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Reactive gliosis and the effect on Aquaporin-4 distribution within astrocytes – an experimental study on ischemic stroke

Degree Project in Medicine

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Abstract

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Degree Project in Medicine, Programme in Medicine, Gothenburg, Sweden 2021
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Background: Ischemic stroke is a major cause of death and disability all over the world and is often accompanied by cerebral edema. Astrocytes are one of the most abundant cells in the central nervous system and have a major role in acute stress management. Astrocytes responding to injury or disease is referred to as reactive (astro)gliosis.

Aquaporin-4 is a water-channel protein mostly located in the vascular astrocyte endfeet, involved in the cerebral water-balance and the formation of cerebral edema. The intracellular distribution of Aquaporin-4 is altered in ischemic stroke and the mechanism behind this is not yet fully understood.

Aim: The primary purpose of this study is to assess whether the intracellular distribution of Aquaporin-4 is altered when reactive gliosis is attenuated in ischemic stroke.

Methods: An experimental preclinical study on nine wild-type mice and seven mice with attenuated reactive gliosis assessed four weeks after photothrombotic stroke induction. Double immunofluorescence immunohistochemistry was performed with markers for Aquaporin-4 and Podocalyxin to visualise blood-vessels. Images was captured by Confocal microscopy and analysed in Image J using intensity plotting and colocalization.

Results: The results of this study showed no significant difference ($p>0.05$) in Aquaporin-4 distribution between wild-type mice and mice with attenuated reactive gliosis.

Conclusion: The results of this study suggest that reactive gliosis might have a limited impact on Aquaporin-4 intracellular distribution four weeks after ischemic stroke, at least in the cerebral cortex.

Key words: Ischemic stroke, Reactive gliosis, Aquaporin-4, Cerebral edema.

1. Background

This experimental study is set out to investigate the role of reactive astrogliosis on the Aquaporin-4 distribution in ischemic stroke. In a previous study within the research group, the role of astrocyte activation and reactive gliosis was assessed in hemorrhagic stroke. Preliminary data suggests that, in sharp contrast to ischemic stroke, attenuation of reactive gliosis in hemorrhagic stroke has major beneficial effects already at the acute stage. Altered intracellular distribution of Aquaporin-4 is implicated, at least in part, to be responsible for this effect in hemorrhagic stroke. Aquaporin-4 is considered a possible future target for treatment of stroke and various therapeutic attempts has been made, although so far mostly unsuccessful and more research in this field is required(1, 2).

1.1 Epidemiology of stroke

Stroke is defined by the World Health Organization (WHO) as “rapidly developing clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24 hours or longer, or leading to death, with no apparent cause other than of vascular originis” and can be divided into two groups - ischemic stroke and hemorrhagic stroke(3). Globally, about 80% of all strokes are ischemic but in high-income countries the number is closer to 90%(3). Hemorrhagic strokes can be divided in two sub-groups, intracerebral hemorrhage (ICH) and subarachnoid hemorrhage.

Stroke is one of the main cause of mortality and morbidity around the world(3, 4). In fact, around 15 million people suffer from a stroke each year(5, 6), it is the second largest cause of death in the world(4) and 50% of the surviving patients are chronically disabled(3, 6).
The burden of stroke is major. Not only are there massive consequences for patients and their families but also big costs for the public health economy. Moreover, it is believed to be an increased burden in the future due to demographic shifts, especially in low-income countries(3, 7).

1.2 Pathophysiology of stroke

The pathophysiology of ischemic stroke and hemorrhagic stroke is in many ways similar, however there are some differences.

1.2.1 Pathophysiology of ischemic stroke

Thrombosis or embolization causes a blood vessel to get occluded and the blood flow to be interrupted. Neurons are very sensitive to hypoxia and are rapidly affected when homeostasis is disturbed(8). The ischemic situation leads to a cascade of events, among these; release of damaging excitatory neurotransmitters (excitotoxity) and increased free radical and NO production. Furthermore, the ATP-dependent Na/K pump failure results in an accumulation of intracellular potassium, chloride and calcium. The increase of intracellular ions results in an osmotic gradient, an increase of intracellular water and intracellular swelling inside neurons and astrocytes, referred to as cytotoxic edema(9, 10). As a result, the cells undergo apoptosis or necrosis(8, 11) and there is a loss of neuronal function(5). Later on, the blood-brain barrier

(BBB) is disrupted by the disturbance of endothelial cell tight junctions(12) and extracellular accumulation of water (vasogenic edema) is formed, causing the intracranial pressure to rise(8, 12). Moreover, the ischemic event initiates an immunologic response and activates glial cells which will be further addressed later in this report.

1.2.2 Pathophysiology of intracerebral hemorrhage

The brain is surrounded by the skull, creating a closed room with limited possibilities to increase its volume(13). A blood vessel rupturing is mostly due to trauma or hypertension and as the hematoma grows, the mass effect causes the intracranial pressure (ICP) to rise, tissue to be compressed and primary brain injury to occur(14). Formation of cerebral edema leads to compression of structures and vasculature with risk of herniation and disruption of blood flow. The extent of vasogenic edema is suggested to be correlated to the increase of ICP, recovery-time and is a major contributor to mortality in stroke patients(13). Moreover, edema, inflammatory response and toxic blood-components causes secondary brain injury(15). The cascade of molecular mechanisms in response to injury seems to be similar as for ischemic stroke; excitotoxicity, ion influx, dysfunction of the BBB, creation of cytotoxic and vasogenic edema and a prominent activation of glial cells known as reactive astrogliosis(5).

1.3 Reactive astrogliosis

Neuroinflammation is a crosstalk between cells from the innate and adaptive immune system, glial cells, endothelial cells, neurons and ependymal cells. The inflammation in the central nervous system (CNS) starts in the acute phase of disease by damage associated molecules

binding to receptors on glial cells and the inflammation prolongs for weeks. Leukocytes entre the damaged brain as the BBB is disrupted(16). This thesis paper focus more exclusively on the reactive glial cells.

1.3.1 Glial cells

There are many different cell-types in the brain, and they all work together as a collaborative network. Glial cells are a group of cells that support neurons, regulate our brains immune system, release proinflammatory mediators and interact with other immune cells in the CNS. The different types of glial cells are; microglia, astrocytes, oligodendrocytes and NG2-cells(17).

Microglial cells function much like vacuum cleaners, clearing the brain from microbes and dead cells, but microglia also participate in the immune response. Oligodendrocytes are involved in myelinization(17). Astrocytes have a broad spectrum of functions as modulators of homeostasis, assistance with myelinization, formation and maintenance of synapses and Blood-Brain Barrier (BBB) maintenance, supporting neurons with nutrients and responding to CNS-injury(18, 19). Reactive astrogliosis is a term for the altered gene expression, morphological changes and functional changes that takes place in astrocytes as a response to CNS-injury or disease(18).

1.3.2 Mechanisms of reactive astrogliosis

Brain injury or disease lead to an increase of damage associated proteins which microglial cells reacts to by releasing cytokines and proinflammatory mediators(8, 16, 17). Astrocytes exposed to proinflammatory mediators starts the immunological response possibly through the STAT3-signalling pathway(19). Upon activation, astrocytes change their gene expression causing an upregulation of certain proteins and changes in their morphology. Reactive astrocytes have both general features such as hypertrophy of cellular processes and astrocyte migration, and disease-specific components(20). Studies have suggested several possible proteins involved in this mechanism such as glial fibrillary acidic protein (GFAP), vimentin but also the water-channel protein Aquaporin-4 and some has the potential to be therapeutic targets in the future(18, 20, 21). In fact, the research for drugs affecting astrocytes is already looking promising(18).

Reactive astrocytes have been shown to play a pivotal role in many disease situations; neurotrauma, cerebral ischemia and neurodegenerative diseases such as Amyotrophic lateral sclerosis (ALS) and Alzheimer’s disease. Evidence suggest that reactive astrogliosis have a crucial role inhibiting the formation of amyloid plaques in Alzheimer’s disease, reduce the size of the infarction in focal brain ischemia and decrease the loss of synapses in neurotrauma, but can also have detrimental effects such as limiting functional recovery(18).

