The study of AQP4-mediated cell volume regulation in astrocytoma cells

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LIST OF ABBREVIATIONS

AM: acetoxymethyl ester

AQP: aquaporin

AVP: Vassopressin

BBB: blood-brain barrier

BSA: bovine serum albumin

cAMP: cyclic adenosine monophosphate

CSF: cerebrospinal fluid

DABCO: 1,4-diazabicyclo[2.2.2]octane

DAG: diacylglycerol

DMEM: Dulbecco's modified Eagle's medium

EDTA: ethylenediamine tetraacetic acid

EGTA: Ethylene Glycol Bis-2-Aminoethyl Ether-N,N',N",n'-Tetraacetic Acid

ER: endoplasmic reticulum

FCS: fetal calf serum

GA: glutaraldehyde

ICP: intracranial pressure

iGluR: ionotropic glutamate receptor

IMP: intramembrane particles

IP_3: inositol-1,4,5-triphosphate

NPA: asparagines-proline-alanine

mGluR: metabotropic glutamate receptor

mRNA: messenger ribo nucleic acid

NGS: normal goat serum

PBS: phosphate-buffered saline

PDZ: PSD95-Discs large-ZO1

PIPES: $C_2H_{18}N_2O_6S_2$

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

OAP: orthogonal arrays of particles

RT-PCR: real time-polymerase chain reaction

SEM: standard error of the mean

TBI: traumatic brain injury

TSSV: C-terminal sequence Serine-Serine-Valine

TX100: Triton X-100

PREFACE

I would like to thank many people for making this research project for my thesis possible.

First of all, I thank Prof. Dr. Theo Meert, my promoter and research leader of the Pain and Neurology group (Johnson & Johnson, Beerse) for giving me the opportunity to do my internship in his department.

I would especially like to thank Dr. Rony Nuydens, my co-promoter, for his guidance and good advice during my internship here.

A special thank to Dr. Raf De Jongh, my second co-promoter, for his critical view and suggestions for my thesis.

I would also like to thank Prof. Dr. Jean-Michel Rigo, my promoter at the university of Hasselt, for his critical view for my thesis.

I thank An, my student-colleague, for her help and cooperation in the study of AQP4.

I also thank Susanne, Anneleen, Kirsten, Eve and many other colleagues at Johnson & Johnson for the pleasant time here.

Finally, a special thank to my family and Anthony, my boyfriend, for their support during my internship.

ABSTRACT

The aquaporin family (AQP0-AQP12) are water channels that allow bi-directional water transport driven by osmotic and hydrostatic forces. In the brain, AQP4 is the predominant water channel, mainly expressed at endfeet of astrocytes facing the pia and blood vessels. The polarized expression of AQP4 and the high water permeability, suggest an important role of AQP4 in water homeostasis in the brain. AQP4 may play a crucial role in formation, maintenance and resolution of brain edema, a complication after brain injury. Following traumatic brain injury, various mediators are released which may enhance brain edema formation. These mediators include histamine and glutamate. An in vitro model for cell volume regulation by applying a hypotonic shock to human 1321-N1 astrocytoma cells to induce cell swelling, was developed to investigate the effects of histamine and glutamate. Histamine and glutamate both induced a dose-dependent inhibition of hypotonicity induced cell swelling. Immunocytochemistry experiments showed that both mediators caused internalization of AQP4 from the membrane to the cytoplasm. This reduction of AQP4 in the membrane reduces hypotonicity induced cell swelling. To unravel the molecular mechanism of histamine and glutamate, intracellular calcium was investigated. Histamine induced an initial increase in intracellular calcium followed by a decline of $[Ca^{2+}_i]$ or a prolonged elevated plateau. Glutamate induced a small transient rice in intracellular calcium. It is not yet known how calcium causes AQP4 internalization. But the results indicate that histamine and glutamate have an effect on AQP4 mediated water fluxes, which could have important consequences during the course of brain edema. A fast removal of the excess brain fluid is crucial. High levels of AQP4 may be beneficial for rapid clearance of brain edema. Further research on AQP4 and mediators released after traumatic brain injury can lead to an appropriate treatment.

ABSTRACT

De aquaporine familie (AQP0-AQP12) bestaat uit water kanaaltjes die instaan voor watertransport gedreven door osmotische en hydrostatische krachten. De AQP-4 expressie in de hersenen is hoog in de membranen van de eindvoetjes van de astrocyten die contact maken met microcapillairen en de pia. Deze gepolarizeerde expressie van AQP4 en de hoge water permeabiliteit dat het bezit, suggereren een belangrijke rol voor AQP4 in de hersen waterhomeostase. AQP4 kan een belangrijke rol spelen in de vorming, behoud en klaring van hersenoedeem. Hersenoedeem is een belangrijke complicatie die kan optreden na een trauma. Na een hersentrauma worden verschillende neuronale mediatoren, zoals histamine en glutamaat, vrijgezet in het extracellular vocht die hersenoedeem kunnen versterken. Om de effecten van histamine en glutamaat te onderzoeken, werd een in vitro model voor hersen oedeem ontwikkeld. Een hypotone shock werd toegediend aan 1321-N1 menselijke astrocytoma cellen om op deze manier celzwelling te induceren. Histamine en glutamaat induceren een dosis-afhankelijke inhibitie van de celzwelling. Immunocytochemie toonde aan dat deze mediatoren een internalisatie van AQP4 van het membraan naar het cytoplasma veroorzaken. Deze vermindering van AQP4 in het membraan kan verantwoordelijk zijn voor de daling in celzwelling. Om het moleculair mechanisme achter de effecten van histamine en glutamaat te achterhalen, werd er gefocusd op calcium als mogelijke mediator. Histamine induceerde een dosis-afhankelijke stijging in intracellulair calcium gevolgd door een daling of een plateau van de $[Ca^{2+}_i]$. Bij glutamaat werd slechts een kleine stijging gezien van het intracellulair calcium. Uit de bekomen resultaten kan worden besloten dat histamine en glutamaat een effect hebben op AQP4 gemedieeerde waterfluxen. Dit kan gevolgen hebben voor het verloop van hersenoedeem. Een snelle klaring van het overvloedige vocht is dan cruciaal. Een hoge expressie van AQP4 in de celmembraan zou dus voordelig zijn om zo tot een snelle klaring van hersenoedeem te komen. Verder onderzoek zou dan kunnen leiden naar een geschikte behandeling voor hersenoedeem.

CHAPTER 1: INTRODUCTION

1.1 Aquaporins

The movement of water across cell membranes is fundamental to life. Water constitutes roughly 70 % of the human body, so a good water homeostasis is required. Water permeates through hydrophobic, lipid layers, but this diffusion is not sufficient to sustain many physiological processes. Therefore, a family of membrane channel proteins, aquaporins, evolved for the rapid transport of water across biological membranes (1).

1.1.1 Family of aquaporins

The aquaporins (AQP) are a family of small, hydrophobic, integral membrane proteins, of which 13 members (AQP0-AQP12) have been identified so far in animals. In most cell types, the AQPs reside constitutively in the plasma membrane.

Aquaporins allow passive water transport driven by an osmotic gradient (aquaporins) and in some cases also small solutes, such as glycerol (aquaglyceroporins). AQP1, AQP2, AQP4, AQP5 and AQP8 are primarily water selective (2).

1.2 Aquaporin in the brain

Aquaporins are present in many organs including: brain, eye, ear, lungs, kidneys and digestive tract. (3) In the brain, AQP4 is strongly expressed. Other water channels present in the brains are AQP1 and AQP9, but in significant lower amounts compared to AQP4 (4).

1.2.1 AQP4 in the brain

In astrocytes, AQP4 is polarized in membranes facing capillaries, so-called astrocyte endfeet and facing neuronal synapses. AQP4 is also expressed in ependymal and pial surfaces in contact with CSF. The glial lamellae associated with osmosensoric areas also show high expression of AQP4 (4, 5). Brain endothelial cells have a low expression of AQP4. Neurons, microglia in resting state and oligodendrocytes do not express AQP4.

1.2.2 AQP1 and AQP9 in the brain

AQP1 is predominantly expressed in the epithelial cells of the choroid plexus. This suggests that AQP1 plays a role in CSF secretion (4). AQP9 is expressed in endothelial cells, neurons and at lower levels in astrocytes. AQP9 is also permeable for small solutes, such as lactate. The exact role of AQP9 in the brain remains unclear. It may be implicated in brain energy metabolism as a neutral solute channel and could facilitate the diffusion of lactate from the astrocyte to the neuron. These hypotheses about the function of AQP9 are still speculative (6).

1.3 Expression and structure of AQP4

The AQP4 gene is located on chromosome 18. The AQP4 protein is a small, hydrophobic, intrinsic membrane protein that forms a heterotetramer. Two isoforms that differ at their N termini of AQP4 are found: M1 (323 amino acids) and the shorter M23 (301 amino acids). These isoforms are the result of translation initiation at the first methionine (M1) or the second methionine (M23). Both isoforms are present in the brain, but M23 is more abundant (7,8).

1.3.1 AQP4 is a tetramer

Each AQP4 monomer has 6 membrane-spanning alpha-helical domains interconnected by 5 loops. Two NPA (asparagines-proline-alanine) signature motifs are located on the longest loops B and E as shown in figure 1.1. These loops fold into the lipid bilayer and form the water selective transmembrane pore. These units aggregate in the membrane to form a more stable tetramer (9).
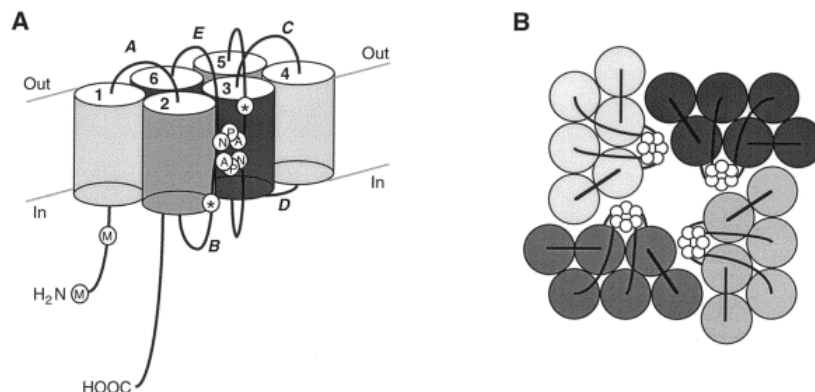

Fig 1.1: Molecular structure of AQP4 A) 3-D structure relating to AQP showing the bilayer-spanning domains, interconnecting loops and the highly conserved NPA motifs located on loops B and E. B) The arrangement of AQP4 monomers into stable tetramers in the membrane.

1.3.2 AQP4 is assembled in orthogonally arranged particles

Before the water channels were described, aggregates of intramembrane particles (IMP) were characterized during freeze-fracture electron microscopy. As these IMP aggregates were found in membranes of cells with increased cell permeability. Researchers proposed that AQPs formed an important protein of IMP (10).

Orthogonal arrays of particles (OAP) are regular square arrays of IMPs and are also visualized by electron microscopy of freeze-fractured membranes of several cell types. These cell types correspond to cells expressing AQP4. Transfection of oocytes with AQP4 resulted in formation of OAPs, which were not found in non-tranfected cells indicating that OAPs are formed by AQP4.

Large OAPs were found on cells transfected with M23mRNA, while cells transfected with M1mRNA did not show OAPs. Transfection with both isoforms of AQP4 resulted in abundant but small OAPs. The water permeability was the highest in cells transfected with M23mRNA, moderate high in cells transfected with both isoforms and lowest if transfected only with the M1 isoform. Thus, assembly into arrays may be necessary for the functionality the water channel (11).

1.4 Regulation of AQP4

The capacity of transmembrane passive water transport through AQP4 depends on the single channel water permeability and the number of channels present in the membrane. The short-term regulation of AQP4 water permeability is further described.

1.4.1 Regulation by PKC

AQP4 has a phosphorylation site for protein kinase C (PKC) at Ser-180. Activation of PKC induces phosphorylation of AQP4, which results in allosteric alterations of AQP4. This leads to a decreased water permeability of AQP4. Thus PKC has the ability to inhibit the activity of AQP4 (12).

