al., 2019; Vorisek and Sykova, 1997a). As mentioned above, the OGD model of ischemia in tissue slices cannot fully mimic real ischemic conditions *in vivo*, as it allows the removal of toxic metabolites, and the perfusing solution contains 5 % oxygen. Furthermore, it could be associated with the previously seen protective effect of hypothermic preconditioning (32-33°C) that is present *in situ* (Jiang et al., 2018; Kreisman et al., 2020). Other *in situ* studies have also reported a much lower increase in $[K^+]_e$ during OGD than in ischemia *in vivo* (Helleringer et al., 2017; Jiang et al., 2018; Perez-Pinzon et al., 1995; Rice and Nicholson, 1991).

5.5 Does AQP4 and TRPV4 channel deletion have a protective or deteriorating effect *in vivo*?

Surprisingly, the quantification of MRI images following pMCAO revealed that mice lacking either the AQP4 or TRPV4 channels display significantly enlarged ischemic lesions at day 1 and day 7, when compared to mice carrying both channels. In addition,

immunohistochemical analyses employing antibodies against GFAP and Iba1 revealed the presence of reactive astrocytes and microglia within the damaged hemisphere. These data suggest a protective role of AQP4 and TRPV4 during the development of brain edema, especially in the subacute phase after ischemic injury (D1). Furthermore, at D7, the deletion of either AQP4 or TRPV4 still had a negative impact on the extent of ischemic damage. Similar findings were demonstrated before, seen as aggravation of ischemia/reperfusion injury in AQP4 knockout mice (Shi et al., 2012a; Zeng et al., 2012) and TRPV4 knockout mice (Pivonkova et al., 2018). In contrast, other studies showed a reduced lesion size in TRPV4^{-/-} mice (Tanaka et al., 2020) and reduced brain edema and infarct volume in AQP4-deficient mice (Yao et al., 2015).

In the literature, controversies concerning the protective or detrimental effect of AQP4 deletion on the development of brain edema are common. Katada and colleagues (Katada et al., 2014) showed reduced brain swelling after bilateral transient carotid artery occlusion, and Manley and colleagues (Manley et al., 2000) demonstrated less pronounced cerebral edema in response to stroke, which was accompanied by improved neurological deficit scores. An improved long-term outcome associated with decreased lesion volume, neuronal cell death, and neuroinflammation was also described in the AQP4^{-/-} mice after transient cerebral ischemia (Hirt et al., 2017). In studies in which the AQP4 inhibitor TGN-020 was implemented, there has been reduced cerebral edema in rats following MCAO (Pirici et al., 2018; Sun et al., 2022). However, the opposite effect of AQP4 deletion was shown during the development of brain edema associated with bacterial infection, where deletion of AQP4 led to an increase in the brain swelling (Bloch et al., 2005). Such discrepancies are likely caused by the different mechanisms of tissue swelling employed in each model and by the different contribution of vasogenic and cytotoxic types of brain edema. AQP4^{-/-} mice have been shown to swell less than control animals in conditions associated with cytotoxic brain edema, but worse in experimental approaches that cause vasogenic brain edema (Clement et al., 2020; MacAulay, 2021; Papadopoulos et al., 2004). In addition, AQP4 expression responds to ischemia. In rodent stroke models, early increases in AQP4 expression parallel the development of cytotoxic and ionic brain edema (Risher et al., 2009). In mice treated with transient MCAO, AQP4 expression was rapidly upregulated in the perivascular astrocyte endfeet, peaking at 1 h after stroke onset in the infarct core and penumbra (Fukuda et al., 2012; Hirt et al., 2009). However, increased AQP4 expression was not detected in more severe stroke models (Friedman et al., 2009), prompting the hypothesis that under

severe ischemia, the brain is not able to synthesise AQP4 during the early phase of reperfusion. Interestingly, Hirt et al. have shown, using a thrombin preconditioning paradigm, that upregulated AQP4 expression attenuated the early phase of hemispheric enlargement 1 hour after MCAO (Hirt et al., 2009).