1.3.3 Reactive astrogliosis and intermediate filaments

Astrocyte intermediate filaments are a part of the cells cytoskeleton and seem to have a major role in the response to CNS-injury and acute, subacute or chronic stress(18, 22). Glial fibrillary acidic protein (GFAP) and Vimentin are two intermediate filament proteins that are strongly upregulated in astrocytes in response to injury. In fact, GFAP and Vimentin serves as hallmarks for reactive astrogliosis. Moreover, GFAP/Vimentin null mice show attenuated reactive astrogliosis and glial scarring. $GFAP^{-/-}Vim^{-/-}$ mice is an established genetic mouse model for investigating the function of reactive astrogliosis and are used in this study as a model for attenuated astrogliosis(17, 18).

1.3.4 Reactive astrogliosis and stroke

Brain ischemia as well as brain hemorrhage leads to prominent astrocyte activation(17, 20). In the area surrounding the infarction, glial cells are the dominating cell-type(17). In fact, some astrocytes migrate to the core of the lesion, forming a glial scar together with pericytes and microglial cells which creates a wall around the lesion. However, activated glial cells are believed to be both beneficial and harmful in the acute stage of ischemia. Reactive astrogliosis may have negative effects when releasing cytokines, neurotoxins and matrix metalloproteinases (MMPs), contributing to increased inflammatory response and disruption of the BBB(13, 17) but positive since astrocytes have the ability to counteract brain edema and efflux ions to decrease the hypo-osmolar environment(20). Moreover, astrocytes are crucial for maintenance of homeostasis, reconstruction of the BBB, reducing brain damage and increasing neuronal survival(17, 19).

In the recovering brain, reactive astrocytes seem to be inhibiting axonal sprouting and may also inhibit synaptic plasticity(20). On the other hand, reactive glial cells are beneficial for recovery by reducing brain damage, cleaning up dead cells and releasing growth factors(17). Recent evidence suggest reactive astrocytes to be beneficial for vascular repair and remodeling after ischemic stroke(23). Furthermore, astrocytes secrete vascular endothelial growth factor (VEGF) and thrombospondins stimulating angiogenesis and synaptogenesis(19).

1.4 Aquaporin-4

Aquaporin-4 seem to have a broad role in the CNS. Not only does Aquaporin-4 control the cerebral water-balance, but was also found participating in neural signal transduction, astrocyte migration, potassium ion clearance, glial cell swelling, brain tumor growth and affecting our memory(2, 24). Considering this, Aquaporin-4 is most likely involved in many cerebral diseases such as stroke, epilepsy, neuromyelitis optica, glioblastoma, meningitis and neurodegenerative diseases(21, 24, 25). However, this report will further investigate Aquaporin-4 in a specific disease situation, cerebral infarction.

1.4.1 Aquaporin-4 - Structure and function

Aquaporin-4 is a member of the large family of Aquaporins – water channel proteins fluxing water bidirectional in response to osmotic pressure. Many aquaporins have specific organ and cell localization, for some aquaporins this spectrum is broad(1). The structure of Aquaporins are similar, six transmembrane and two shorter helical segments forming a channel.

Aquaporin-4 often assemble in multimers called orthogonal arrays of intramembranous particles (OAPs) and have mainly two isoforms, the bigger M1 and smaller M23(26). Aquaporin-4 is strongly expressed in the brain and mostly located at the end foot-processes of the astrocyte enwrapping the cerebral blood vessels(1, 27), often referred to as polarized Aquaporin-4. Polarized Aquaporin-4 is anchored by the interaction with α-Syntrophin and the dystrophin-associated glycoprotein complex(28-30). OAPs are essential for polarization(26) and polarization of Aquaporin-4 is essential to ensure proper function and is suggested to be important for clearance of neurotoxins(31). Loss of polarization is associated with formation of vasogenic edema and does also counteract cytotoxic edema formation(32, 33), the polarization of Aquaporin-4 could therefore be a mechanism to consider for future therapeutic targets.

Moreover, aquaporin-4 is also lining the Subarachnoid space(1) and astrocyte endfeet forms the Blood-brain barrier (BBB) together with endothelial cells and pericytes. Aquaporin-4 facilitates the flow of water in and out of the parenchyma and across the Blood-brain barrier(34), affecting the cerebral water-balance and subsequently brain edema formation(27). Aquaporin-4 has been shown to be important for the vasogenic edema formation as well as the cytotoxic edema and also to affect the potassium ion homeostasis(1). Furthermore, Aquaporin-4 is suggested to be involved in the flow of cerebrospinal fluid(24). However, Aquaporin-4 and its passive transport of fluid is not alone responsible for the cerebral water balance, co-transporting proteins are needed for active transport and the importance of this is not to be forgotten(29).

1.4.2 Aquaporin-4 and stroke

The expression of Aquaporin-4 tends to be upregulated after ischemic stroke, however opposing results have also been suggested which seem to depend on study design(35-38). Increased expression of Aquaporin-4 is beneficial in late stage ischemic stroke due to its role in clearing the vasogenic edema(27). In comparison, deletion of Aquaporin-4 in mice with acute ischemic stroke lead to smaller lesions, less parenchymal edema, lower ICP, better functional outcome and higher survival-rate(9, 39, 40). In conclusion, Aquaporin-4 seems to be on one hand beneficial, but on the other hand harmful, depending on the type of edema and stage of disease (Fig.1). Considering that cerebral edema leads to elevated ICP, tissue damage and is a major cause of deaths related to stroke(29), it is no wonder that the interest for Aquaporin-4 in medical research has exploded over the last decade.

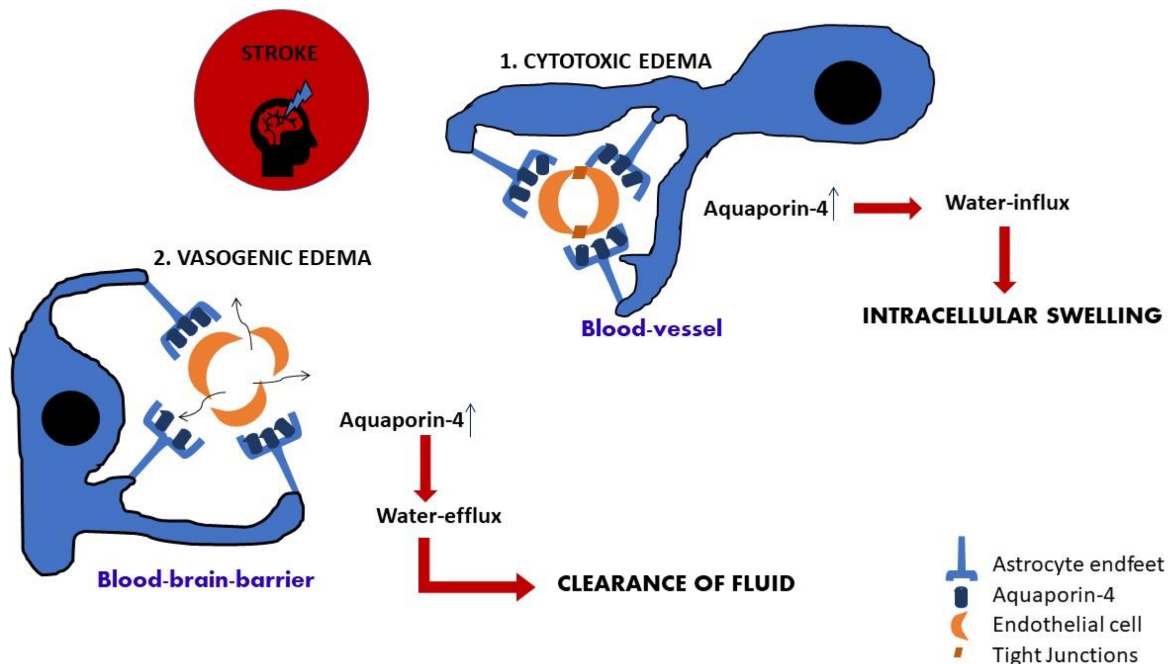

Figure 1. The dual role of Aquaporin-4 in ischemic stroke. In ischemia and hypoxia, the lack of ATP causes the ATP-dependent Na/K-pump to fail. The increase of intracellular ions creates an osmotic gradient and the passive influx of water into the cell is increased and facilitated by Aquaporin-4. Increased expression of Aquaporin-4 in ischemic stroke affects the water-permeability and water-influx to the cell. In conclusion, Aquaporin-4 enhances formation of cytotoxic edema. Furthermore, reactive astrocytes release vascular permeability factors and cytokines affecting the endothelial tight junctions and consequently leads to disruption of the Blood-Brain-Barrier (BBB). Extracellular fluid leaks into the parenchyma and vasogenic edema is formed. Aquaporin-4 facilitate clearance of the vasogenic edema and Aquaporin-4 is therefore detrimental in early stages of ischemic stroke but beneficial in later stages.