1.4.2 Regulation by cAMP

Vassopressin (AVP) and other agents that stimulate cAMP production cause the activation of protein kinase A (PKA). This activation results in an increase in water permeability (13).

1.5 AQP4 in astrocytes

AQP4 is mainly expressed in the membrane domains near blood vessels and the pia. This polarized expression pattern indicates that the perivascular endfeet and glia limitans are important sites for water fluxes. Also the membrane domains in contact with synapses have a higher expression of AQP4. In vitro, AQP4 has no polarized expression. This suggests a possible role for endothelial cells and neuronal cells in the polarization of AQP4 to perivascular endfeet and synapses respectively (6, 14).
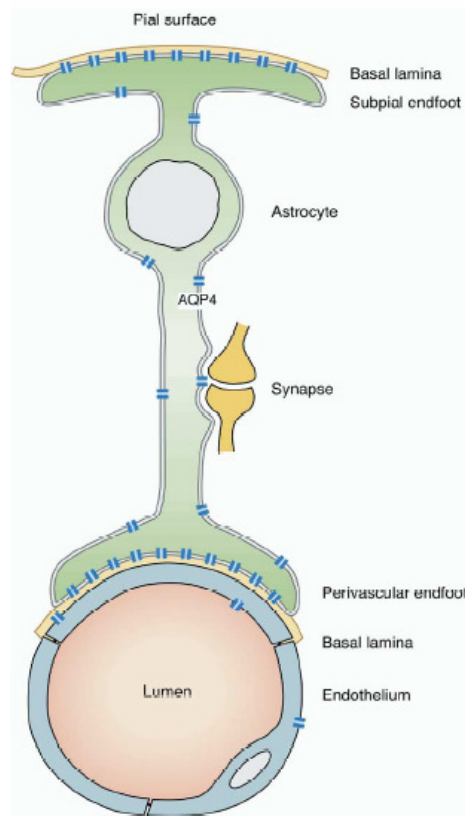
Fig 1.2: Expression of AQP4 in astrocyte-endfeet   AQP4 is mainly expressed in the membrane domains near blood vessels and the pia. This polarized expression pattern indicates that the perivascular endfeet and glia limitans are important sites for water fluxes.

AQP4 and the dystrophin protein complex

AQP4 is concentrated in the perivascular astrocyte endfeet. It seems to colocalize with the dystrophin protein complex. Dystrophin is part of a large membrane assembly connecting the cytoskeleton to the extracellular matrix. Dystrophin forms a connection between filamentous actin and the transmembrane protein β-dystroglycan. On the cytoplasmic side of the complex, dystrophin binds to dystrobrevin and both can bind two syntrophin molecules. Syntrophins are

a family of five proteins (α, β_1, β_2, γ_1, γ_2). They contain a PSD95-Discs large-ZO1 (PDZ) domain. This domain is responsible for recruiting membrane channels, receptors, kinases, … thus the dystrophin protein complex forms a platform, where several types of membrane or submembrane proteins can be localized. Localization of the dystrophin protein complex in perivascular astrocyte endfeet suggests association with AQP4. AQP4 has the C-terminal sequence Ser-Ser-Val (tSSV) that is capable of binding to PDZ domains (14, 15).

1.6 Function of AQP4

The expression pattern of AQP4 suggests that it plays an important role in water fluxes. But it also has some unexpected roles.

1.6.1 Water permeability

Water fluxes through aquaporins are bidirectional and are driven by osmotic and hydrostatic forces. Astrocytes show a remarkably polarized expression of AQP4 in the perivascular endfeet and glia. This implies that these membrane domains are specialized for rapid transmembrane water exchange.

Transmembrane water fluxes can be divided into constitutive and activity dependent fluxes. The constitutive fluxes are composed of water derived from the glucose metabolism and the net influx of water from the blood. Activity dependent fluxes occur during high neuronal activity when astrocytes are responsible for removing excess extracellular K^+ (16).

1.6.2 Coexpression

In the astrocyte endfeet membranes, AQP4 shows a precise colocalization with Kir4.1, an inward rectifying K^+ channel. This suggests that the two molecules could be part of the same supramolecular complex. Nagelhus et al. (2004) observed that shrinkage of the extracellular space at the site of neuronal activity was accompanied by an increase of the extracellular space volume. They suggested that these two transport processes are functionally coupled and that AQP4 and Kir4.1 are part of the same supramolecular complex (17).

1.7 Brain edema

Brain edema is the accumulation of water in the brain parenchyma. Because the adult skull is mechanically rigid, an increase in brain volume results in displacement of fluid from low-pressure cerebrospinal fluid (CSF), low-pressure venous and high-pressure arterial

compartments. Brain swelling increases the intracranial pressure (ICP), which impairs vascular perfusion and can lead to brain ischemia and death. Brain edema is seen in primary brain diseases such as stroke, head injury, brain tumor and brain abscess. But it is also seen in important systemic infections that involve the brain and in conditions that affect the brain indirectly, e.g. hypotonic failure (4, 18).

1.7.1 Water homeostasis in the brain

The volume of the intracranial cavity contains the brain parenchyma, CSF and blood and is about 1200 to 1400 ml in humans. The exchange of fluid between these compartments occurs at the blood-brain barrier (BBB), ventricular ependyma, choroid plexus and arachnoid granulations. Up to 30 ml is produced every day from the glucose metabolism. Water movement between cerebrospinal fluid (CSF) and blood is driven by osmotic gradients and hydrostatic pressure differences (4).

1.7.2 Vasogenic and cytotoxic brain edema

In the 1960s, Klatzo described 2 major types of brain edema: vasogenic and cytotoxic edema. The functional integrity of the BBB is important for the maintenance of a normal cerebral water homeostasis. Vasogenic edema occurs when the BBB becomes leaky, which permits the entry of plasma fluid into the brain parenchyma. BBB damage can be caused by mechanical injury, autodestructive mediators, … Vasogenic edema fluid is extracellular and accumulates primarily in the white matter (18, 19).

Cytotoxic edema is due to intracellular fluid accumulation, involving both astrocytes and neurons. This type of edema occurs independently of the BBB integrity. Three mechanisms account for the cell swelling: an increased Na^+ and K^+ permeability of the membrane; energy depletion followed by failure of the active ion-pumps or sustained uptake of osmotically active solutes and the intracellular water production by the glucose metabolism (4, 18).

1.7.3 Traumatic brain injury

Following traumatic brain injury (TBI), various mediators are released which enhance vasogenic and cytotoxic brain edema. These mediators include glutamate, histamine, lactate, H^+, K^+, Ca^{2+}, nitric oxide, arachidonic acid and its metabolites, free oxygen radicals and kinins. Cytotoxic edema is the predominant cause of brain swelling early after a TBI. Propagation of traumatic cytotoxic edema may arise due to direct mechanical injury inducing

mitochondrial impairment. Following a TBI, neuronal activation and cell lysis leads to an increased uptake of Na^+, which cannot be compensated by the active Na^+/K^+-ATPase. Sustained disturbance in electrogenic and electrochemical stability is influenced by glutamate. Activation of glutamate receptors and glutamate transporters is associated with an influx of Na^+, H^+, Ca^{2+} and Cl^- ions, which contributes to cytotoxic edema formation. This influx causes a shift of water from the extracellular to the intracellular space. Neuronal and glial efforts to maintain electrolyte equilibrium lead to energy depletion. This energy depletion results in failure of the ion pumps and breakdown of the membrane stability, which leads to uncontrolled cell swelling of neurons and astrocytes. AQP4 may play a crucial role in formation, maintenance or resolution of traumatic cytotoxic edema (18).

1.7.4 The role of AQP4 during brain edema

Vasogenic edema is probably cleared by bulk flow of fluid through the extracellular space and glia limitans into the ventricles and subarachnoid space and to a lesser extent through astrocyte foot processes and capillary endothelium into the blood. Experiments done on AQP4 null mice, demonstrated an impaired clearance of brain edema fluid after intraparenchymal fluid infusion, freeze injury and brain tumor implantation. These results suggest that AQP4 provides a low-resistance transcellular route, which allows edema fluid to move across the astrocyte cell membranes of the glia limitans into the CSF (21). There is experimental evidence for altered AQP4 expression in brain edema, providing indirect evidence for a role of AQP4 in the pathophysiology of brain edema. In humans, AQP4 is upregulated in astrocytes around edematous brain tumors, traumatic brain injury and brain ischemia (20). However, a down-regulation of AQP4 is seen in rodents after experimental TBI combined with hyponatremia (4). Increased expression/function of AQP4 should accelerate the elimination of edema fluid from the brain parenchyma, where a decreased expression/function would slow cytotoxic edema formation. Thus, augmentation in AQP4 expression or function may be beneficial in reducing brain swelling in vasogenic edema and in the resolution of cytotoxic edema (21).

1.7.5 The potential role of histamine during brain edema

Histamine is one of the mediators released in the brain after a TBI. The source of histamine remains speculative. Overactivation of the histaminergic system after a TBI is one possible explanation. The histaminergic system consists of a group of neurons that projects to all parts

of the CNS and are located in the tuberomamillary nucleus, a part of the posterior hypothalamus. These neurons are involved in many functions, such as alertness, sleep, metabolism, neuroendocrine and cardiovascular as well as learning and memory. Other possible sources of histamine are the mast cells. Histamine is released from these cells during inflammation. The turnover of histamine in mast cells is much slower than in neurons (22).

Four histamine receptors are known: H_1, H_2, H_3 and H_4. H_1, H_2 and H_3 are the most important receptors in the brain. G-proteins and various second messengers' pathways mediate the actions of histamine. The H_1 receptor is located on neurons and astrocytes and is coupled to G_{q11}. Binding of histamine leads to the activation of phospholipase C (PLC). PLC cleaves $PI(4,5)P_2$ in to diacylgycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). IP_3 binds to the IP_3 receptor of the endoplasmic reticulum (ER), which releases Ca^{2+}. H_2 receptors are coupled to G_s and activate adenylyl cyclase, which leads to the formation of cAMP. cAMP activates PKA. H_3 receptors play a role in the regulation of the synthesis and release of histamine (22).

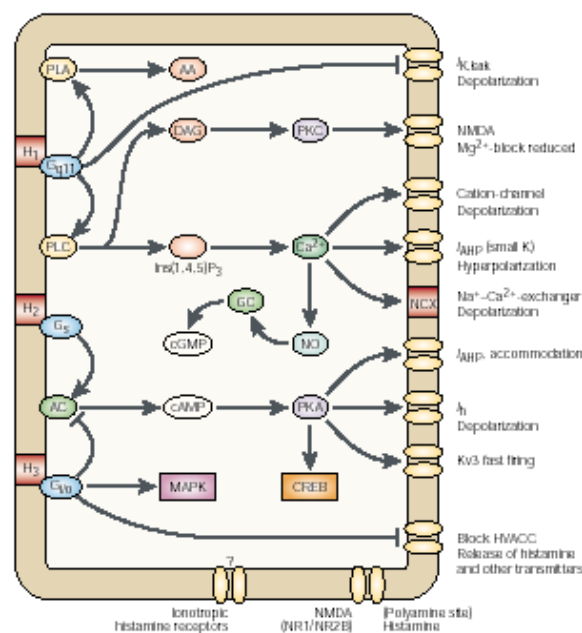**Fig. 1.3: Signaling pathways activated by histamine receptors** Histamine receptors and their coupling with G-proteins are shown at the left; the membrane targets are shown on the right. The box contains the signaling pathways.

Carmosino et al. (2001) showed that histamine reduces the number of OAPs in AQP4-expressing gastric cells. Therefore, histamine may have an effect on the membrane location of AQP4 (28).

1.7.6 The potential role of glutamate during brain edema

Glutamate, an excitatory neurotransmitter, is also released after a TBI. Most areas of the brain are innervated by glutamate-containing neurons. Ionotropic glutamate receptors (iGluR), ligand-gated ion channels, and metabotropic glutamate receptors (mGluR), coupled to second-messenger systems, are present on glial cells. iGluR has been demonstrated in all macroglial cells, while mGluR appear to be expressed only in astrocytes. Activation of iGluR, causes intracellular Ca^{2+} transients due to transmembrane influx of calcium through Ca^{2+}-permeable iGluR and voltage-activated Ca^{2+} channels. The mGluR is coupled by a G-protein and binding of glutamate activates PLC. PLC cleaves $PI(4,5)P_2$ into DAG and IP_3. IP_3 triggers calcium release from the ER (23, 24)

1.8 The goal of the study

The effect of histamine and glutamate on the expression and function of AQP4 in the brain and its influence on brain edema has not yet been determined.