Several studies have shown that AQP4 co-localises with various channels/transporters. In AQP4 knockout the function of Kir4.1, ClC2, VRAC and glutamate uptake was deteriorated (Benfenati et al., 2007; McCoy et al., 2010; Reed and Blazer-Yost, 2022; Zeng et al., 2007). As these channels/transporters are involved in ion, volume and glutamate homeostasis, the resulting detrimental or beneficiary effect of AQP4 deletion might depend on the prevailing activity of different transport mechanisms. It has been proposed that during ischemia, SUR1-TRPM4 and AQP4 can form a novel heteromultimeric water/ion channel complex, which synergistically regulates astrocyte swelling (Jha et al., 2021; Stokum et al., 2018). The change in the AQP4 cooperating partner from TRPV4 to SUR1-TRPM4 after ischemia induction (Stokum et al., 2018) may explain the different findings in the acute and early chronic phase of ischemia. Currently, a SUR1-TRPM4 channel inhibitor is the only drug that has advanced to clinical trials for treating cerebral edema after ischemic stroke (Yao et al., 2021). Inhibition of this complex by glibenclamide has been shown to reduce cerebral edema (Pergakis et al., 2019; Sheth et al., 2016). However, it is not known how AQP4 deletion affects the function of SUR1-TRPM4 complex.

Furthermore, Chu and colleagues (Chu et al., 2023) demonstrated that AQP4 and Cx43 were co-expressed in astrocytes after MCAO and their expression was increased in the ipsilateral subventricular zone and the peri-infarct cortex, suggesting that Cx43 and AQP4 participated in neurogenesis. Besides, higher levels of inflammatory cytokines and lesser neurogenesis were detected in AQP4 and Cx43 knockout mice. The extent of inflammation could explain the increased volume of lesion in AQP4 knockout. Moreover, in another study, it has been also detected that AQP4 channels play an inhibitory role in post-ischemic inflammation caused by microglia (Shi et al., 2012a, 2012b). Aggravated inflammation in AQP4^{-/-} may thus significantly enlarge the post-ischemic lesion, especially in the early chronic phase of ischemia (D7). Furthermore, pro-inflammatory mediators released by activated microglia have been found to release MMPs in *in vitro* astrocyte cultures (Candelario-Jalil et al., 2009), which are partially responsible for BBB disruption (Rosell et al., 2006) and degradation of agrin or dystroglycan (Wolburg-Buchholz et al., 2009), both of

which play a pivotal role in the formation of OAP and AQP4 assembly in astroglia endfeet membranes (Fallier-Becker et al., 2011; Noell et al., 2009).

Localisation of AQP4 loses its polarization to the astrocytic endfeet following ischemia induction. The positioning primarily depends on its binding to the α -syntrophin/dystroglycan complex and its integration into OAPs through binding to the ECM molecule agrin (Noell et al., 2009), which subsequently reduces its lateral movement. A more detailed analysis of α -syntrophin impact on edema formation was provided in the discussion of the RTI results (see Chapter 5.1 to 5.4). The disintegration of OAPs and changes in the ratio between AQP4-M1 and AQP4-M23 are observed early after stroke (Iwasaki et al., n.d.; Lisjak et al., 2017); however, although the consequences of the changes in OAPs in brain injury are still unknown, it could potentially influence water movement.

The role of TRPV4 in the brain edema formation was previously demonstrated in the TRPV4 knockout animals, where TRPV4 deficiency led to the reduction of cell swelling and amelioration of peri-infarct depolarisation (Hoshi et al., 2018; Jie et al., 2015; Rakers et al., 2017), as well as to the decrease in lesion volume and milder neurological symptoms (Tanaka et al., 2020). On the contrary, a recent study showed that TRPV4 deletion aggravated the extent of brain edema as evaluated 1 day and 7 days after pMCAO (Pivonkova et al., 2018). The results of our experiments are in agreement with the deteriorating effect of TRPV4 channel deletion. Similarly, in a recent work, Rosenkranz and colleagues observed no protection in mice lacking TRPV4 in various pathological models, including transient MCAO (Rosenkranz et al., 2020), when experiments were performed on the same mouse strain as Pivonkova et al. implemented (Pivonkova et al., 2018). The discrepancies could be related to the used mice strain, the different MCAO model, the method of quantification (2,3,5-Triphenyltetrazolium Chloride (TTC) staining vs. T2-weighted MRI in Tanaka et al., 2020 and Pivonkova et al. 2018, respectively).