Evidence suggest that ischemia not only affects the expression of Aquaporin-4 but also alter the Aquaporin-4 polarization resulting in insufficient edema clearance(31, 33) and possibly also affecting the clearance of neurotoxic proteins, causing increased secondary neurodegeneration(31). Ischemia leads to a redistribution of Aquaporin-4 away from the endfeet in grey matter and increased polarization in white matter, but the mechanism behind this remain unclear(41). Research in this field present several possible mechanisms behind the altered distribution of Aquaporin-4. Firstly, ischemia is suggested to change the M23/M1 ratio resulting in more M1, the larger M1 size limits OAP formation and decrease polarization(26).

Secondly, the abutting cells are suggested to contribute, more specifically capillary cell-types facing the astrocyte endfeet. Pericytes in particular seem to have positive effect on the polarization of Aquaporin-4(42). Thirdly, a recent experimental study on rats with middle cerebral artery occlusion (MCAO) suggest hyperglycemia to affect Aquaporin-4 distribution. Hyperglycemia decreased the polarization of Aquaporin-4 and aggravated the cerebral edema. Moreover, the study indicated regional differences, where striatum was more affected by hyperglycemia than the cortex(43). Fourthly, a study on the brain glymphatic system and Aquaporin-4 distribution suggested polarization of Aquaporin-4 to be dependent on circadian rhythm. The authors suggest polarization of Aquaporin-4 to be peaking at mid-day due to rhythmic gene-expression of proteins in the complex anchoring Aquaporin-4(44). Lastly, another possible mechanism to affect polarization of Aquaporin-4 is degradation of membrane proteins serving as anchors(31, 33), but it is also suggested to be related to reactive astrogliosis. In response to ischemia astrocytes release MMPs and MMPs have been suggested to inhibit OAP formation consequently affecting the polarization of Aquaporin-4(33, 45).

Despite the clinical importance of cerebral edema and the damage that follows, not all seems to be known about the complex mechanism behind the formation of edema and cerebral water-balance, reflecting the lack of effective treatments in this field. Treatments targeting the formation of edema rather than decreasing already existing edema would without doubt be helpful. Researchers seem to agree that Aquaporin-4 is a promising target for treatment of cerebral edema. So far, different approaches have been tried to affect Aquaporin-4; regulation of gene-expression, regulation on the channel gating and regulation of permeability(46, 47).

Although modulation of Aquaporin-4 looks promising in vivo, it seems to be difficult to apply in humans(16, 48).

In a recent animal study on spinal cord injury the authors suggested a slightly different approach, to decrease the hypoxia-induced Aquaporin-4 translocation to the cell-surface by inhibition of Protein kinas A or Calmodulin. Inhibition of these proteins resulted in significantly less Aquaporin-4 translocation to the cell surface, decreased water permeability and less cerebral edema(6).

In conclusion, much is now known about Aquaporin-4 and its involvement in the brain's management of edema, but clear understanding of the regulation and distribution of Aquaporin-4 is crucial. The regulation of Aquaporin-4 is a complicated process and many questions remain. Few studies have been done on reactive astrogliosis and the effect on Aquaporin-4 distribution. This master student project focuses on elucidating the Aquaporin-4 intracellular distribution in experimental ischemic stroke as an attempt to contribute to this growing area of research.

2. Aim

The primary purpose of this study is to assess whether the Aquaporin-4 intracellular distribution is altered when reactive gliosis is attenuated in ischemic stroke - and to what degree. The aim is to quantify and compare the Aquaporin-4 intracellular distribution four

weeks after photothrombotic stroke using wild-type mice and mice with attenuated reactive gliosis ($GFAP^{-/-}Vim^{-/-}$).

3. Methods and materials

Using male mice at eight weeks carrying a null mutation in GFAP and Vimentin ($GFAP^{-/-}Vim^{-/-}$) genes and wild-type (WT) control mice with the same genetic background, our lab partners induced a photothrombotic stroke in the left somatosensory cortex and primary motor cortex using the Rose Bengal technique. The Rose Bengal technique uses a photoactive dye and photo-oxidation, resulting in endothelial damage, platelet activation and thrombosis. This is a well-known and minimally invasive method(49). Moreover, this method generates a lesion with clear boundaries, no penumbra and is efficient for cell characterizing or functional studies(8, 49). This model also shares inflammatory response to middle cerebral artery occlusion (MCAO)(50). Mice were then sacrificed by our lab partners at 4 weeks and the brains were preserved using paraformaldehyde and paraffin, coronal sections 8µm thick.

Aquaporin-4 distribution was then assessed on the available paraffin tissue sections by double-immunofluorescence immunohistochemistry as described below, using antibodies for Aquaporin-4 and Podocalyxin; a protein in vascular endothelial cells incorporated to visualise blood vessels.

3.1 Tissue selection

Equivalent 8µm thick paraffin tissue sections on slides from 16 mice was carefully selected, with eight sections on each slide. All slides contained coronal brain sections from mice four weeks after lab-induced photothrombotic stroke and withheld good visualization of the peri-infarct area. Lastly, three randomly chosen sections were set as negative controls lacking primary antibodies.

3.2 Immunohistochemistry

3.2.1 Deparaffinization and Antigen retrieval

First, slides were preheated in 60C for 30 minutes followed by deparaffinization by SAKURA robot. Second, antigen retrieval was done with heat in a pressure cooker. Slides were placed in rack containing 0.01 M citrate buffer and 2 mM Ethylenediaminetetraacetic acid - EDTA (pH 6.2 with 0.05% Tween in phosphate-buffered saline). Third, slides were cooled down and washed in Tris buffered saline (TBS) two times.

3.2.2 Blocking the unspecific binding

Excessive water was dried off and a hydrophobic pen was used to draw around the tissue sections, creating a hydrophobic border. Blocking/permeabilization was done with Blocking buffer: 2% Normal Donkey serum, 2% Bovine serum albumin (BSA), in TBS containing 0.5% of Tween 20, for 30 minutes in a closed box in room temperature with a dampen paper towel beneath.

3.2.3 Incubation with primary antibodies

Chosen primary antibodies were Goat anti Podocalyxin with a concentration of 1:500 and Rabbit anti Aquaporin-4 with a concentration of 1:500. Antibodies were diluted in Dilution buffer with 2% Donkey serum, 2% BSA, in TBS containing 0.05% of Tween 20. Slides were incubated with 300 µL of primary antibody solution in a fridge overnight, in a closed box with a dampen towel underneath.

3.2.4 Incubation with secondary antibodies

First, slides were washed in TBST (Tris buffered saline with Tween 20) three times. Second, slides were incubated with secondary antibodies diluted in Dilution buffer, 300 µL on each, for one hour in room temperature. Secondary antibodies were chosen to be Donkey anti goat IgG-conjugated Alexa 488 in a concentration of 1:300 and Donkey and Rabbit IgG-conjugated Alexa 555 in a concentration of 1:555. Cell nuclei were identified using 4′,6-diamidino-2-phenylindole (DAPI) in a concentration of 1:2000.