In the study, a hypotonic shock is applied to 1321-N1 wild-type and AQP4 transfected human astrocytoma cells to induce cell swelling. First, the role of AQP4 during hypotonicity induced water fluxes will be determined. In the second part of the study, the effect of histamine and glutamate on the expression and cellular distribution of AQP4 and the effect of hypotonicity induced cell swelling will be examined. If these mediators would have an effect on the function of AQP4, the molecular mechanism underlying these effects would deserve further investigation.

2.1 Cell culture of 1321-N1 cells

Wild-type 1321-N1 human astrocytoma cells and cells transfected with M1 and/or M23 were kept in culture. These cells were grown at 37°C and 5 % CO_2 in 75 cm^2 plastic culture flasks with Dulbecco's modified Eagle's medium (DMEM; Gibco, UK) enriched with 10 % fetal calf serum (FCS; Hyclone, USA), 2 % L-Glutamine 200 mM (Gibko, UK), 2 % sodium bicarbonate (Gibco, UK) and dependent on the cell type 100 µg/ml Hygromycine (Invitrogen, USA) or 400 µg/ml Geneticin (Gibco, UK). Medium containing Hygromycine or Geneticin was used only for the AQP4 transfected clones, M23 and M1 respectively, which have a resistance marker. This guarantees that only the transfected cells survived. The cells were split two times a week. Cells were first trypsinizised with trypsine (0.05 %)-EDTA (0.53 mM) (Gibco, UK); 7 ml trypsine was added to the cells during 30 seconds. After removing the trypsine, the culture flasks were incubated at room temperature for 2 minutes. The cells were then suspended in the appropriate growth medium and divided into two new culture flasks.

2.2 Measuring cell volume

A live cell imaging system was used to detect changes in cell volume after applying a hypotonic shock to the cells.

2.2.1 Calcein

Calcein AM (Invitrogen, Molecular Probes, USA) is a dye that enters living cells and makes these cells fluorescent. Calcein is a negatively charged, green fluorescent molecule that is not able to enter the cells. Therefore calcein is coupled to acetoxymethyl esters (AM), which marks the negative charge. The resulting uncharged molecule can permeate the cell membranes. Once inside the cell, calcein AM is hydrolysed by esterases, resulting in a charged, fluorescent dye that cannot leak outside of the cell. This requires the action of esterases, which are active in living cells only. Consequently, calcein AM is retained in the cytoplasm but is rapidly lost under conditions that cause cell lysis. Because of its properties, calcein AM can be used to monitor changes in cell volume in living cells. Calcein has an excitation wavelength of 494 nm and emits at 517 nm.

2.2.2 Preparation of the cells

The cells were trypsinized and a cell suspension of 100000 cells per ml Dulbecco's Phosphate-Buffered Saline (D-PBS, GIBCO, UK) enriched with 1 g/l glucose was prepared. The cells were loaded with 1 µM calcein AM (1 µl/ml cell suspension) and 1 ml of the suspension is added to each well of a 12-well plate coated with poly-L-lysine (10 µg/ml). After 15 minutes, the cells have adhered and the wells could be washed with PBS.

2.2.3 Cell swelling experiments

Experiments of 9 minutes were performed. The cells are suspended in iso-osmotic PBS, which was refreshed after 3 minutes. After an additional 3 minutes, a hypotonic shock was applied. The iso-osmotic PBS (138 mM Na^+) was 50 % diluted with distilled water. This resulted in hypotonic buffers containing 69 mM Na^+. An example of the time course of this experiment is shown in fig 2.1.

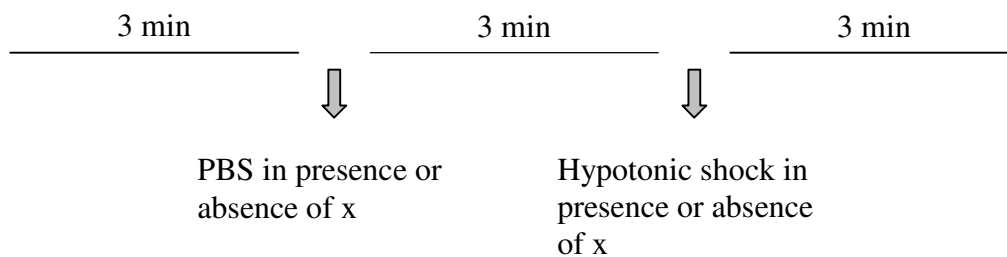| 3 min	3 min	3 min
⇩	⇩	
PBS in presence or absence of x	Hypotonic shock in presence or absence of x	

Fig 2.1: General protocol for a cell swelling experiment of 9 minutes. X represents for example 0.1-10 mM histamine (Fluka Biochemika, Switzerland) or glutamate (Sigma, USA). Each arrow indicates aspiration of the buffer followed by an addition.

2.2.4 Live cell imaging

The image recording system consists of an Axiovert S135 inverted microscope (Zeiss, Germany) equipped with a motorized (x,y-stage (Märzhauser, Germany), focus-drive and CCD-camera (Photonics, USA). Proprietary software allows automated focusing, image acquisition, and storage for each well (25). A 12-well plate was put on the stage and of each well, two fields containing cells were selected and followed over time. One field corresponds with a grid of nine squares containing about 100 cells. When the desired fields are selected, images were recorded automatically every 30 seconds during a preset time (9 minutes). A 40x objective lens is used.

Image analysis was performed using Morfo software (26). First, the cells are segmented from the background. For each cell, its area, volume and total intensity are computed and a gallery image is created. This gallery contains a picture of the cell extracted on each time point. The volume is estimated from the segmented area by assuming that this area is a circular cross-section from the cell from which the mean diameter can be computed. The volumes over time are put in a text file that can be exported to a spreadsheet where curves of each cell volume over time can be made. In these curves, the relative cell volumes are presented. The mean cell volume of the first 3 time points is calculated and the cell volume at each time point is divided by this mean value to obtain the relative cell volume.

2.3 Immunocytochemistry

To visualize AQP4 in the human astrocytoma cells, immunofluorescence was used. The cells plated on cover glasses were placed in petri dishes at a density of 100 000 cells per ml. The cells were placed in the incubator at 37 °C overnight.

Each step corresponds with quantities of 1 ml. After removing the medium, the cells were treated for 15 minutes with PBS in absence or presence of histamine or glutamate. After the treatment, 0.5 % glutaraldehyde (GA, Fluka Biochemika, Switzerland), and 0.5 % Triton-X100 (TX100; Sigma, USA) in PHEM buffer (2 l PHEM contains 1.01 M PIPES, 0.76 M EGTA, 0.19 M $MgCl_2$, 1 l Double Hanks buffer containing 2.0 M glucose, 0.8 M KCl, 15 M NaCl, 0.12 M KH_2PO_4, 0.7 M $NaHCO_3$) was added for 10 minutes. GA is used to fix the cells and TX100 permeabilizes the cells. Next, the cells were washed with PHEM. Then 0.5 % TX100 in PHEM was applied for 30 minutes, where after the cells were washed with PHEM. 1 mg/ml $NaBH_4$ (Sigma, USA) in PHEM was added during 10 minutes and was followed by a washing with PHEM. $NaBH_4$ reduces free aldehyde groups. 5 % normal goat serum (NGS, Sigma, USA) in PBS with 0.1 % bovine serum albumin (BSA, Sigma, USA) was applied for 30 minutes. NGS eliminates aspecific binding of the antibody. Then 1 % NGS and 1/200 primary antibody in PBS with 0.1 % BSA was added. A polyclonal rabbit AQP4 antibody (Santa Cruz Biotechnology, USA) was used as primary antibody. This antibody recognizes M1 and M23 clones as epitope. After an overnight incubation at room temperature, the cells were washed 4 times with PBS with 0.1 % BSA during 10 minutes. There after the secondary antibody was applied during 1 hour. 1/200 goat anti-rabbit IgG conjugated to Alexa 555 (Invitrogen, Molecular probes, USA) (excitation/emission maxima ±555/565 nm) was used as secondary antibody.

After labeling the cells, the cover glasses were monted on microscope slides with 100 mg 1,4-diazabicyclo[2.2.2]octane (DABCO; Acro Organics, Belgium) in 1 ml gelvatol. After a short centrifugation, a droplet of the mixture was applied to the glass and the cover glass was placed with the cells facing the slide. Gelvatol is a medium that allows samples to be kept for a long period of time and DABCO eliminates bleaching of the fluorescent signal.

Finally the cells were analyzed with the confocal microscope (Zeis LSM 510) to determine the distribution of AQP4 in the cell. An Argon laser with an excitation wavelength of 514 nm was used.

2.4 Determination of changes in intracellular Calcium concentrations

Fluo-4, a fluorescent calcium indicator was used to examine the effect of histamine or glutamate on the intracellular Ca^{2+} concentration. Fluo-4 (Invitrogen, Molecular Probes, USA) is a cell-permeant acetoxymethyl ester (AM). Fluo-4 emits at 516 nm. The more calcium that is present in the cell, the more calcium can bind fluo-4 and the higher the fluorescence intensity.

The 1321-N1 astrocytoma cells were plated in a 96-well plate at a density of 10000 cells/100 µl complete medium and were incubated overnight at 37 °C. After the incubation, the cells were loaded with 1 µg/ml fluo-4. After 30 minutes incubation, the cells were washed with PBS ($+Ca^{2+}$ and Mg^{2+}, + 1g/l glucose).

With the use of a microplate reader (Fluoroskan Ascent FL, Thermo), fluorescence intensities are measured during 4 minutes. The fluorescence was measured every 5 seconds. Fluorescence intensities at each timepoint are put into a text file and exported to a spreadsheet where the relative fluorescence intensities over time are calculated for each well. The mean fluorescence intensities of the first 3 time points is calculated and the fluorescence intensity at each time point is then divided by this mean value to obtain relative fluorescence intensities.

2.5 Statistics

The results are shown as the mean +/- standard error of the mean (SEM) from n experiments. To determine whether the mean values are significantly different, unpaired Student's t-test with equal variances was performed.

3.1 AQP4 transfection in 1321-N1 human astrocytoma cells

1321-N1 human astrocytoma cells have a low expression of AQP4. To obtain a higher expression of AQP4, the cells were transfected with human cDNA AQP4 (M1 and/or M23 isoform).