The increased activity of TRPV4 channel affects the expression or function of several membrane proteins such as BK_{Ca}, NMDA receptors, α -amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptors (AMPA_Rs), IP₃Rs, RyRs, AQP4, and other potential cooperative receptors in the brain (Liu et al., 2020). Since TRPV4 co-localizes with AQP4 (Benfenati et al., 2011; Jo et al., 2015), TRPV4 knockout may also affect the function of the AQP4-co-expressing channels (Mola et al., 2021). Activated TRPV4 channels in endothelial and smooth muscle cells mediated the activation of BK_{Ca}, leading to cerebral arterial dilation that improved the hypoperfusion present in the infarcted area (Han et al., 2018; Xia et al.,

2002). However, activation of TRPV4 and subsequent increase in perfusion paradoxically caused vasogenic edema and worsening of damage, as shown in traumatic brain injury (Szarka et al., 2018). Moreover, inhibition of TRPV4 channels using the antagonist HC-067047 reduced cerebral ischemic injury in mice by preventing glutamate excitotoxicity induced by NMDA receptor activation (Li et al., 2013).

TRPV4 channels in astrocytes are sensitive to mechanical changes and when activated by experimentally induced increases in blood pressure, they trigger Ca^{2+} signaling and ATP through connexins (Turovsky et al., 2020). ATP then amplifies astroglial Ca^{2+} excitation by activating the purinergic G protein-coupled receptor P2Y1, which recruits Ca^{2+} from intracellular stores. We may speculate that TRPV4 activity can block the activation of astrocytic K-ATP channels, which are normally activated by a decrease in cellular ATP levels (Tureckova et al., 2023). K-ATP channels respond to low ATP or high ADP levels, and their activation leads to cell hyperpolarization, inhibiting voltage-gated Ca^{2+} channels, and reducing excessive glutamate release (Wang et al., 2004; Busija et al., 2004). Structurally, K-ATP channels contain sulfonylurea receptor subunit SUR1 in astrocytes (Sun and Hu, 2010), which is the same as in SUR1-TRPM4 channels, key player in brain edema formation (Reed and Blazer-Yost, 2022). Inactivating K-ATP channels through TRPV4 signaling or using inhibitors such as glibenclamide could help prevent brain edema (Maqoud et al., 2022; Woo et al., 2020).

The potential role of changes in TRPV4 expression and its activity as treatments for cerebral ischemia remains unclear. It appears that several channels/transporters can serve as TRPV4 effector proteins and a dual effect of TRPV4 blockage (especially in the acute or delayed phase of ischemia) may be caused by a 'switch' between various effectors. Moreover, it has been shown that under some pathological conditions, such as ischemia, TRPV4 channels were upregulated (Butenko et al., 2012) and sensitised via cellular signalling pathways causing additional tissue damage.

Unlike AQP4, which is expressed predominantly in astrocytes (Saadoun et al., 2005), TRPV4 channels are expressed by a wide variety of cell types. In neurons, TRPV4 is involved in modulating neuronal excitability (Shibasaki et al., 2015). In astrocytes, they are abundant in astrocytic endfeet processes around blood vessels and thus might play a key role in brain volume homeostasis (Benfenati et al., 2011, 2007). Interestingly, recent studies discovered that only a subset of astrocytes expressed TRPV4 and responded to neuronal signals (Milich et al., 2021; Pivonkova et al., 2018; Zhang et al., 2014). However, these cells