3.2.5 Mounting

Slides were washed in TBST three times and Sudan black solution was added to top on the sections for 10 minutes to decrease autofluorescence. After washing two times with TBST, a coverslip was manually placed using mounting medium containing 2.5% DABCO (1,4-diazabicyclo-[2,2,2]-octane).

3.3 Visualization

Tiled images of Aquaporin-4, Podocalyxin and DAPI covering a large part of the cerebral cortex was captured using laser scanning confocal microscopy (LSM 700, Carl Zeiss). Images were then used to quantify the immunoreactivity of Aquaporin-4 within the perivascular glial endfeet (polarized Aquaporin-4) relative to the immunoreactivity in the tissue outside vessel regions (unpolarized Aquaporin-4) using Image J software (www.imagej.net, NIH).

Genotypes was blinded throughout the image analysis. In total, 3 sections from each mouse was captured and quantified. Four regions were used in this study, all 400µm wide. Region A was placed at the peri-infarct area, 250µm from the lesion edge covering cortical layer II-V. Region B was marked ipsilateral to the lesion in the medial cortex covering cortical layer II-III. Region Bb and Aa was marked at the corresponding position in the opposite hemisphere; contralateral medial cortex and contralateral lateral cortex (Fig.2).

Regions Of Interest (ROIs)

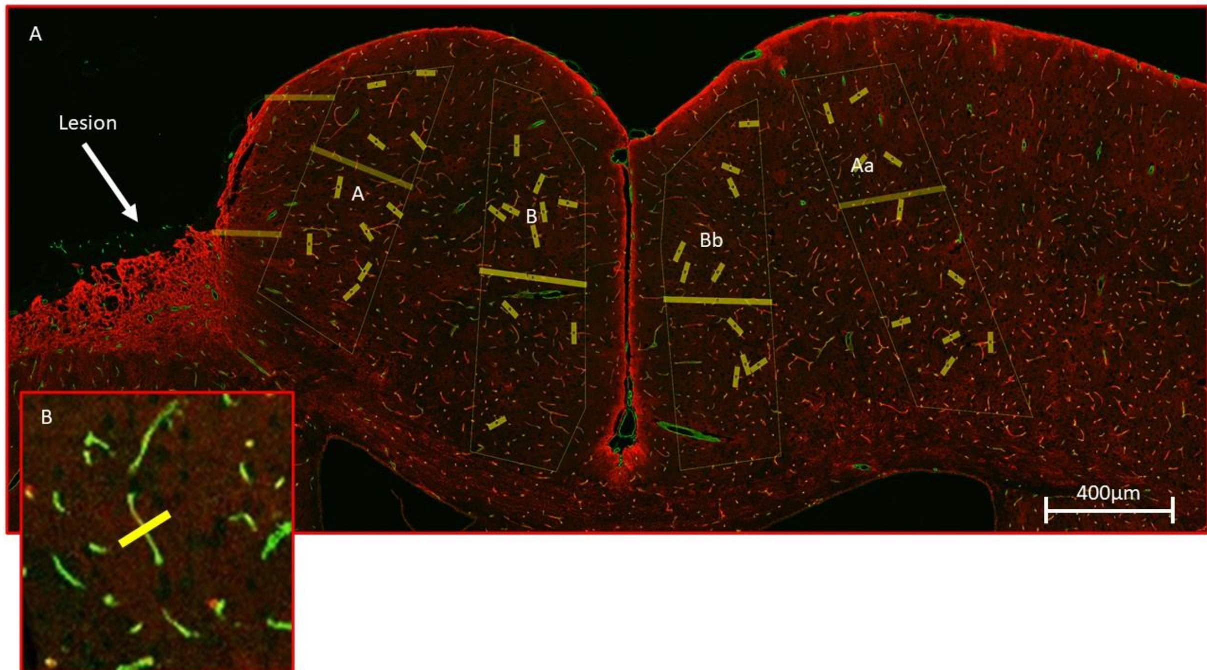

Figure 2. Example of regions of interest (ROIs) in 40x objective. In red: Aquaporin-4 and in green: Podocalyxin. ***A)*** *Region A represents the peri-infarct area, region B is the ipsilateral medial cortex, region Bb is the contralateral medial cortex and region Aa is the contralareral lateral cortex.* ***B)*** *A close-up image of the vessel and the line drawn to measure staining intensity.*

Using the Podocalyxin image to visualise vessels, a total of 10 vessels 2.5-7µm in diameter was selected for each region (Fig 2). Line width was 33µm and length 50µm and the vessel was placed as close to the middle of the line as possible. Each intensity-profile was plotted for both the Podocalyxin image and the Aquaporin-4 image and Excel was used to calculate means. Moreover, the colocalization threshold plugin in Image J was used for the same regions to observe the spatial overlap, whether Aquaporin-4 and Podocalyxin co-occur in location and to what degree of correlation.

4. Ethics

All experiments with animals were performed before the start of this project and the mouse tissue material used in the project was already available at the research lab as fixed paraffin tissue sections. Ethical permits are available at the lab, and all experiments were performed in compliance to animal care laws and guidelines.

5. Statistical methods

5.1 Data collection procedures

For each vessel, the Podocalyxin data was interpretated as well as the Aquaporin-4 data using Excel. The location of the vessel was marked and defined as all Podocalyxin data points higher than two times the median value (> 2*MEDIAN). All values > 2*MEDIAN was counted, generating a measure of the diameter of each vessel.

As demonstrated in Figure 3, the five highest Podocalyxin-values was set to represent the vessel for mean value calculations of both Podocalyxin and Aquaporin-4 vessel or endfeet intensity profile. The mean-intensity in the parenchymal tissue surrounding the vessel was calculated by using all values 23,99 µm from the maximum value to ensure a solid distance from the capillary.

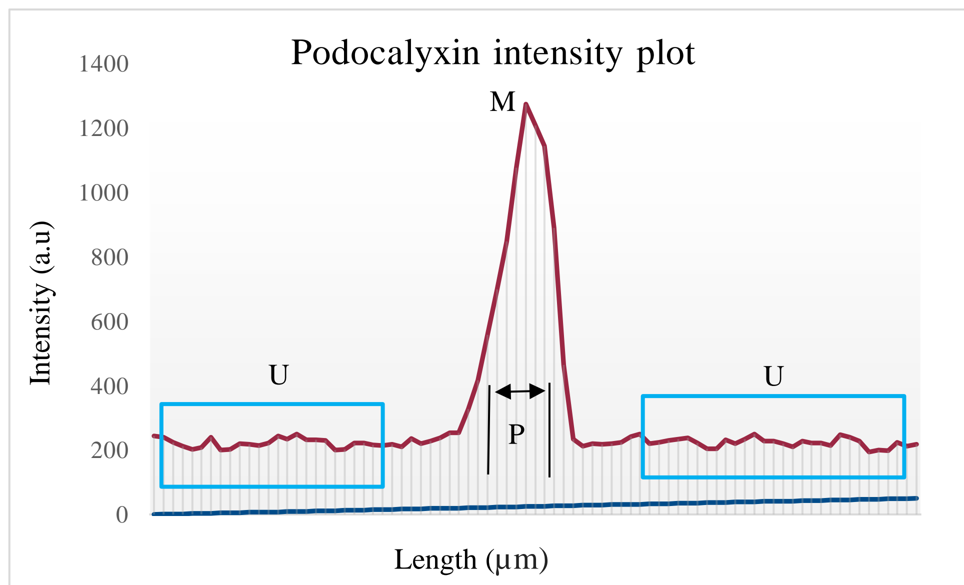

Figure 3. Example of a Podocalyxin intensity histogram. The maximum (M) value, values representing Podocalyxin at the capillaries - polarized (P) and values in the surrounding parenchyma not directly in contact with any capillary - unpolarized (U) exemplified.