3.1.1 Expression and distribution of AQP4 in the transfected 1321-N1 cells

With the use of confocal microscopy, the distribution of AQP4 in the transfected cells was determined (Fig.3.1). In the non-transfected human astrocytoma cells (control), the overall expression of AQP4 is low and is situated in the cytoplasm and on the membrane. M1 clone 10 and M23 clone 8 show a higher expression of AQP4. In the M1 cl10 cells, the protein is mainly situated at the cell membrane, while in the M23 cl8 cells, AQP4 is mostly found in vesicles in the cytoplasm. The double transfected (M1 clone 10 and M23 clone 6) cells have the highest membrane staining of AQP4.
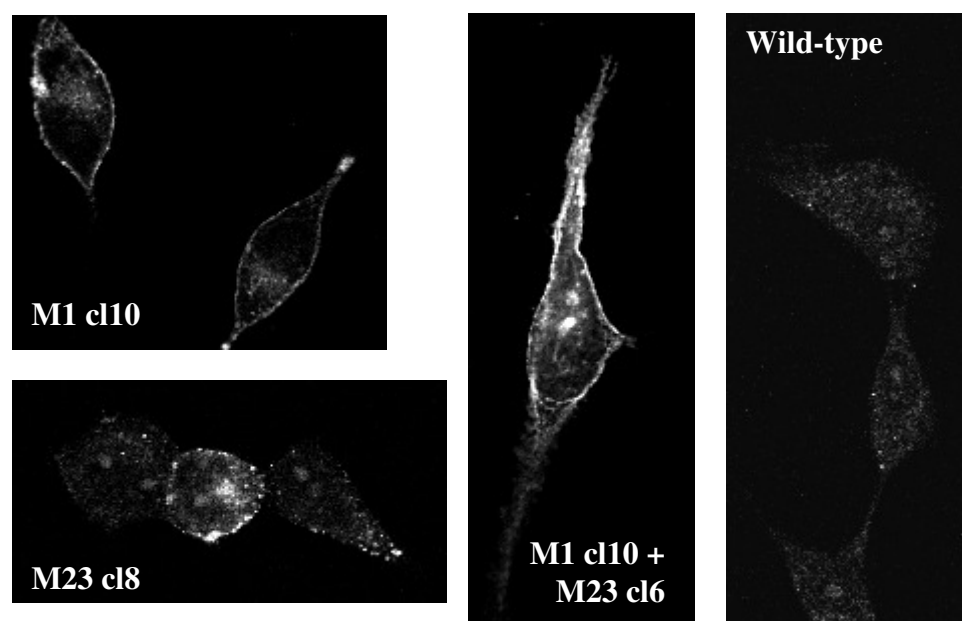

Fig. 3.1: Distribution of AQP4 in human astrocytoma 1321-N1 cells Wild-type cells and transfected clones were labeled for AQP4. After fixation and permeabilization the cells were labeled for AQP4. Rabbit polyclonal anti-AQP4 and goat anti-rabbit conjugated to Alexa 555 were used as primary and secondary antibody respectively. Digital images were obtained using the confocal microscope.

3.2 The influence of AQP4 expression and distribution on cell swelling during a hypotonic shock

To investigate whether the hypotonicity induced cell swelling is correlated with the expression level and distribution of AQP4, cell swelling experiments were performed (Fig. 3.2). The non-transfected as well as the transfected cells were investigated. After 6 minutes of measuring in iso-osmotic PBS, a hypotonic shock of 69 mM Na^+ was applied to the cells during an additional 3 minutes.

No cell volume changes were observed in the different cells during iso-osomotic conditions (0-6 min). After applying a hypotonic shock, the increase in cell volume varies between the clones. There is a difference in the cell volume change and also the time course of swelling is different. The cells with a higher expression of membrane associated AQP4 show a faster and higher degree of swelling after hypotonic shock compared to the wild-type cells. Cells transfected with M1 cl10 and the double transfected cells show a significantly higher degree of cell swelling after a hypotonic challenge and the increase in cell volume occurred faster in these cells compared with the wild-type cells and those transfected with M23 cl8. The 1321-N1 M23 cl8 cells show a slight increase in hypotonicity induced cell swelling, but this increase is not significant compared with the wild-type cells.
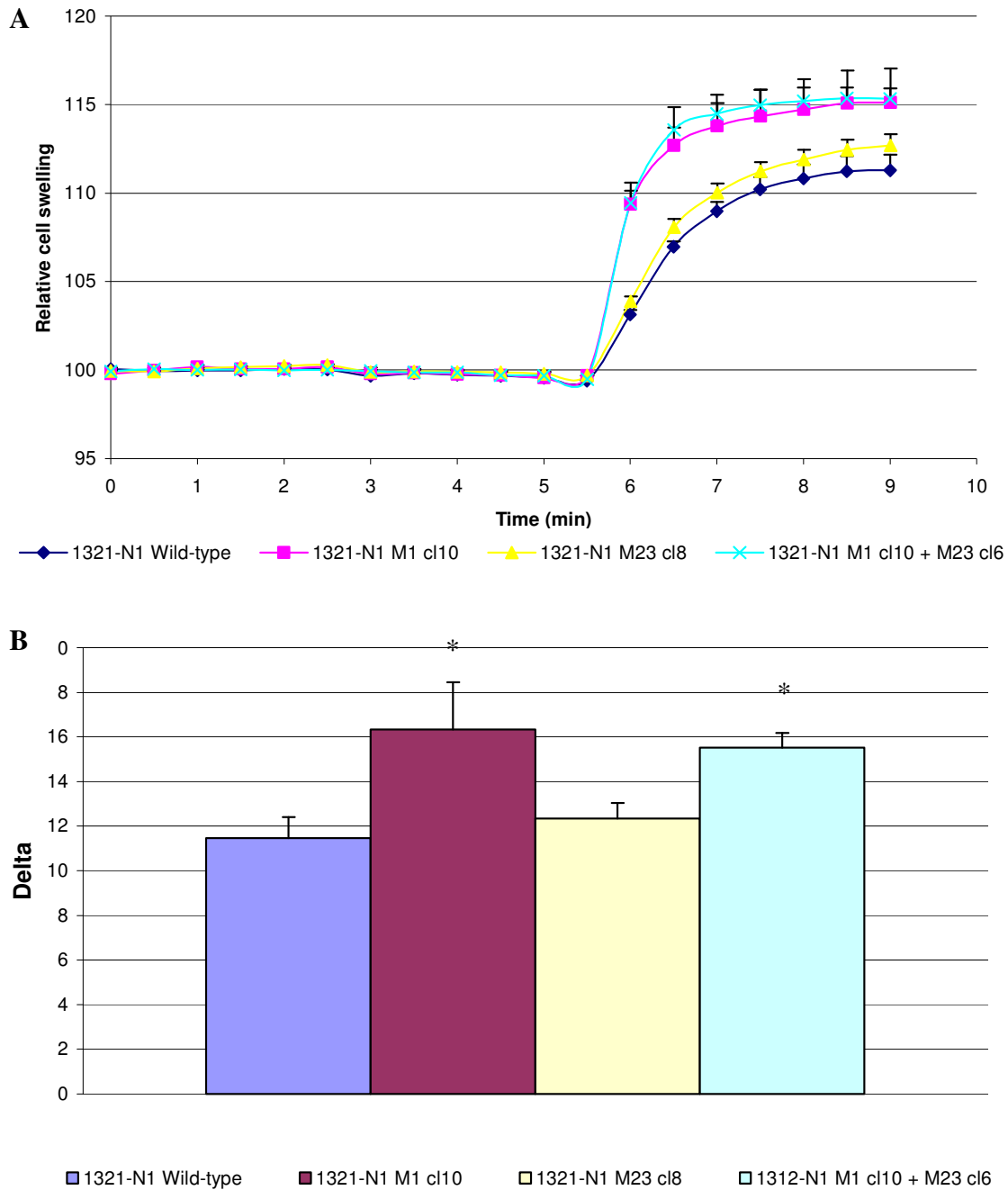
Fig. 3.2: Hypotonicity induced increase in cell volume varies between wild-type and transfected 1321-N1 cells 1321-N1 cells were seeded in 12 well plates at a density of 100000 cells/ml PBS with 1 ml per well. A) Relative cell volumes over time during hypotonic shock. After 6 minutes in iso-osmotic PBS, a hypotonic shock of 69 mM Na^+ was applied. B) Average increase in cell diameter (Δ) (6-9 min). Shown is the mean +/- SEM of 6 experiments. P-values were obtained via Student's t-test (* $p < 0.05$ versus Wild-type).

3.3 Effect of histamine on expression and function of AQP4

Histamine may alter AQP4 mediated water fluxes. To investigate this hypothesis, the effect of histamine on AQP4 distribution and function was examined.

3.3.1 Histamine stimulates internalization of AQP4 from the cell membrane

To examine the effect of histamine on the distribution of AQP4, 1321-N1 wild-type and transfected cells were treated with histamine (0.1, 1 and 10 mM). After 15 minutes of treatment, the cells were fixed and permeabilized. The cells were labeled for AQP4 using the same protocol as described in the legend of figure 3.1.

In control conditions, a clear membrane staining is visible in all the transfected cells, as shown in fig. 3.3A. When the cells were treated with 10 mM histamine, this resulted in a translocation of AQP4 from the membrane to the cytoplasm. This translocation is more abundant in the cells transfected with M1 cl10 and the double transfected cells compared with M23 cl8 cells. To quantify this translocation induced by histamine, the number of cells with a clear membrane staining present in at least 100 cells were counted in the four different conditions (control, 0.1, 1 and 10 mM histamine). Figure 3.3B shows a dose-dependent decrease in the percentage of cells with a clear membrane AQP4 staining. This is more pronounced in the 1321-N1 M1 cl10 cells.
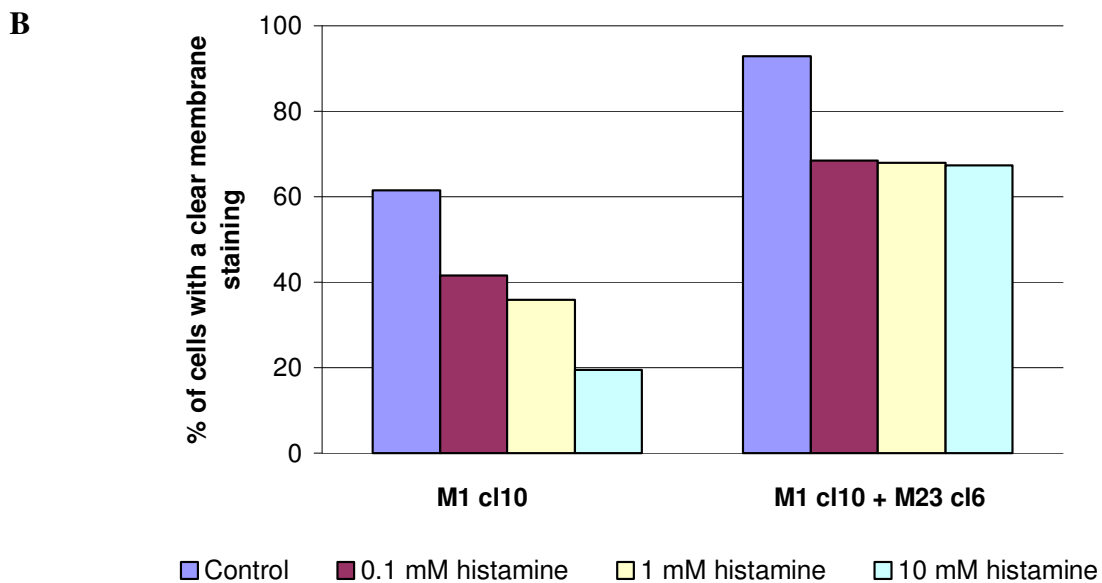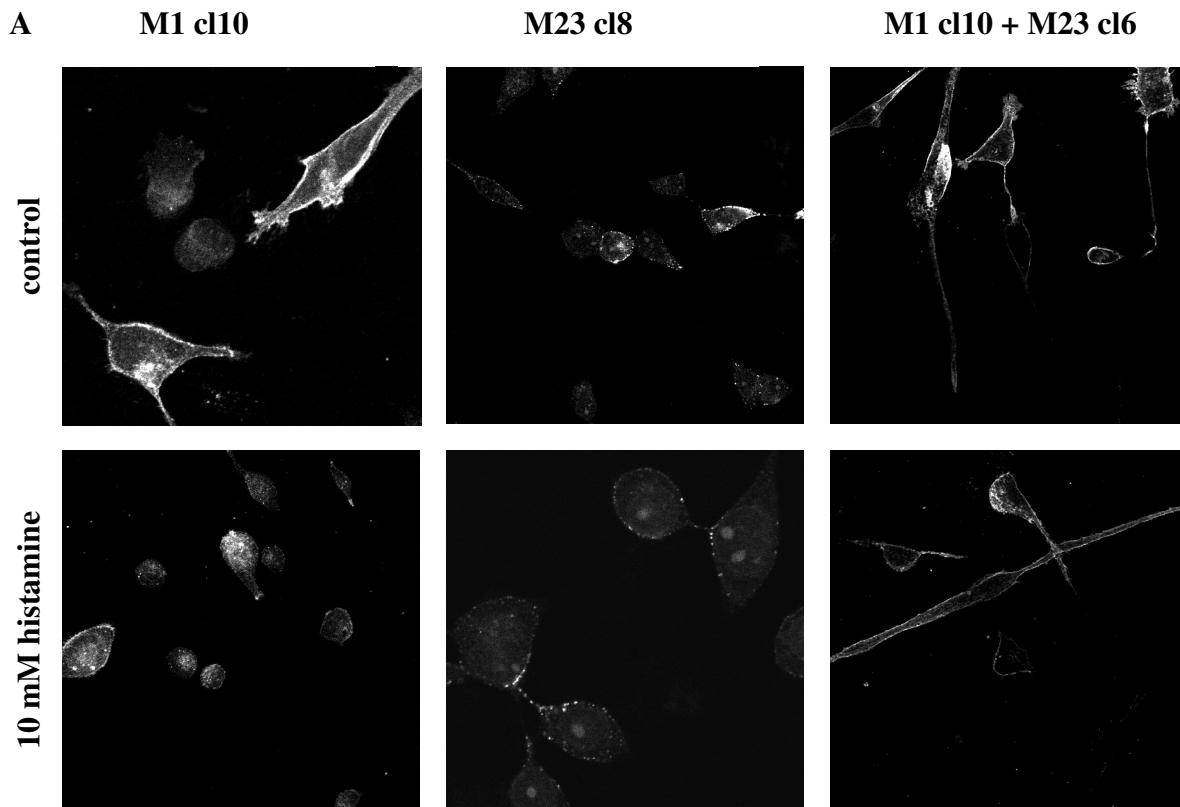
Fig. 3.3: The effect of histamine on the distribution of AQP4 Transfected 1321-N1 cells were treated with histamine during 15 minutes. As a control, the cells were treated with PBS during 15 minutes. The cells were labeled for AQP4 as described in fig. 3.1. A) Digital images were made using the confocal microscope. The cells were treated with 10 mM histamine. B) Percentage of cells with a clear membrane staining. 1321-N1 M1cl10 and double transfected cells were treated with histamine (0.1, 1 and 10 mM histamine) and with PBS as a control. The percentage of cells with a clear membrane staining was determined by counting the number of cells with a clear membrane staining in at least 100 cells per condition.