activate neighbouring TRPV4-negative astrocytes via ATP and gap junction-mediated signalling (Shibasaki et al., 2014). An issue that should be considered when interpreting single-cell polymerase chain reaction (PCR) or ribonucleic acid sequencing (RNAseq) data may be that treatment of cell suspensions may result in the loss of locally expressed proteins and mRNA, predominantly in small processes and astrocytic endfeet (Boulay et al., 2017; Sakers et al., 2017). Recent research discovered TRPV4 channels on OPCs, where their activation promoted proliferation (Ohashi et al., 2018). It has also been shown that TRPV4 channels regulated microglial activity (Chakraborty and Goswami, 2022; Konno et al., 2012) and played a role in the regulation of cerebral perfusion through their effect on smooth muscles (Diaz-Otero et al., 2019). Interestingly, Redmon and colleagues demonstrated that TRPV4 channels may represent a primary retinal microglial sensor of osmochallenges under physiological and pathological conditions and suggested that TRPV4 inhibition might be a useful strategy to suppress microglial overactivation in the swollen and edematous CNS (Redmon et al., 2021). In summary, due to its broad expression in various cell types, TRPV4 channels may be activated during ischemia by multiple stimuli and involved in various functions.

5.5.1 The simultaneous deletion of AQP4 and TRPV4 strongly attenuates brain edema formation in the subacute post-ischemic phase, but not in the early chronic phase

Interestingly, simultaneous deletion of AQP4 and TRPV4 produced an effect that was quite the opposite of what was observed in the single knockouts. We detected significant reduction of the size of ischemic lesion 1 day after pMCAO and less affected ADC_w in AQP4^{-/-}/TRPV4^{-/-} mice, when compared to the Ctrl mice. However, after 7 days the size of the lesion in AQP4^{-/-}/TRPV4^{-/-} mice was comparable to that in Ctrl animals. Our results indicate that there is indeed an interplay between AQP4 and TRPV4, which was previously suggested in experiments using cultured glia (Benfenati et al., 2011; Jo et al., 2015) or in meningiomas (Faropoulos et al., 2021), where AQP4-mediated water fluxes promoted TRPV4 activation, while Ca²⁺ entry through TRPV4 channels reciprocally modulated volume regulation, swelling, and *Aqp4* gene expression (Jo et al., 2015). Their cooperation is probably more prominent during the acute phase of ischemia, because 7 days the damage detected in the AQP4^{-/-}/TRPV4^{-/-} mice was comparable to that observed in the Ctrl mice. Therefore, we can conclude that the simultaneous deletion of both channels may play a protective role during the development of brain edema. Despite many studies, the

mechanism underlying the interaction between TRPV4 and AQP4 is still poorly understood. It is well established that TRPV4 and AQP4 are co-expressed in astrocyte plasma membranes (more often in the subarachnoid and perivascular spaces where they are likely to play a role in the export of osmolytes to the CSF) and they are important in triggering the RVD (Benfenati et al., 2011). Eilert-Olsen et al. described rapid Ca^{2+} oscillations in subpial endfeet critically dependent on IP₃R2 receptors, but also showed some dependence on TRPV4 and dystrophin (Eilert-Olsen et al., 2019). In the study, rapid entry of water through AQP4 induced Ca^{2+} influx via TRPV4 channels and Ca^{2+} -induced Ca^{2+} release from the endoplasmic reticulum modulated by IP₃R2 receptors that has been seen in different astrocytic territories (Eilert-Olsen et al., 2019). In contrast, another study reported that TRPV4 activation was independent of osmolality-associated AQP4 permeability (Wang and Parpura, 2018). The upregulation of SUR1-TRPM4 observed in traumatic brain injury and ischemia (Gerzanich et al., 2019; Woo et al., 2020) or the replacement of TRPV4 with SUR1-TRPM4 as a partner of AQP4, as previously noted in cold brain injury (Stokum et al., 2018), could clarify the differing outcomes observed on day 7 post-ischemia during the early chronic phase. In the subacute phase (D1), TRPV4 appears to play a greater role in astrocyte swelling through its interaction with AQP4. However, as ischemia progresses, replacement of the TRPV4 with SUR1-TRPM4 may change the dynamics of water and ion transport, resulting in distinct effects on brain edema and cellular responses (Stokum et al., 2018). This shift in molecular interactions may underlie the variations in ischemic injury progression and recovery between the subacute and early chronic phases. Alternatively, as AQP4 and TRPV4 co-operate with other channels/transporters (Benfenati et al., 2007; McCoy et al., 2010; Reed and Blazer-Yost, 2022; Zeng et al., 2007), the resulting detrimental or beneficiary effect of their deletion might depend on the prevailing activity of distinct proteins in different phases of ischemia. Further research into the TRPV4-AQP4 interaction is essential to expand our understanding.