Data from each section was then used to calculate mean values for the Podocalyxin and Aquaporin-4 images, at the capillaries and in the brain parenchyma not directly in contact with any capillary. The Mander's colocalization coefficient was also used to calculate a mean value for each section. The mean values from each section was then used to calculate mean values for each mouse. Data from all 16 mice was then statistically tested using Prism Graphpad (www.graphpad.com). Significance levels were set at $p<0.05$ and data normality testing was done using Shapiro Wilk test and Kolmogorov-Smirnov test.

5.2 Statistical testing of the Podocalyxin immunoreactivity in capillary wall

All intensity data for the Podocalyxin layer was normally distributed. However, one group (tissue region) was not normally distributed in Shapiro Wilk test but when using

Kolmogorov-Smirnov test the group was indeed normal distributed. For another group, data was normally distributed in Kolmogorov-Smirnov test but not in Shapiro Wilk, in conclusion, all data were tested normally distributed depending on choice of test but to avoid incorrect significance nonparametric testing was performed.

To calculate differences in Podocalyxin intensity, evaluate the immunohistochemistry and compare the capillary diameter we used paired Friedman test and Dunn's multiple comparison test to test differences between genotypes, and Mann Whitney-test to test differences between regions within genotypes.

5.3 Statistical testing of the Aquaporin-4 distribution

All data for polarized/end feet Aquaporin-4 was normally distributed. Data for unpolarized/parenchymal Aqpuaporin-4 and for the ratio between polarized and unpolarized Aquaporin-4 was not normally distributed.

To calculate differences in polarized Aquaporin-4 within genotypes we used one-way ANOVA with the Geisser-Greenhouse correction and Tukey's multiple comparisons test with individual variances computed for. To calculate differences in polarized Aquaporin-4 between genotypes we used unpaired t-test with Welch correction.

Statistical testing of the unpolarized Aquaporin-4 and the ratio unpolarized/polarized Aquaporin-4 we used Friedman test, Dunn's multiple comparison test and Mann-Whitney test.

5.4 Statistical testing of Mander's colocalization coefficient (MCC)

The mean values of Mander's colocalization coefficient were not normally distributed. Differences between genotypes was distinguished with Mann-Whitney and differences between regions within the genotype was tested using paired Friedman test and Dunn's multiple comparisons test.

6 Results

The immunohistochemistry (IHC) performed with markers for Aquaporin-4 and Podocalyxin to visualize blood-vessels showed some variations in staining intensity between sections and between mice. Figure 4 demonstrates an example of the IHC staining.

Immunohistochemistry staining

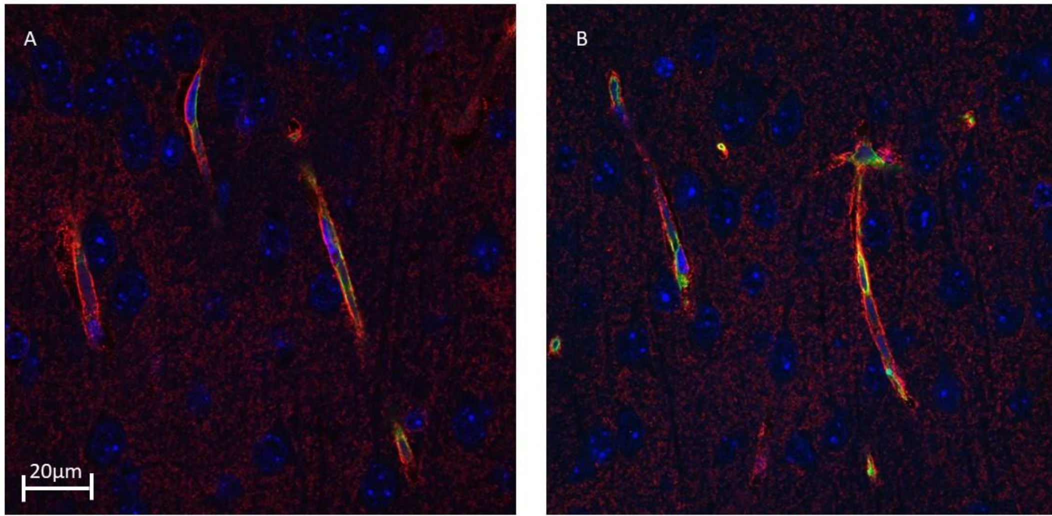

Figure 4. Demonstration of the immunohistochemistry (IHC) staining. In green: Podocalyxin, in red: Aquaporin-4 and in blue: DAPI (nuclei). ***A)*** *Example of a close-up image from a $GFAP^{-/-}Vim^{-/-}$ mouse.* ***B)*** *Example of a close up-image from wild-type (WT) mouse.*

6.1 Results for the Podocalyxin immunoreactivity in capillary wall

For the Podocalyxin immunoreactivity there was no significant difference in intensity in the capillary wall within genotypes or between genotypes. As a secondary finding, there was a statistically significant difference in diameter of capillaries between regions in both genotypes as shown in Figure 5. For Wild-type mice (WT, n=9) the largest difference was seen between the peri-infarct area and the contralateral medial cortex (*P=0.0001, adjusted for multiple comparisons*) but there was also a difference between the contralateral medial cortex and the contralateral lateral cortex (*P=0.0084, adjusted for multiple comparisons*). For $GFAP^{-/-}Vim^{-/-}$mice (n=7) there was a significant difference in capillary diameter between the peri-infarct area and the contralateral medial cortex(*P=0.0038, adjusted for multiple*

comparisons). However, there was no statistically significant difference in capillary diameter between the genotypes.

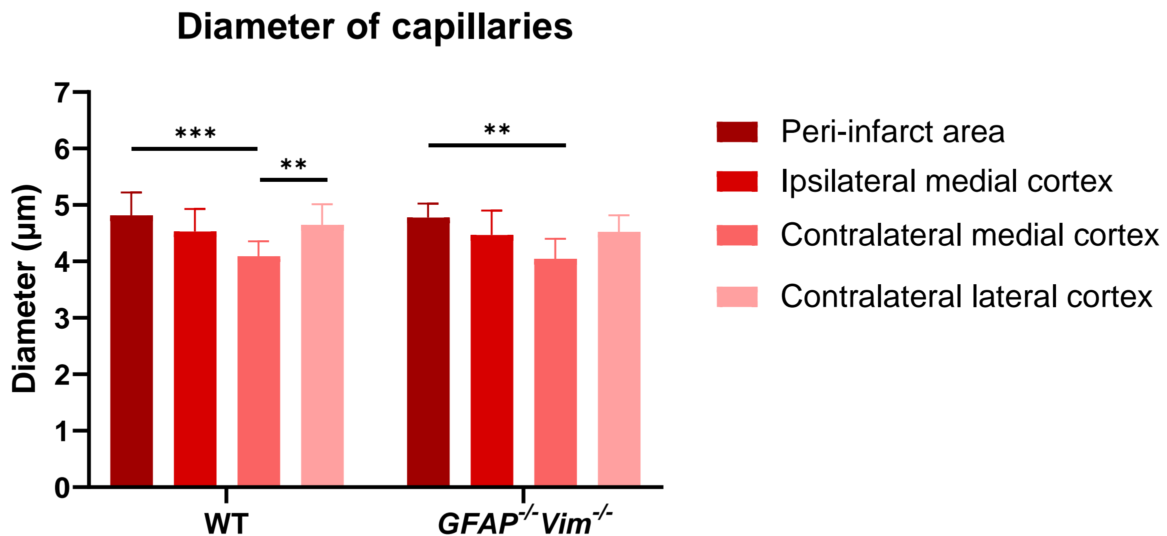

*Figure 5. Differences within genotypes in the diameter of capillaries (mean with SD). Mice with attenuated reactive gliosis ($GFAP^{-/-}Vim^{-/-}$, n=7) and wild-type mice (WT, n=9) show a significant difference in diameter between the peri-infarct area and the contralateral medial cortex. WT also show a significant difference between the contralateral medial cortex and the contralateral lateral cortex (*** $p<0.001$, ** $p< 0.01$).*

6.2 Results of the Aquaporin-4 distribution

6.2.1 Polarized Aquaporin-4

The intensity of Aquaporin-4 immunoreactivity in the astrocyte endfeet, referred to as Polarized Aquaporin-4 was compared between genotypes and between regions within genotypes. As presented in figure 6, there was no statistically significant difference between the genotypes which was also supported with the comparable result by the testing of Mander's colocalization coefficient.