3.3.2 Histamine induced a dose-dependent inhibition of cell swelling

To investigate the effect of histamine on the cell swelling during a hypotonic challenge, different concentrations histamine (0.1, 1, 10 mM histamine) were tested in wild-type and transfected 1321-N1 cells.

After 3 minutes, histamine in iso-osmotic PBS was added for an additional 3 minutes. Next a hypotonic shock of 69 mM Na^+ was applied in the presence of histamine. As a control, the experiment was performed in the absence of this mediator.

A dose-dependent inhibition of cell swelling during hypotonic challenge was observed in the wild-type and transfected 1321-N1 cells (see Fig. 3.4). If the effect of histamine is AQP4-dependent, a different effect should be present in all the cells because of their different distribution/expression level of AQP4. 10 mM histamine resulted in a significant reduction of cell swelling compared with the control in all the 1321-N1 cells. 0.1 and 1 mM histamine resulted in the 1321-N1 M1 cl10 and double transfected cells in a significant reduction.

These results agree with our previous observations: the hypotonicity induced cell swelling is higher in the 1321-N1 M1 cl10 and double transfected cells than in wild-type and M23 cl8 cells.
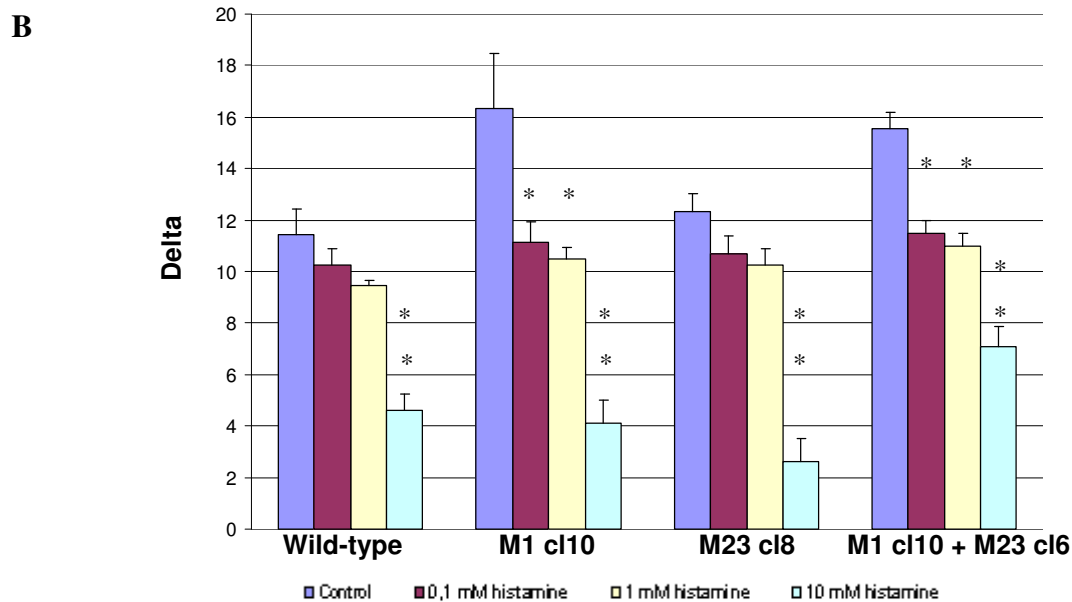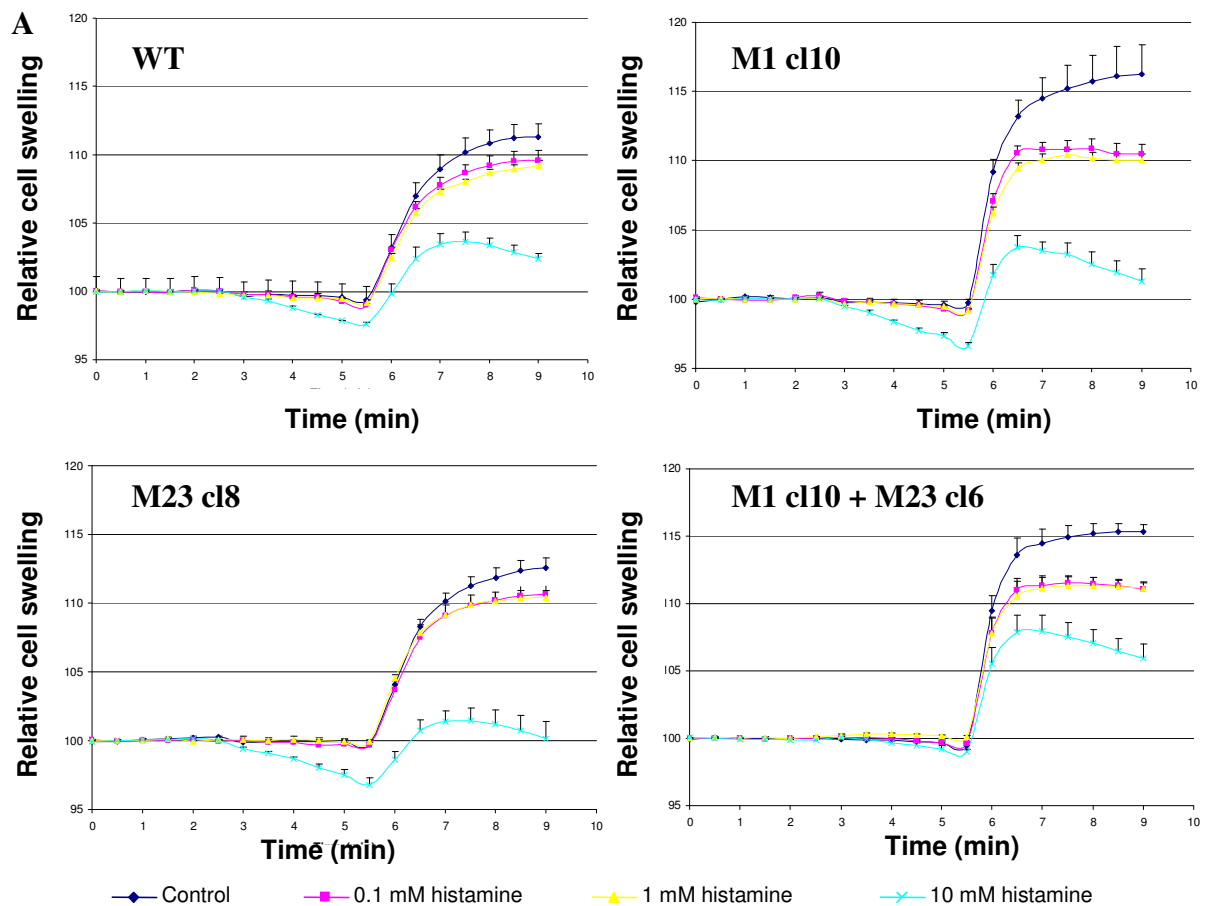
Fig. 3.4: Histamine induced a dose-dependent inhibition of cell swelling after a hypotonic shock 1321-N1 astrocytoma cells (wild type and transfected) were seeded in 12 well plates at a density of 100000 cells/well. After 3 minutes histamine in iso-osmotic PBS was added for an additional 3 minutes. Next a hypotonic shock of 69 mM Na^+ was applied in the presence of histamine. As a control, the experiment was performed in the absence of the mediator. A) The increase in relative cell volume over time. B) Shown is the average increase in cell diameter (Δ) and SEM from 6 experiments. P-values obtained via Student's t-test (* $p < 0.05$, * * $p < 0.001$)

3.4 Effect of glutamate on expression and function of AQP4

Glutamate may have an effect on the expression and function of AQP4 in human astrocytoma cells. To test this hypothesis, the effect of glutamate on the expression and function of AQP4 was investigated.

3.4.1 Glutamate stimulates internalization of AQP4 from the cell membrane

To examine the effect of glutamate on the intracellular distribution of AQP4, wild-type and transfected human astrocytoma 1321-N1 cells were treated with different concentrations of glutamate (0.5, 1 and 10 mM). As a control, the cells were treated with PBS. After 15 minutes of treatment, the cells were fixed and permeabilized. The cells were then labeled for AQP4 using the same protocol as described before.