5.6 Perineuronal nets are attenuated in control and AQP4^{-/-}/TRPV4^{-/-} mice 7 days after pMCAO

Before ischemia, PNNs were present in the cortex with comparable distribution in all studied genotypes. However, at D7, the ipsilateral hemisphere, relative to the pMCAO injury, was characterised by decreased WFA positivity in the control and AQP4^{-/-}/TRPV4^{-/-} mice as compared to the non-ischemic and contralateral cortex. Furthermore, the total number of PNNs detected decreased in the ipsilesional cortex in all experimental groups.

After an ischemic stroke induced by MCAO, PNNs undergo degradation, especially in the peri-infarct region. This degradation is mediated by the increased activity of enzymes such as MMPs and other proteases that break down the ECM components of PNNs (Dzyubenko et al., 2023; Montaner et al., 2019). PNN degradation is associated with increased synaptic plasticity, which is critical for recovery; however, PNNs are also thought to provide neuroprotective functions by stabilising synapses and protecting neurons from excitotoxicity (Li et al., 2024).

In the work of Dzyubenko and colleagues cortical hypoperfusion moderately decreased the intensity of WFA staining, while an evident loss of PNNs was observed only in the ipsilesional cortex following MCAO and was not associated with neuronal cell death (Dzyubenko et al., 2018). Similarly, the study of Hartig et al. analysed brain tissues obtained 1 day after pMCAO and observed largely decomposed and nearly erased PNNs (stained with WFA) in infarcted neocortical areas and a relative decrease in the directly ischemia-affected neocortex (Hartig et al., 2017). Decomposition and reduced expression of PNN components were also reported after MCAO in spontaneously hypertensive rats. In the cortical periinfarct region, delineated by an astrocytic scar, the number of WFA-stained PNNs was permanently reduced even a month after MCAO (Hobohm et al., 2005; Karetko-Sysa et al., 2011). In the work of Karetko-Sysa et al., photothrombosis led to a 42% reduction in the density of PNNs stained with WFA as early as 4 h after stroke, while in the contralateral cortical regions, PNNs densities also decreased. At D30 post-stroke, the number of WFA-stained PNNs was still 70% lower than in control tissue (Karetko-Sysa et al., 2011). Recently, Dzyubenko et al. proposed that the loosening of PNNs creates permissive regions supporting GABAergic synapse plasticity at D7 and D14 post-stroke. PNN loosening and synaptic remodelling was observed in both ipsi- and contralesional hemispheres, which suggests bilateral rewiring of brain circuits after stroke (Dzyubenko et al., 2023). The beneficial effect of PNNs removal by exogenous enzymes on post-traumatic recovery was shown in several studies (Bradbury et al., 2002; Bradbury and Carter, 2011; Rosenzweig et al., 2019). The downregulation of PNNs in ischemic brains could thus be considered an attempt to create conditions favourable for synaptic remodelling and the onset of regeneration.