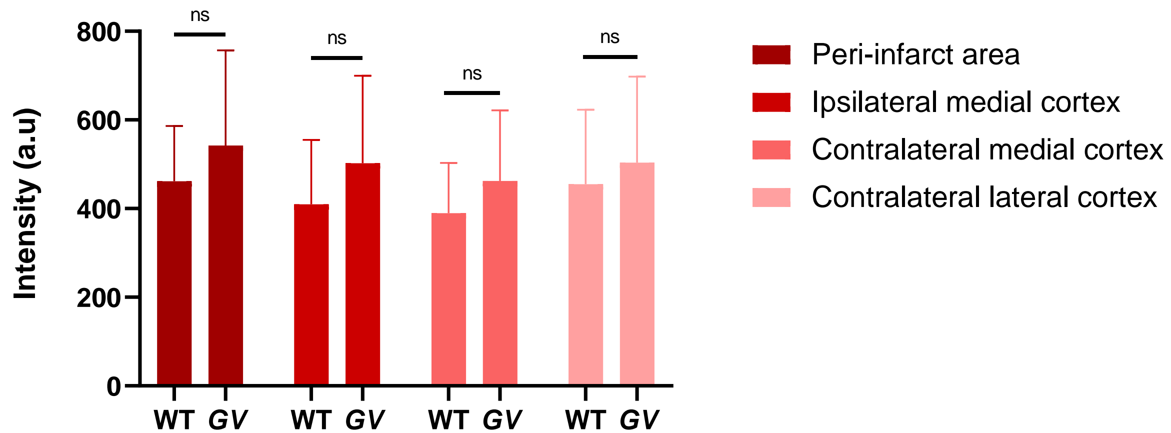

Figure 6. Differences in polarized Aquaporin-4 (mean with SD) between Wild-type mice (WT, n=9))and $GFAP^{-/-}Vim^{-/-}$*mice (GV, n=7). There was no significant difference (ns; not significant) between genotypes.*

As presented in Figure 7 there was a significant difference in the intensity of polarized Aquaporin-4 in the peri-infarct region compared to the ipsilateral medial cortex in WT mice (*P=0.0196, 95% CI of diff. 9.096-95.24. P-value adjusted for multiple comparisons*). There was also significantly more polarized Aquaporin-4 in the peri-infarct region compared to the contralateral medial cortex (*P=0.0233, 95% CI of diff. 10.49-133.0. P-value adjusted for multiple comparisons*). However, this difference was not seen in $GFAP^{-/-}Vim^{-/-}$mice and was not supported by the Mander's colocalization coefficient testing.

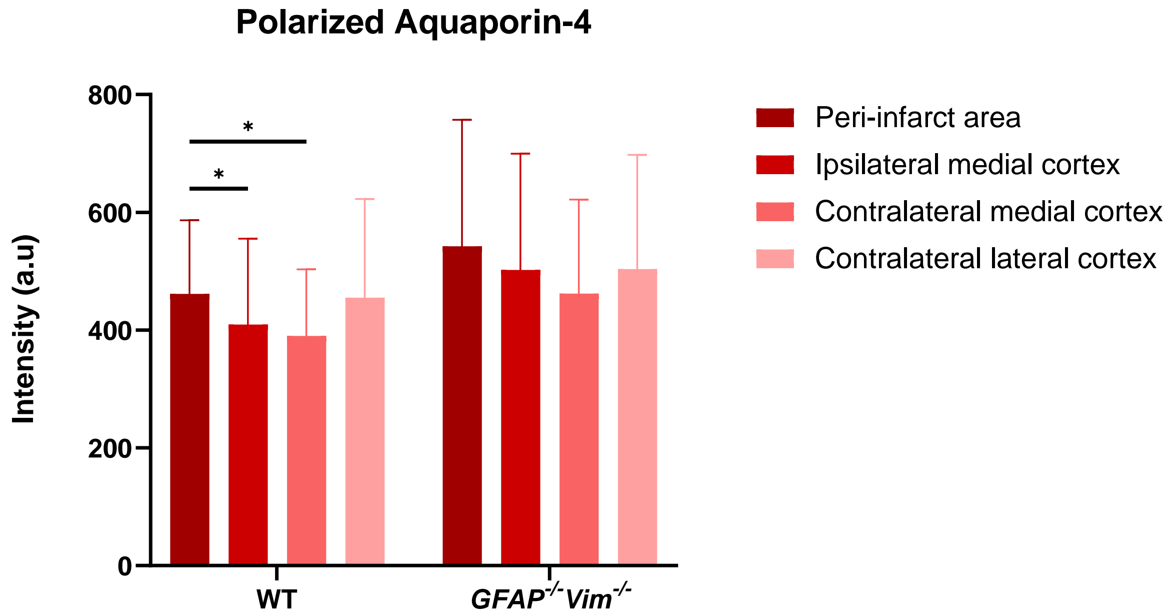

Figure 7. Polarized/endfeet Aquaporin-4 (mean with SD) and differences between regions within genotypes. In wild-type mice (WT, n=9) there are significantly more polarized Aquaporin-4 in the peri-infarct area than in the ipsilateral medial cortex and the contralateral medial cortex (p<0.05). In $GFAP^{-/-}Vim^{-/-}$ mice (n=7) there are no significant difference between regions.*

6.2.2 Unpolarized Aquaporin-4

No significant difference in unpolarized Aquaporin-4 was seen between genotypes. However, there was significantly more unpolarized Aquaporin-4 in the peri-infarct area compared to the contralateral medial cortex for both WT mice (*P=0.0002, adjusted for multiple comparisons*) and $GFAP^{-/-}Vim^{-/-}$ mice (*P=0.0005, adjusted for multiple comparisons*). As presented in Figure 8, there was also significantly higher unpolarized Aquaporin-4 in the peri-infarct area compared to the ipsilateral medial cortex in WT mice (*P=0.0370, adjusted for multiple comparisons*) and in $GFAP^{-/-}Vim^{-/-}$ mice (*P=0.0427, adjusted for multiple comparisons*). Moreover, in $GFAP^{-/-}Vim^{-/-}$ mice the contralateral cortex showed significantly more

unpolarized Aquaporin-4 than the contralateral medial cortex (*P=0.0427, adjusted for multiple comparisons*).