In the control conditions, all the transfected cells show a clear membrane staining. When the cells were treated with 10 mM glutamate during 15 minutes, a translocation of AQP4 from the membrane to the cytoplasm is observed. This is more pronounced in the M1 cl10 1321-N1 cells (figure 3.5A). To quantify this endocytosis of AQP4, the number of cells with a clear membrane staining present in at least 100 cells were counted in the four different conditions (control, 0.5, 1 and 10 mM glutamate). A dose-dependent decrease in the percentage of cells with a clear membrane AQP4 staining is shown in figure 3.5B. This decrease is more pronounced in the M1 cl10 transfected 1321-N1 cells compared with the double transfected cells.
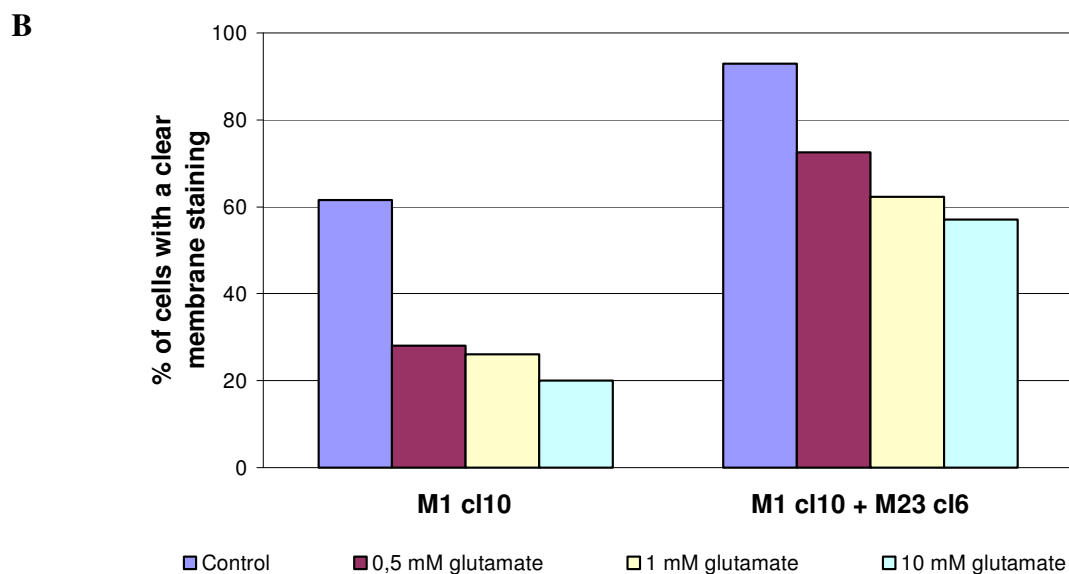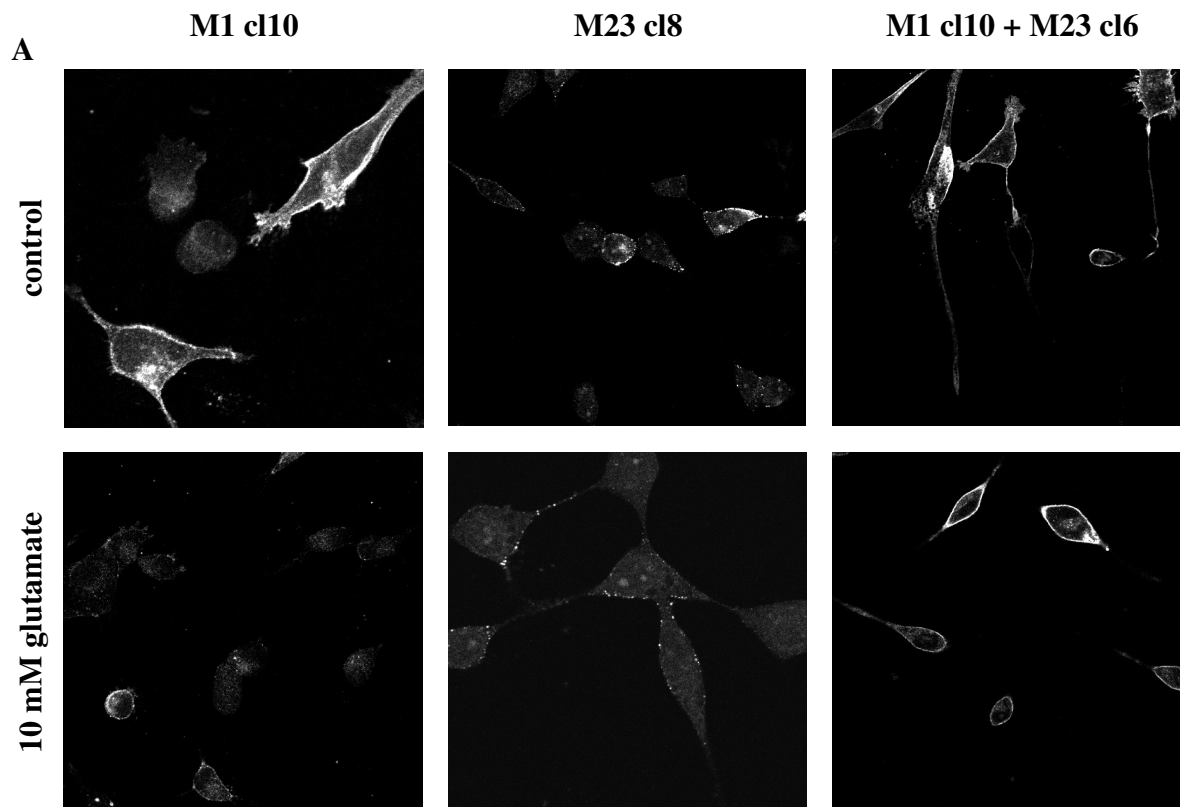
Fig. 3.5: The effect of glutamate on the distribution of AQP4 Transfected 1321-N1 cells were treated with glutamate during 15 minutes. As a control, the cells were treated with PBS during 15 minutes. The cells were labeled for AQP4 as described in fig. 3.1. A) Digital images were made using the confocal microscope. The cells were treated with 10 mM glutamate. B) Percentage of cells with a clear membrane staining. 1321-N1 M1cl10 and double transfected cells were treated with glutamate (0.1, 1 and 10 mM glutamate) and with PBS as a control. The percentage of cells with a clear membrane staining was determined by counting the number of cells in at least 100 cells per condition.

3.4.2 Glutamate induced a dose-dependent inhibition of cell swelling

To test whether glutamate induce an inhibition of cell swelling after a hypotonic shock, different concentrations glutamate (0.5, 1, 10 mM) were investigated in wild-type and transfected 1321-N1 cells.

After 3 minutes in PBS, glutamate in iso-osmotic PBS was added for an additional 3 minutes. Next a hypotonic shock of 69 mM Na^+ was applied in the presence of glutamate. As a control, the experiment was performed in the absence of glutamate.

A dose-dependent inhibition of cell swelling during hypotonic challenge was observed in the wild-type and transfected human astrocytoma cells (Fig. 3.6). The effect of glutamate varies between the different cell types, which correlates with the different expression levels of AQP4 in each cell. 10 mM glutamate gave a significant reduction of cell swelling in all the 1312-N1 cells compared to the control condition. 0.5 and 1 mM glutamate resulted in the M1 cl10 and double transfected astrocytoma cells in a significant reduction. In the 1321-N1 M23 cl8 cells, only the highest concentration had a significant effect on the cell swelling.

The effect of glutamate on hypotonicity-induced cell swelling is higher in the 1321-N1 M1 cl10 and double transfected cells than in wild-type and M23 cl8 cells.
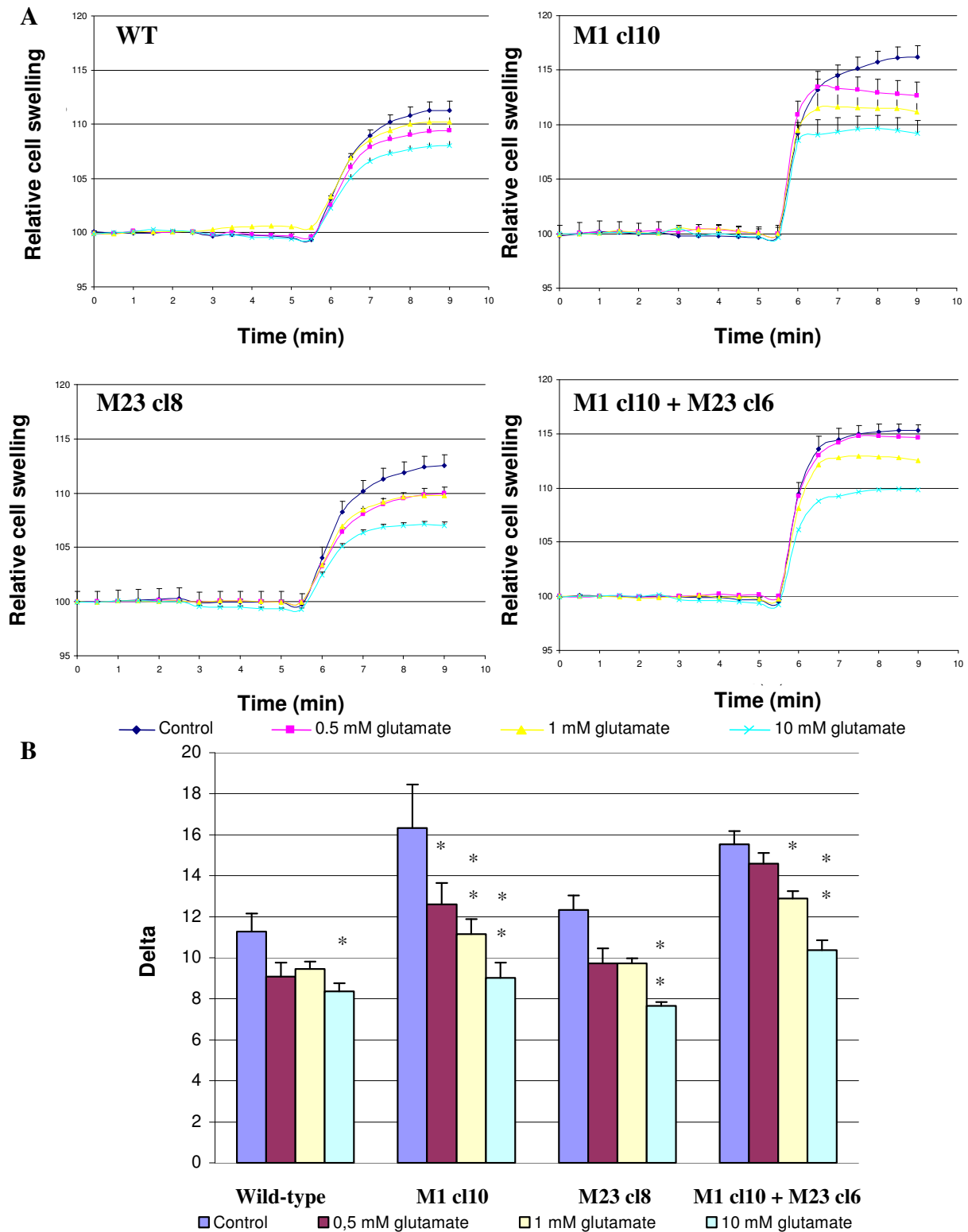
Fig. 3.6: Glutamate induced a dose-dependent inhibition of cell swelling after a hypotonic shock 1321-N1 astrocytoma cells (wild type and transfected) were seeded in 12 well plates at a density of 100000 cells/well. After 3 minutes glutamate in iso-osmotic PBS was added for an additional 3 minutes. Next a hypotonic shock of 69 mM Na+ was applied in the presence of glutamate. As a control, the experiment was performed in the absence of the mediator. A) The increase in relative cell volume over time. B) Shown is the average increase in cell diameter (Δ) and SEM from 6 experiments. P-values obtained via Student's t-test (* p < 0.05, * * p < 0.001)

3.5 Molecular mechanisms of histamine and glutamate

To investigate the molecular mechanisms of histamine and glutamate to inhibit the cell swelling during hypotonic challenge, intracellular calcium as second messenger was measured.

3.5.1 Histamine induced a dose-dependent increase in intracellular calcium

1321-N1 wild-type and the transfected cells were seeded in a 96-well plate and loaded with Fluo-4 to detect changes in the concentration of intracellular calcium ($[Ca^{2+}]_i$) using the microplate reader. Different concentrations of histamine were tested (0.1, 1 and 10 mM). After 1 minute in PBS, histamine was applied to the cells. The whole experiment lasted 4 minutes and every 5 seconds the fluorescence was measured. As a control, the same protocol was done with PBS in absence of histamine.

Histamine induced a dose-dependent increase in $[Ca^{2+}]_i$ in all cells, as shown in figure 3.7; 0.1 and 1 mM histamine resulted in a transient rise in $[Ca^{2+}]_i$ and 10 mM resulted in a sustained calcium increase.

Fig. 3.7: Histamine induced a dose-dependent calcium response in wild-type and transfected 1321-N1 cells
1321-N1 astrocytoma cells were seeded in a 96 well plate at 10000 cells/well in complete medium. After an overnight incubation the cells were loaded with 1 µg/ml Fluo-4 during 30 minutes at 37 °C. After 1 minute, histamine in PBS was applied. Using the microplate reader, fluorescence was measured every 5 seconds. Relative fluorescence increase over time. Shown is the mean +/-SEM of 9 experiments.

3.5.2 Glutamate induced a small intracellular calcium increase

1321-N1 wild-type and the transfected cells were seeded in a 96-well plate and loaded with Fluo-4 to detect changes in the concentration of intracellular calcium ($[Ca^{2+}]_i$) using the microplate reader. Different concentrations of glutamate were tested (0.5, 1 and 10 mM). After 1 minute PBS was replaced with glutamate in PBS. The whole experiment lasted 4 minutes and every 5 seconds the fluorescence was measured. As a control, the same experiment was done with PBS.