In our study, AQP4 and TRPV4 knockout mice, that exhibit larger lesions after pMCAO, had more pronounced PNN expression at D7. This finding suggests a potential compensatory response to an extensive neuronal damage, where the brain might attempt to stabilise and protect the remaining neurons by enhancing PNN formation. Alternatively, the

presence of PNNs could reflect a maladaptive response, where increased PNN expression inhibits plasticity in the peri-lesional areas, potentially limiting recovery. Assuming that ischemia-evoked PNN degradation enhances synaptic plasticity, we may hypothesise that control and AQP4^{-/-}/TRPV4^{-/-} mice may have more favourable conditions for regeneration. Further investigation into the functional outcomes associated with PNNs is necessary to determine details of their protective/detrimental effect on tissue regeneration in subacute and early chronic post-ischemic tissue.

6 CONCLUSIONS

We evaluated the role of AQP4 and TRPV4 channels, and their possible cooperation in the development of cytotoxic edema *in situ* and *in vivo* using transgenic mice lacking either AQP4, TRPV4 or both channels concomitantly.

From our results we can conclude:

1. We confirmed our hypothesis that both the AQP4 and TRPV4 channels are involved in the development of cytotoxic edema *in situ* and *in vivo*. However, their effect varies depending on the severity of the insult, time from the ischemia onset (acute/early chronic phase) and selected ischemic model. Our results indicate that AQP4 or TRPV4 promote cell swelling, especially during the acute phase of severe insults (terminal ischemia *in vivo*, OGD *in situ*), where their deficit had an attenuating effect. However, since both proteins are also involved in cell volume regulation during recovery phase, their deficits aggravated tissue damage in early chronic phase after induction of pMCAO.
2. The effect of AQP4 and/or TRPV4 channels on cell swelling can indirectly be also mediated by their influence on the $[K^+]_e$ elevation during ischemia. The impact of their deficiency depends on the severity of the insult. While the deficit of a single AQP4 or TRPV4 slowed down the $[K^+]_e$ rise during terminal ischemia, a concomitant deletion of both proteins was necessary to attenuate $[K^+]_e$ rise during OGD.
3. We confirmed that AQP4 and TRPV4 channels functionally cooperate in cell volume changes *in situ* and *in vivo*. Their concomitant deletion strongly potentiated the protective effect during acute OGD-induced cell swelling and even reversed the detrimental influence of a single deletion of AQP4 or TRPV4 deletion on the lesion size after induction of pMCAO into a protective effect observed in AQP4^{-/-}/TRPV4^{-/-} mice. However, the discrepancy in the impact of deletion of AQP4 and TRPV4 between the single- and double-transgenic animals during the early chronic phase after pMCAO indicates that both proteins may also cooperate also with other partners.
4. Our results confirmed the hypothesis that ischemia evokes an alteration of the ECM that can be affected by the AQP4 or TRPV4 deficit. The enzyme decomposition of PNNs detected in the early chronic phase (D7) after pMCAO was more pronounced in control and AQP4^{-/-}/TRPV4^{-/-} mice than in single knockouts. Assuming that

ischemia-evoked PNN degradation enhances synaptic plasticity and thus promotes tissue regeneration, these findings are in agreement with smaller lesions in control and AQP4^{-/-}/TRPV4^{-/-} mice compared to AQP4^{-/-} or TRPV4^{-/-} animals.

7 SUMMARY

In this thesis we evaluated the role of AQP4 and TRPV4 channels, and their possible cooperation in the development of cytotoxic edema using transgenic mice lacking either AQP4, TRPV4 or both channels (AQP4^{-/-}, TRPV4^{-/-}, AQP4^{-/-}/TRPV4^{-/-}, respectively). We compared the changes in the ECS diffusion parameter (volume fraction, α ; tortuosity, λ) changes in the *in vivo* model of terminal ischemia and *in situ* models of cytotoxic edema/ischemia (hypoosmotic stress, hyperkalemia, oxygen-glucose deprivation - OGD) using the real-time iontophoretic (RTI) method. Magnetic resonance imaging (MRI) was performed to quantify the brain lesions one day (D1) and seven days (D7) after permanent middle cerebral artery occlusion (pMCAO) complemented by the analysis of the perineuronal net (PNN) expression at D7.