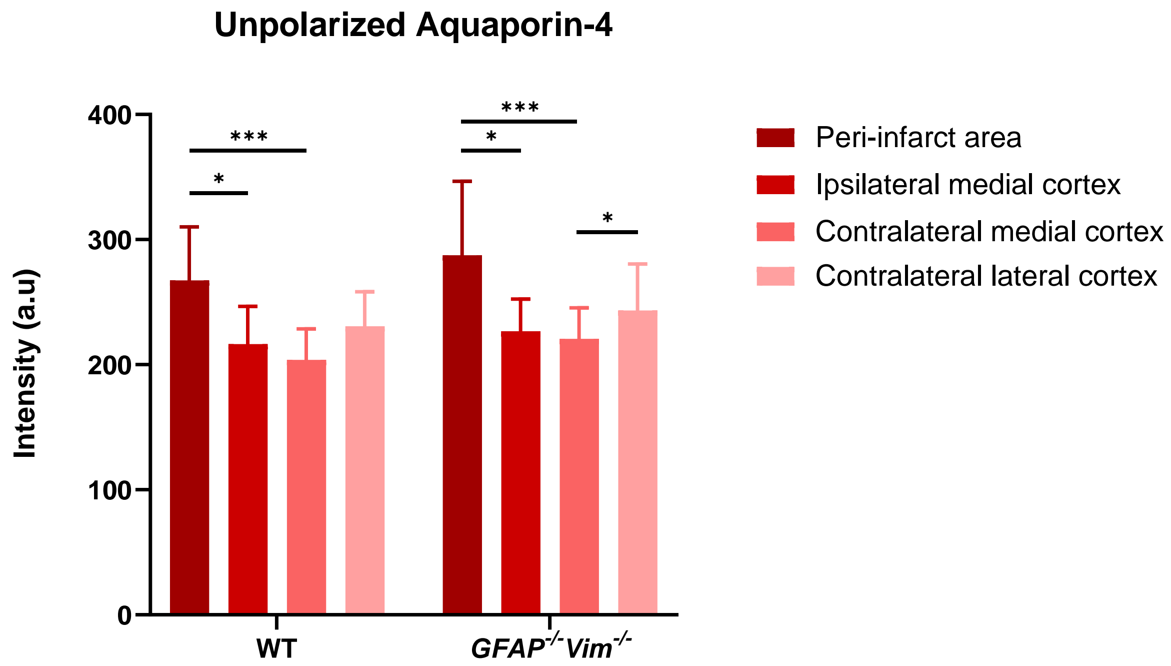

*Figure 8. Differences in unpolarized Aquaporin-4 between regions within genotypes (mean and SD). In wild-type mice (WT, n=9) and $GFAP^{-/-}Vim^{-/-}$ mice (n=7) there are significantly more unpolarized Aquaporin-4 in the peri-infarct area than in the ipsilateral and contralateral medial cortex. In $GFAP^{-/-}Vim^{-/-}$ mice there are significantly more unpolarized Aquaporin-4 in the contralateral lateral cortex than in the contralateral medial cortex (*** p<0.001, * p<0.05).*

6.2.3 Unpolarized Aquaporin-4 in relation to polarized Aquaporin-4

Comparison of the ratio Unpolarized/Polarized (U/P) Aquaporin-4 showed no significant difference between WT mice and $GFAP^{-/-}Vim^{-/-}$ mice. As presented in Figure 9 there is a significantly higher ratio (U/P) in the peri-infarct area compared to the contralateral lateral cortex in WT mice (*P=0.0115, adjusted for multiple comparisons*) and compared to the

ipsilateral medial cortex in $GFAP^{-/-}Vim^{-/-}$mice (*P=0.025, adjusted for multiple comparisons*).

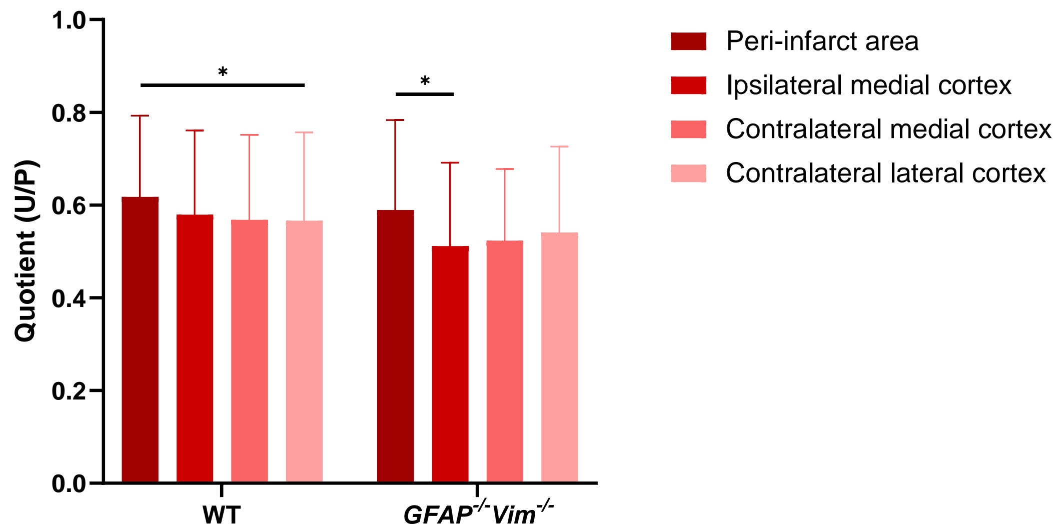

Figure 9. Ratio (U/P, mean with SD) of unpolarized Aquaporin-4 (U) divided with Polarized Aquaporin-4 (P). For wild-type mice (WT, n=9) the ratio is significantly higher in the peri-infarct area compared to the contralateral lateral cortex. For $GFAP^{-/-}Vim^{-/-}$mice (n=7) there was a significant difference of the ratio in the peri-infarct area compared to the ipsilateral medial cortex (p <0.05).*

7 Discussion

This experimental study examined the effect of attenuated reactive gliosis on the Aquaporin-4 intracellular distribution in ischemic stroke by using a photothrombotic stroke model and immunohistochemistry.

Contrary to expectations, this study did not find a significant difference in Aquaporin-4 distribution between wild-type mice and mice with attenuated reactive gliosis, suggesting that reactive astrogliosis might have a limited impact on the intracellular distribution of Aquaporin-4 four weeks after stroke. To our knowledge few studies has been done on reactive gliosis and the effect on Aquaporin-4 distribution, making the results not yet supported by other studies.

The results of our study showed differences in Aquaporin-4 distribution between the four regions in cortex in both genotypes. First, it seems to be more unpolarized Aquaporin-4 in areas close to the lesion in both wild-type mice and $GFAP^{-/-}Vim^{-/-}$mice. Second, the results also suggest more polarized Aquaporin-4 in the peri-infarct area compared to the ipsi- and contralateral medial cortex in wild-type mice. Third, when looking at the ratio of unpolarized and polarized aquaporin-4, the result suggests that the distribution of Aquaporin-4 is shifted away from the astrocyte endfeet to a higher extent in the region closer to the infarction.

Possible mechanisms behind the results of this study could be many considering the complex regulation of Aquaporin-4 and its many interactions. Nevertheless, regional differences in the ratio between unpolarized and polarized Aquaporin-4 could be explained by changes in the expressional ratio of M1/M23 isoforms. Data suggest changes in this ratio in response to ischemia, which could explain the result of less polarized Aquaporin-4 closer to the lesion. M1 limits the formation of OAP due to its greater size, M23 is smaller and a decrease in M23

results in less possibilities for Aquaporin-4 to polarize at the astrocyte endfeet(41, 51, 52). Moreover, in a study on hyperglycemia and the effect on Aquaporin-4 distribution the authors suggest regional differences including cortex and striatum, indicating that differences could exist between regions of the brain(43).

Moreover, we found no difference in Aquaporin-4 distribution between genotypes and this may be due to the variability of the samples and the small sample size, as well as only looking four weeks after stroke and only in grey matter cortical regions. Data suggest that astrocytes react differently to ischemia in white and grey matter and that the change in Aquaporin-4 polarization is bigger in white matter followed by more cerebral edema(41). In conclusion, it is possible that reactive gliosis has a larger impact on Aquaporin-4 distribution in white matter, striatum or at other time-points post stroke which would not be shown in this study.

Our study found more unpolarized Aquaporin-4 close to the lesion which goes in line with previous studies and the report that ischemia causes a shift in polarization away from the endfeet possibly due to changes in anchoring proteins(31, 53). However, these changes in polarization is suggested to normalize four weeks after diffuse stroke which of course could affect the results of our study even though the experimental stroke model is different(53).

In contrast to these studies we additionally found more polarized Aquaporin-4 close to the lesion. One possible explanation to the stronger immunoreactivity of both unpolarized as well as polarized Aquaporin-4 in the peri-infarct area compared to other regions could be the

general increase of aquaporin-4 expression in response to ischemia and that the expression might be different in different areas of the brain. It is possible that this increased expression is greater closer to the lesion, in the superficial brain tissue or at the lateral areas of the cortex? In a study using photothrombotic stroke in mice the authors suggested a decreased expression of Aquaporin-4 in the peri-infarct area(31), opposing this suggestion. Another experimental study indicate that the believed increased expression of Aquaporin-4 in fact is post-translational changes or redistribution since only a slim increase in mRNA was observed(54).