Glutamate induced a small increase in $[Ca^{2+}]_i$ in all cells. All the concentrations of glutamate applied to the cells resulted in a transient calcium peak, as shown in figure 3.8.
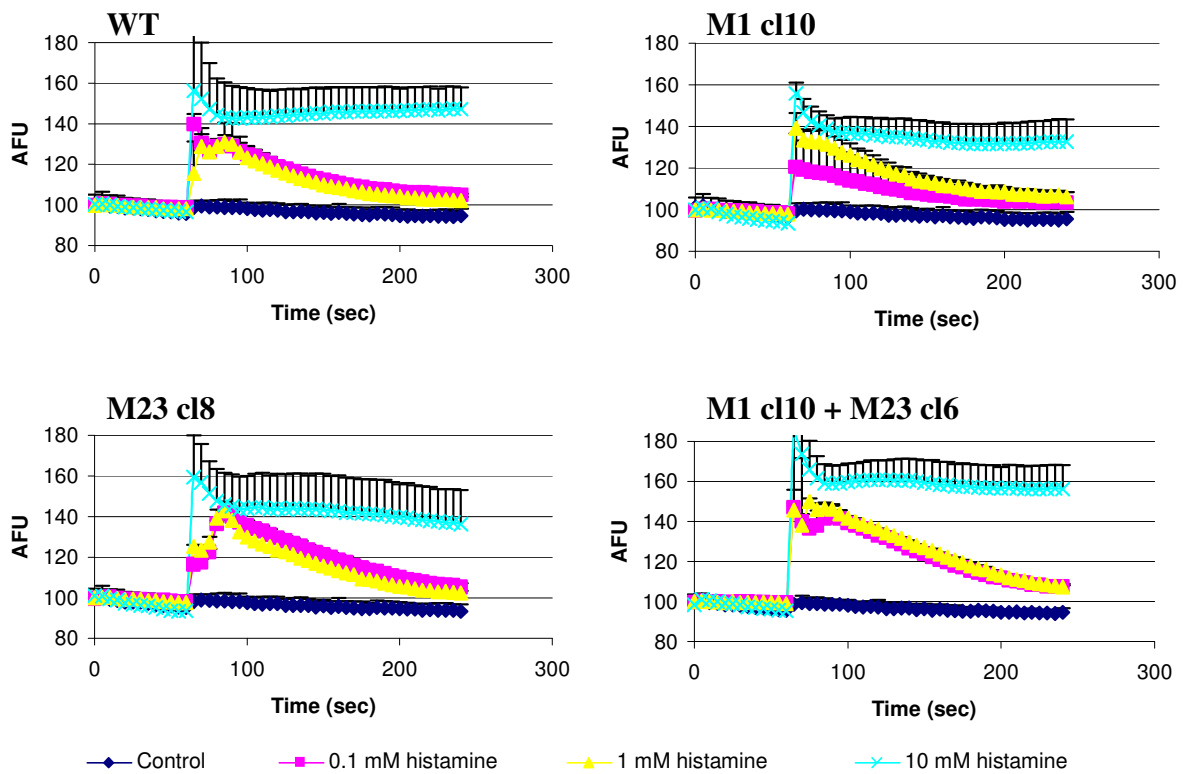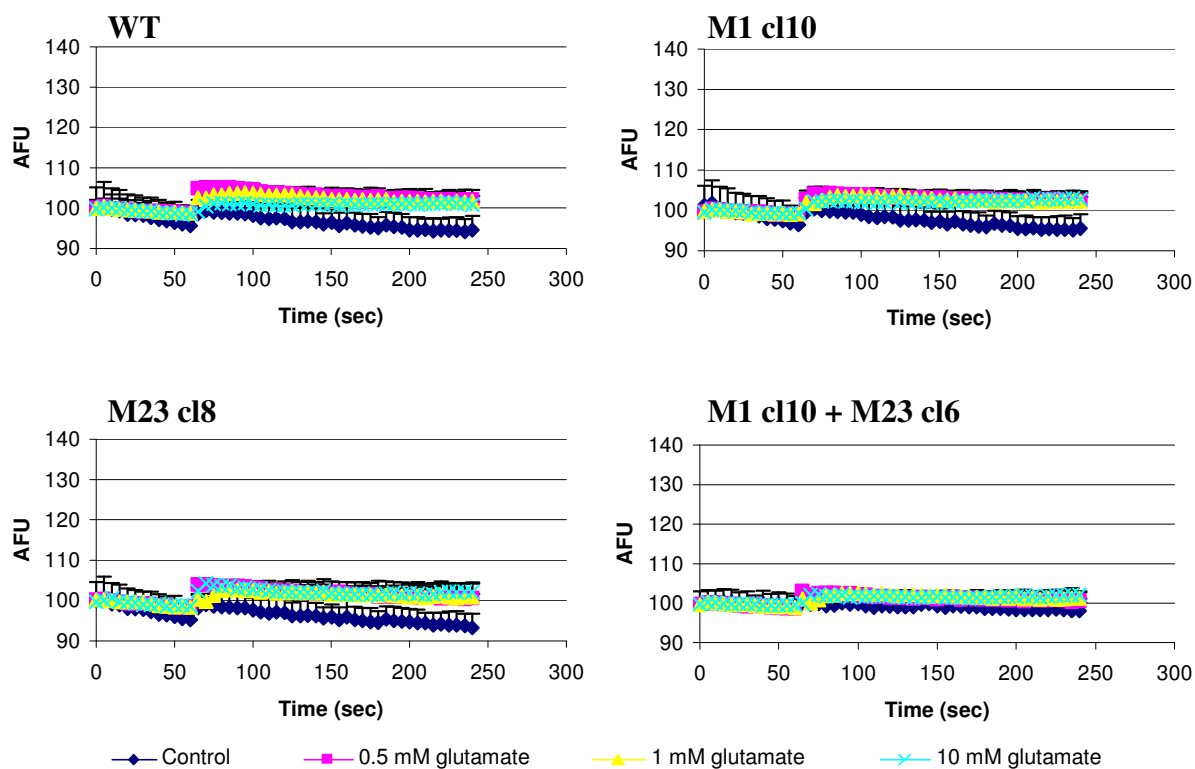
Fig. 3.8: Glutamate induced a small calcium response in wild-type and transfected 1321-N1 cells   1321-N1 astrocytoma cells were seeded in a 96 well plate at 10000 cells/well in complete medium. After an overnight incubation the cells were loaded with 1 µg/ml Fluo-4 during 30 minutes at 37 °C. After 1 minute, glutamate in PBS was applied. Using the microplate reader, fluorescence was measured every 5 seconds. Relative fluorescence increase over time. Shown is the mean +/-SEM of 9 experiments.

After traumatic brain injury, various neuronal mediators are released including histamine, glutamate, lactate, arachidonic acid and kinins. The release of these mediators can contribute to the formation of brain edema. Brain edema is the accumulation of water in the brain parenchyma. Brain swelling increases the intracranial pressure, which impairs vascular perfusion and could lead to brain ischemia, herniation and death (4). Klatzo classified brain edema as vasogenic or cytotoxic. Vasogenic edema occurs when the blood-brain barrier integrity is impaired. Cytotoxic edema consists of intracellular fluid accumulation. In cytotoxic edema, it is believed that the astrocytes play a major role (18). In the brain, AQP4 is the predominant water channel. It is a small hydrophobic protein that is mainly expressed at the endfeet of astrocytes facing the pia mater and blood vessels. AQP4 plays an important role in the regulation of brain water homeostasis and may be implicated in the formation, maintenance and resolution of brain edema as seen after traumatic brain injury (20, 21). Because of the possible role of AQP4 in brain edema, we looked at possible effects of neuronal mediators released after a TBI on AQP4 mediated water fluxes. Therefore an in vitro model for cell volume regulation was developed to investigate the effect of histamine and glutamate on cell swelling. Our results pointed out that these mediators have an effect on AQP4 mediated cell swelling and could be important in the formation, maintenance and resolution of brain edema.

4.1 Aquaporin 4 in human astrocytoma cells

AQP4 is the predominant water channel in the brain and has a high water permeability. AQP4 is expressed in astrocyte foot processes near capillaries and the pia mater and in ependymal cells lining the ventricles. These are key sites for water movement between cellular, vascular and ventricular compartments. AQP4 plays an essential role in the regulation of brain water homeostasis and could have an important role during brain edema.

In the present study, human astrocytoma 1321-N1 cells were used. The wild-type astrocytoma cells have a low expression level of AQP4. To obtain an overexpression of AQP4, the 1321-N1 cells were transfected with cDNA of the 2 isoforms, M1 and M23. The experiments were done with M1 cl10, M23cl8 and double (M1 cl10 and M23 cl6) transfected and wild-type 1321-N1 cells. All the cells were tested for the cellular distribution of AQP4. The wild-type

cells had more cytoplasm AQP4 staining, while the transfected cells had a clear membrane AQP4 staining. M1 cl10 and double transfected cells had a more pronounced membrane AQP4 staining then M23 cl8 cells.

Cell swelling experiments were done with the live cell imaging system. The experiments were performed with all types of astrocytoma cells (wild-type and transfected cells). After 6 minutes in iso-osmotic PBS (138 mM Na^+) a hypotonic shock (**69** mM Na^+) was applied. The subsequent increase in cell volume varied between the clones. In addition, there was a difference in the time course of swelling. Cells with the higher expression of AQP4 (M1 cl10, M23 cl8 and double transfected cells) showed a more pronounced swelling after the hypotonic shock compared to the wild-type cells. The increase in cell volume occurred also faster in the transfected cells. M23 cl8 1321-N1 cells showed a less pronounced hypotonicity induced swelling then M1 cl10 and double transfected cells. This is also in agreement with previous observations because M23 cl8 cells showed less membrane AQP4 staining then the other transfected cells. The higher increase in cell swelling indicates that the degree of cell volume change after a hypotonic shock is dependent on the expression level of AQP4. These results show that AQP4 plays an important role in the movement of water across the membrane. This conclusion is in agreement with the literature. Nicchia et al. (2003) determined alterations in water transport in AQP4-knockdown astrocytes. They used a stopped-flow light scattering method to examine the osmotic water permeability of the AQP4-knockdown astrocytes. Their results show that AQP4 gene silencing induces alteration in the water transport properties of the astrocyte cell membrane. This represents direct evidence that the high water permeability of astrocytes is AQP4 mediated (27).

The AQP4 transfected human astrocytoma cells illustrated high water permeability and AQP4 was mainly expressed in the plasma membrane, while the wild-type cells have a more cytoplasmic distribution of AQP4. The current in vitro model for brain edema is suitable for further testing the influence of neuronal mediators on the function and expression of AQP4.

4.2 The effect of histamine on cell swelling

To determine the effect of histamine on the function and expression of AQP4 in astrocytoma cells, the cells were treated with different concentrations histamine (0.1, 1 and 10 mM) during 15 minutes. After fixation and labeling for AQP4, images were made using the confocal microscope. The non-treated cells showed a clear membrane AQP4 staining, but the cells

treated with histamine showed a less pronounced membrane staining. There was a translocation of AQP4 from the membrane to the cytoplasm. The percentage of cells with a clear membrane staining was determined and a dose-dependent reduction was observed. The cells treated with the highest concentration histamine showed a reduction of clear membrane staining for AQP4. This is in agreement with the literature. Carmosino et al. (2001) observed endocytosis of AQP4 in histamine treated gastric cells. They found that histamine treatment resulted in a reduction of OAPs in human AQP4-overexpressing gastric cells. After 20 minutes of treatment with 100 µM histamine, the cell surface biotinylated experiments showed a internalization of AQP4. The higher concentrations of histamine in our study may be due to the different cell type used (28).

Because histamine is able to translocate AQP4 from the membrane into the cytosol, although AQP4 shedding cannot be excluded, histamine may affect hypotonicity-induced cell swelling as well. The absence of AQP4 in the membrane should lead to a reduction of water fluxes across the cell membrane.

To determine the functionality of the AQP4 water channels after histamine treatment, cell swelling experiments were done using wild-type and transfected 1321-N1 cells. The experiments showed that treatment with different concentrations of histamine (0.1, 1 and 10 mM) results in a dose-dependent inhibition of cell swelling during a hypotonic challenge. These results correlate with previous findings. Because of the histamine induced reduction of AQP4 in the membrane, less water fluxes across the membrane are possible. If less AQP4 is present in the membrane, less water can enter the cell during hypotonic challenge. Previous studies in vitro support these findings. Solenov et al. (2003) found a 7 fold reduced water-permeability in astrocytes obtained from AQP4 knock-out mice compared to control astrocytes. There is a correlation with the expression level of AQP4 and hypotonicity-induced cell swelling and this agrees with the histamine findings. If less AQP4 is present in the membrane, less water can move across the membrane. This suggests that AQP4 forms the main route for water fluxes across the cell membrane (29).