The baseline values of α and λ , did not differ between the control and transgenic strains, except for AQP4^{-/-} mice that exhibited increased α values. Hypoosmotic stress and hyperkalemia did not reveal different responses between control and transgenic mice. In contrast, the deletion of the TRPV4 channel resulted in less pronounced OGD-induced cytotoxic edema, while in mice with concomitant deletion of AQP4 and TRPV4, the application of OGD did not provoke any volume changes. The RTI measurements *in vivo* revealed that AQP4 or TRPV4 deficit slows down the rate of α decrease during terminal ischemia/anoxia. Moreover, the absence of AQP4, TRPV4 or AQP4/TRPV4 complex affected the rise of $[K^+]_e$ during terminal ischemia or OGD, respectively. After the pMCAO induction, a single deletion of AQP4 or TRPV4 channels exacerbated the ischemic brain injury at D1 and D7. On the contrary, the simultaneous deletion of both channels resulted in a smaller brain lesion at D1, but similar tissue damage at D7, compared to control animals. This was in agreement with a decreased staining intensity of PNNs that can be associated with enhanced synaptic plasticity and tissue regeneration in control and AQP4^{-/-}/TRPV4^{-/-} mice at D7.

Our findings suggest that the interaction between the AQP4 and TRPV4 channels plays a crucial role in neuronal and non-neuronal swelling, especially during the acute phase of severe ischemic injury, where their deficits had an attenuating effect. In contrast, their involvement in cell volume regulation during recovery phase may aggravate the post-ischemic tissue damage in early chronic phase. We confirmed that AQP4 and TRPV4 channels functionally cooperate in cell volume changes *in situ* and *in vivo*. However, various responses to ischemia induction in different transgenic strains indicate that the AQP4 and

TRPV4 channels may collaborate with other proteins. Notwithstanding, targeting the AQP4-TRPV4 interaction could represent a promising approach for developing new therapeutic interventions for conditions like stroke.

8 SOUHRN

V této práci jsme zkoumali roli AQP4 a TRPV4 kanálů, a jejich možnou interakci při vzniku cytotoxického edému u transgenních myší s deficitem AQP4, TRPV4 nebo obou typů kanálů (AQP4^{-/-}, TRPV4^{-/-}, AQP4^{-/-}/TRPV4^{-/-}, v daném pořadí). Srovnávali jsme změny difúzních parametrů (objemová frakce, α ; tortuositá, λ) v *in vivo* modelu terminální ischemie a *in situ* modelech cytotoxického edému (hypoosmotický stres; hyperkalemie; deprivace kyslíku a glukózy – OGD) pomocí iontoforetické metody v reálném čase (RTI). Pomocí zobrazování magnetickou resonancí (MRI) jsme kvantifikovali mozkové léze první (D1) a sedmý den (D7) po permanentním uzávěru arteria cerebri media (pMCAO) a celkový obraz jsme doplnili analýzou exprese perineurálních sítí (PNN) v D7.

Bazální hodnoty α a λ se u kontrol a transgenních zvířat nelišily s výjimkou AQP4^{-/-} myší, u kterých jsme našli zvýšené hodnoty α . Hypoosmotický stres a hyperkalemie neodhalily žádné významné rozdíly mezi kontrolními a transgenními zvířaty. Oproti tomu při aplikaci OGD, delece TRPV4 kanálu vedla k méně výraznému cytotoxickému edému, zatímco při současné deleci AQP4 a TRPV4 aplikace OGD překvapivě nevyvolala žádné objemové změny. Měření pomocí RTI ukázala, že deficit AQP4 nebo TRPV4 zpomaluje rychlost poklesu α během terminální ischemie/anoxie. Nepřítomnost AQP4, TRPV4 nebo AQP4/TRPV4 komplexu navíc ovlivnila vzestup $[K^+]_e$ při terminální ischemii nebo OGD. Delece samotného AQP4 nebo TRPV4 kanálu zhoršila ischemické poškození jak první, tak sedmý den od začátku pMCAO. Současná delece obou kanálů však vedla první den po MCAO k menší mozkové lézi ve srovnání s kontrolními zvířaty, ale podobnému poškození tkáně v den sedmý. To je v souladu se sníženou intenzitou barvení perineurálních sítí, které je spojeno se zvýšenou synaptickou plasticitou a regenerací tkáně u kontrol a AQP4^{-/-}/TRPV4^{-/-} myší sedmý den po pMCAO.