More questions remain unclear, where does Aquaporin-4 go? When the change in polarization takes Aquaporin-4 away from astrocyte endfeet, does it indeed go to other locations throughout the plasmalemma as some studies suggests(41, 55) or is this dependent on white or grey matter regions(41)?

Lastly, as a secondary finding our study also found that the diameter of capillaries is smaller in the contralateral medial part of cortex. Taking our study-design into consideration, the peri-infarct area and the contralateral lateral cortex cover the full range of cortical layers II-V whereas the ROIs covering the medial cortex mostly covers cortical layers II-III. One possible explanation for our findings is that the diameter of capillaries seem to differ between the cortical layers in humans(56). However, to our knowledge this has not been confirmed in mice. The ipsilateral medial cortex showed no differences to the lateral parts of cortex which could possibly be due to its proximity to the lesion and the hypercapnia-induced dilation of

pericytes(57), although four weeks after stroke the concentration of carbon dioxide would most likely be normalized again.

To properly determine the role of reactive gliosis on Aquaporin-4 intracellular distribution future studies should include larger sample size and possibly also include human tissue for the ability to make sufficient interpretations. Future studies should also assess the role of reactive gliosis on Aquaporin-4 distribution in other stages of disease, such as acute and sub-acute stroke. It would be interesting to further investigate the effect of reactive gliosis on Aquaporin-4 distribution in white matter compared to grey matter and in striatum. Lastly, for future research in this field it is useful to standardize the analysis method to meet methodological variability.

7.1 Methodological considerations

Inducing a photothrombotic stroke by using the Rose Bengal method differs from an ischemic event in humans, making it harder to interpret these results in a clinical point of view. This method triggers occlusion in several arteries in the area, but in general only one artery is occluded in human ischemic stroke. Also, the edema happens simultaneously to the photothrombotic lesion compared to after the occlusion in humans. Moreover, there is no penumbra with this method(49). However, there are also strengths of this stroke model; it creates a clear edge to the lesion, has high survival rates with long-term deficit which is useful when studying recovery and inflammation. In conclusion, each stroke model has their flaws and advantages, and for the purpose of this preclinical study to evaluate inflammation and

cellular and molecular patterns this method seem adequate(50, 58). However, it is important to remember that choice of experimental stroke-model could result in differences in neuroinflammation and should be taken into consideration when comparing results between studies.

Moreover, including human tissue in this study as well as mice would be needed to fully be able to interpret the results in humans since species differ in anatomy, physiology, gene-expression and Aquaporin-4 polarization(59, 60).

A weakness of this study is the statistical power. For this study no power analysis was performed since all mouse tissue was already available and all available mice was used. It is of course a possibility that the results are affected by the sample size, but from an ethical perspective use of animals should be as low as possible. Moreover, some studies suggest that mice recover faster than humans implicating that four weeks post-stroke might be too late to see the differences(60) and other studies may produce different results when looking at other time points post-stroke.

Furthermore, Immunohistochemistry always comes with tissue variability and artefacts. However, it is most likely to the same extent in both wild-type mice and $GFAP^{-/-}Vim^{-/-}$, making the comparison between genotypes more reliable. Background noise can in particular affect the colocalization analysis since it affects the threshold settings resulting in an underestimation of the actual spatial overlap(61), but again this would be the same for both

genotypes. Furthermore, both the image analyses and the immunohistochemistry were performed manually and there is always a risk of user bias. As seen in the result section there was a large variability in immunoreactivity between mice. Still, in this study all slides were stained at the same time by the same person reducing the risk of bias.

More strengths of this study are that the images were blinded before image analysis reducing the risk of bias and two different methods for data collection was performed (intensity plotting and colocalization). Lastly, all mice were handled in the same environment with the same conditions before sacrificing them.

7.2 Conclusions and implications

In conclusion, it is possible that reactive astrogliosis may have a limited impact on the intracellular distribution of Aquaporin-4 four weeks after ischemic stroke, at least in the cerebral cortex. Moreover, this study suggests regional differences in Aquaporin-4 distribution.

Findings reported here contribute to the current understanding of the molecular mechanisms driving cerebral edema formation and brain swelling. Evidence suggest Aquaporin-4 distribution to affect both cytotoxic and vasogenic edema formation(32, 33, 55) and understanding the underlaying mechanisms is crucial for the development of new effective treatments of cerebral edema caused not only by stroke but also other CNS-pathologies. Cerebral edema is a pressing clinical issue and when severe the condition could be critical,

increasing the risk of a poor outcome(62, 63). At the time, treatment for edema focuses mainly on reducing the intracranial pressure and managing already existing edema. Targeting the formation of edema would be massively helpful for many patients all over the world.

Although the small sample size in this study does not allow clear evidence for these results, the study contributes with additional knowledge to this extensive field of research. Further research with larger sample sizes, other time-points post stroke, with white matter and striatum included for comparisons to grey matter is needed to fully determine the role of reactive gliosis on Aquaporin-4 distribution.

8 Populärvetenskaplig sammanfattning

Hjärnans immunförsvar och dess inverkan på lokalisationen av vattenkanaler – en experimentell studie om stroke

Examensarbetet på Läkarprogrammet vid Göteborgs universitet, Maj 2021
Student: Matilda Rantanen
Handledare: Ulrika Wilhelmsson

Känner du någon som har drabbats av en stroke? Stroke är en sjukdom som drabbar hjärnans blodkärl. Det är en av världens vanligaste sjukdomar och den orsakar stort lidande i samhället. Den som drabbas av en stroke har en risk för bestående skador och riskerar i svåra fall att dö av sin stroke. Det finns två huvudtyper av stroke – hjärnblödning och stroke orsakad av en blodpropp.

När ett blodkärl täpps igen av en blodpropp får det området i hjärnan inget syre. Syrebrist leder till att området i hjärnan dör och dess funktioner faller bort. Beroende på vilket område som drabbas kan det leda till svårigheter att styra sina rörelser, språksvårigheter, sväljsvårigheter och förlamning av en kroppsdel med mera. Syrebrist leder också till en vätskeansamling och svullnad i hjärnan som kallas ödem. Ödem är en stor anledning till att stroke orsakar så stora skador och idag finns få effektiva behandlingar mot hjärnödem. För att kunna ta fram fler mediciner mot hjärnödem behöver man först förstå hur hjärnödem bildas och idag vet man inte riktigt hur det går till. Det finns vissa teorier om att delar av hjärnans immunförsvar, så kallade gliaceller, kan påverka bildandet av hjärnödem genom att styra hur mycket vattenkanaler som finns i hjärnan och var de sitter.

I vår studie undersökte vi en särskild vattenkanal i hjärnan som kallas Aquaporin-4 eftersom man tidigare har sett att den påverkar bildandet av hjärnödem vid en blodpropp i hjärnan. Vi använde oss av 16 möss, nio med normalt aktiverade gliaceller och sju möss med försvagade gliaceller. Mössen hade fyra veckor tidigare fått en blodpropp i hjärnan och vi analyserade sedan tvärsnitt av deras hjärnor med hjälp av ett mikroskop.

I studien såg vi ingen skillnad på vattenkanalens position mellan mössen som hade försvagade gliaceller och mössen med normalt fungerande gliaceller. Resultatet kan tolkas som att gliacellerna inte påverkar den här vattenkanalens position i hjärnan särskilt mycket, och därmed finns en möjlighet att gliacellerna inte heller har så stor inverkan på just den aspekten i bildandet av ödem. För att verkligen vara säker på detta behövs större studier och det skulle också behövas studier i ett mer akut skede av sjukdomen. Forskningen inom det här området pågår för fullt och vem vet, kanske får vi nya och effektiva behandlingar mot hjärnödem vid stroke inom en snar framtid!

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