The next step in the present study was to examine the molecular mechanism of histamine to induce its effects on AQP4 translocation.

4.3 Molecular mechanism of histamine

Histamine binds to metabotropic histamine receptors. H_1 and H_2 histamine receptors are found on astrocytes. H_1 acts through calcium and H_2 acts via cAMP. The astrocytoma cells used in this study are not primary human astrocytes and their receptor expression may differ.

To determine the molecular mechanism of histamine to inhibit the cell swelling during hypotonic challenge, 1321-N1 wild-type and the transfected cells were loaded with Fluo-4 to detect changes in the concentration of intracellular calcium ($[Ca^{2+}]_i$) using the microplate reader. Stimulation of the cells with different concentrations of histamine (0.1, 1 and 10 mM) resulted in a dose-dependent increase in intracellular Ca^{2+} concentration. 10 mM Histamine resulted in a sustained increase of $[Ca^{2+}]_i$. The lower concentrations of histamine induced a transient peak of $[Ca^{2+}]_i$ followed by a decrease of $[Ca^{2+}]_i$.

These increases in $[Ca^{2+}]_i$ may be due to the presence of H_1 on the astrocytes. This was concluded by Jung et al. (2000). They investigated the effects of histamine on intracellular calcium concentration of cultured rat cerebellar astrocytes using fura-2-based Ca^{2+} measurements. In this study, $[Ca^{2+}]_i$ increased after an application of histamine and it was antagonized by the H_1 receptor blocker mepyramine. The H_1 receptor is coupled to the PLC-IP_3 pathway. The initial, large transient increase of $[Ca^{2+}]_i$ is due to Ca^{2+} mobilization from internal storage organelles. The following decline or prolonged elevated plateau was mediated by calcium influx via the plasma membrane. The calcium influx across the membrane results from the opening of Ca^{2+} channels in response to a decrease in the Ca^{2+} concentration in the lumen of the ER (30).

In conclusion, the different concentrations of histamine added to the astrocytoma cells may act via H_1 receptors and result in a initial peak in $[Ca^{2+}]_i$ due to the release of Ca^{2+} from intracellular stores. This peak is followed by a decline of $[Ca^{2+}]_i$ or a prolonged elevated plateau in case of high histamine challenge, probably mediated by Ca^{2+} influxes across the membrane.

4.4 The effect of glutamate on cell swelling

To determine the effect of glutamate on the function and expression of AQP4 in astrocytoma cells, the cells were treated with different concentrations glutamate (0.5, 1 and 10 mM) during

15 minutes. After fixation and labeling for AQP4, images were made using the confocal microscope.

The non-treated cells showed a clear membrane AQP4 staining, but the glutamate treated cells showed a cytoplasmic AQP4 staining. Similar to histamine, glutamate induced a translocation of AQP4 from the membrane to the cytoplasm. This observation was quantified and the percentage of cells with a clear membrane staining was determined. Glutamate induced a dose-dependent reduction of cells with a clear membrane staining. Thus, the cells treated with the highest concentrations of glutamate, showed less membrane associated AQP4 staining. It is not known how glutamate causes this internalization of AQP4.

The observed effects of glutamate on cellular distribution of AQP4 indicate that glutamate may affect cell swelling after applying a hypotonic shock. The reduction of AQP4 in the plasma membrane should lead to a decline of water fluxes across these membranes.

To determine the functionality of AQP4 water channels after glutamate treatment, hypotonicity induced cell swelling was tested on wild-type and transfected 1321-N1 cells. The experiments showed that treatment with various concentrations of glutamate (0.5, 1 and 10 mM) resulted in a dose-dependent inhibition of hypotonicity-induced cell swelling. These results correlate with the previous finding that glutamate induced a translocation of AQP4 from the plasma membrane to the cytoplasm. This translocation was more prominent in M1 cl10 1321-N1 cells and this correlates with the pronounced inhibition of cell swelling. If less AQP4 is present in the membrane, less water can enter the cell during hypotonic challenge. Uckermann et al (2006) also observed an inhibition of osmotic glial cell swelling caused by glutamate release (31).

The next step in this study was to unravel the molecular mechanism of glutamate. We focused on Ca^{2+} as mediator of the glutamate effects.

4.5 Molecular mechanism of glutamate

Glutamate binds to mGluR and iGluR. mGluR seems to be expressed only on astrocytes (24). The mGluR is coupled by a G-protein and binding of glutamate activates PLC. PLC cleaves $PI(4,5)P_2$ into DAG and IP_3. IP_3 triggers calcium release from the ER. This is why we focused on Ca^{2+} as mediator of the glutamate effects.

To unravel the molecular mechanism of glutamate to inhibit hypotonicity induced cell swelling, the 1321-N1 cells were loaded with Fluo-4 to detect changes in $[Ca^{2+}]i$ using the microplate reader. These experiments were done in iso-osmotic PBS. All the concentrations glutamate applied to the cells resulted in a small transient calcium peak. These findings are in agreement with literature found about this topic. Padmashri et al. (2007) investigated Ca^{2+} signaling in processes of astrocyte pairs after glutamate treatment. They observed glutamate induced alterations in calcium signaling where PLC, IP_3, internal Ca^{2+} stores appear to play an important role. This suggests that mGluRs play an important role in glutamate-induced cell swelling (32).

All previous experiments in Ca^{2+} measurement were done in iso-osmotic medium. But Uckermann et al. (2006) examined the role of glutamate as an inhibitory mechanism of osmotic glial cell swelling. Their study observed mGluR activation by glutamate, which evokes a Ca^{2+} independent purinergic-signaling cascade that involves the release of adenosine. Adenosine activates A1 receptors and protein kinase A (PKA). This leads to an efflux of K^+ and Cl^- ions, which is accompanied by water transport out of the cells. Here glutamate induces a calcium independent signaling (31).

4.6 Brain edema

Several studies showed that the expression pattern of AQP4 in astrocytes is changed during a TBI. Ke et al (2001) examined heterogeneous responses of AQP4 in oedema formation in a replicated severe TBI model in rats. In this study, the distribution and mRNA levels of AQP4 were investigated using immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR). They showed a negative immunostaining for AQP4 and a down-regulation of its mRNA in oedematous regions with impaired BBB integrity 1 day after the injury. In oedamatous regions where BBB was intact, no significant change in AQP4 expression level could be detected. This heterogeneous pattern of AQP4 responses was interpreted as follows: brain injury with an impaired BBB integrity resulting in vasogenic oedema is associated with the reduction of AQP4 expression, whereas, in cytotoxic oedema changes in AQP4 expression are not significant (33). Sun et al. (2003) also investigated the regulation of AQP4 in a TBI model in rats. AQP4 mRNA expression was measured by RT-PCR. Data from this study demonstrated that an up-regulation of AQP4 occurs at the site of TBI and that a down-regulation occurs at sites distant from the injury (34). Hu et al. (2005)

investigated AQP4 expression in human brain injury and tumors. Immunohistochemistry was used to detect AQP4. They observed an increase in AQP4 expression in human brains after TBI, within brain-derived tumors and around brain tumors (35).

Following traumatic brain injury, various mediators are released which enhance vasogenic and cytotoxic brain edema. AQP4 is mainly expressed in the membrane domains near blood vessels and the pia. This polarized expression pattern indicates that the perivascular endfeet and glia limitans are important sites for water fluxes (6). The water fluxes through AQP4 are bidirectional, thus it may be involved in the development and the resolution of edema. In the literature, several studies use AQP4 knock-out mouse models to investigate the contribution of AQP4 in the formation and clearance of edema.

Manley et al. (2000) investigated brain edema after acute water intoxication and ischemic stroke in AQP4 null mice. Acute water intoxication gave rise to cytotoxic edema without disruption of the BBB. Focal cerebral ischemia results in both cytotoxic and vasogenic edema formation. AQP4 deletion in mice is associated with a reduced cerebral edema in response to water intoxication and ischemia with improved survival and neurological outcome. Reduced brain edema in AQP4-deficient mice indicates that AQP4 is a potential target for drug discovery (36). AQP4 plays a role in early accumulation of brain water in response to neurological insults, producing cytotoxic brain edema. Verkman et al. (2006) showed that AQP4 deficient mice are protected from cytotoxic brain edema. AQP4 upregulation after TBI may be a maladaptive response, which possibly worsens cytotoxic edema. But AQP4 permits bidirectional water transport, so it can be hypothesized that AQP4 facilitate the removal of excess brain water in vasogenic brain edema. In a freeze-injury model of vasogenic brain edema, AQP4 null mice showed a worse clinical outcome. This impaired clearance of excess brain water in AQP4 deficient mice in vasogenic edema suggests that AQP4 provides a transcellular route, which allows edema fluid to move across the astrocyte membrane of the glia limitans into the CSF. Thus, the upregulation of AQP4 after TBI is a protective mechanism for clearance of vasogenic edema (37). It needs, however, emphasis that for these hypothesizes about the contribution of AQP4 on formation, maintenance and clearance of cytotoxic or vasogenic oedema, AQP4-knockout mice were used. Several studies showed that these mice might use alternative pathways to maintain their central water homeostasis, in addition to altered behavior of neurotransmitters. Caution is needed to extrapolate the results of these studies to rodents or animals without genetic manipulations

Histamine and glutamate are released after TBI and inhibited hypotonicity induced cell swelling (28, 31). This may be beneficial towards astrocyte swelling in cytotoxic edema formation. Both mediators cause internalization of AQP4 and thus reduce the water permeability of the astrocytes. Less water can enter the astrocytes, which leads to less cytotoxic edema formation. However, it needs emphasis that reduced membrane-associated AQP4 may prevent the efflux of metabolically produced water, leading to formation of cytotoxic brain edema. When water has already accumulated in the brain, glutamate and histamine may have a harmful effect. The internalization of AQP4 may decrease the clearance of extracellular water. Stimulation of AQP4 functionality may form an effective mechanism to help in the resolution of vasogenic edema.

4.7 General conclusion

This study used an in vitro model for cell volume regulation to investigate the role of histamine and glutamate on cell swelling. These mediators are released after TBI and this study was designed to test the influence of these mediators on AQP4 mediated water fluxes. The experiments were done using wild-type and transfected human astrocytoma cells. To investigate the possible role of histamine and glutamate on cell swelling, the expression and distribution of AQP4 and hypotonicity induced cell swelling was studied. Experiments have pointed out that AQP4 is the main route for water transport. Histamine and glutamate influence the distribution of AQP4. Treatment with these mediators caused an internalization of AQP4 from the plasma membrane to the cytoplasm. They also induced a dose-dependent inhibition of cell swelling during a hypotonic challenge. To unravel the molecular mechanism of histamine and glutamate, we focused on intracellular calcium concentrations as possible mediator. Histamine evoked a dose-dependent rise in intracellular Ca^{2+}, while glutamate induced a smaller transient rise in intracellular calcium. In the future, more research has to be done on this topic because it is not known how these increases in intracellular calcium result in AQP4 internalization. Our study suggests that these effects caused by histamine and glutamate are AQP4-specific, but future investigations with AQP4 knock-out or knock-down cells are planned. This in vitro model only examined the acute effect of histamine and glutamate on hypotonicity induced cell swelling. In vivo experiments should be done to investigate the effect of histamine or glutamate agonists or antagonists on the neurological outcome in a brain edema model.

Brain edema accounts for a large part of the morbidity and mortality associated with common neurological conditions such as TBI, brain tumors and stroke. A fast removal of excess brain fluid is crucial. Treatment options are limited to osmotic-agents, surgery and others, none of which correct the molecular mechanisms responsible for brain edema. Our results point out that high levels of AQP4 may be beneficial for rapid clearance of brain edema. Further research on AQP4 and mediators released after TBI can lead to an appropriate treatment.

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