Naše data naznačují, že interakce mezi AQP4 a TRPV4 kanály hraje klíčovou roli při neuronálním a neneuronálním formování cytotoxického edému zejména během akutní fáze těžkého ischemického poškození, kde jejich deficit zmírňuje poškození tkáně. Na druhé straně, jejich účast v objemové regulaci buněk při reperfúzi může zhoršit post-ischemické poškození tkáně během časně chronické fáze. Potvrdili jsme, že AQP4 a TRPV4 kanály funkčně spolupracují při změnách buněčného objemu *in situ* a *in vivo*. Rozdílné odpovědi na ischemii, které jsme pozorovali u různých transgenních kmenů, ale naznačují, že AQP4 a TRPV4 kanály mohou kooperovat i s jinými proteiny. Zaměření výzkumu na AQP4-TRPV4

interakci může přesto představovat slibný přístup pro vývoj nových terapeutických intervencí závažných patologií, jako je například cévní mozková příhoda.

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10 LIST OF PUBLICATIONS

Publications related to the thesis:

1. **Sucha P**, Hermanova Z, Chmelova M, Kirdajova D, Camacho Garcia S, Marchetti V, Vorisek I, Tureckova J, Shany E, Jirak D, Anderova M, Vargova L. The absence of AQP4/TRPV4 complex substantially reduces acute cytotoxic edema following ischemic injury. *Front Cell Neurosci.* **2022** Dec 8;16:1054919. doi: 10.3389/fncel.2022.1054919. PMID: 36568889; PMCID: PMC9773096. (IF=5.3, Q1)
2. Chmelova M, **Sucha P**, Bochín M, Vorisek I, Pivonkova H, Hermanova Z, Anderova M, Vargova L. The role of aquaporin-4 and transient receptor potential vanilloid isoform 4 channels in the development of cytotoxic edema and associated extracellular diffusion parameter changes. *Eur J Neurosci.* **2019** Jul;50(1):1685-1699. doi: 10.1111/ejn.14338. Epub 2019 Feb 8. PMID: 30633415. (IF=3.115, Q2)

Other publications:

1. **Sucha P**, Chmelova M, Kamenicka M, Bochín M, Oohashi T, Vargova L. The Effect of Hapln4 Link Protein Deficiency on Extracellular Space Diffusion Parameters and Perineuronal Nets in the Auditory System During Aging. *Neurochem Res.* **2020** Jan;45(1):68-82. doi: 10.1007/s11064-019-02894-2. Epub 2019 Oct 29. PMID: 31664654. (IF=3.996, Q2)

Poster presentations at professional meetings:

1. **13th European meeting on Glial Cells in Health and Disease 2017, Edinburgh, UK**
 - Impact of Bral2 link protein deficiency on the perineuronal nets and extracellular space diffusion parameters in the auditory system during aging
 - Contribution of TRPV4 channels to astrocyte volume regulation and brain edema formation
2. **11th conference of Czech neuroscience society 2017, Prague, CZE**
 - The role of Bral2 link protein on diffusion parameters of extracellular space and perineuronal nets in the auditory system during aging
3. **Cold Spring Harbor laboratory meeting: Glia in Health & Disease 2018, USA**
 - The effect of Bral2 link protein deficiency on extracellular space diffusion parameters and perineuronal nets in the auditory system during aging
 - Importance of AQP4 and TRPV4 channels in development of cytotoxic brain edema