AQP4 expression in striatal primary cultures is regulated by dopamine – implications for proliferation of astrocytes

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Abstract

Proliferation of astrocytes plays an essential role during ontogeny and in the adult brain, where it occurs following trauma and in inflammation and neurodegenerative diseases as well as in normal, healthy mammals. The cellular mechanisms underlying glial proliferation remain poorly understood. As dopamine is known to modulate proliferation in different cell populations, we investigated the effects of dopamine on the proliferation of striatal astrocytes in vitro. We found that dopamine reduced proliferation. As proliferation involves, among other things, a change in cell volume, which normally comes with water movement across the membrane, water channels might represent a molecular target of the dopamine effect. Therefore we studied the effect of dopamine on aquaporin 4 (AQP4) expression, the main aquaporin subtype expressed in glial cells, and observed a down-regulation of the AQP4-M23 isoform. This down-regulation was the cause of the dopamine-induced decrease in proliferation as knockdown of AQP4 using siRNA techniques mimicked the effects of dopamine on proliferation. Furthermore, stimulation of glial proliferation by basic fibroblast growth factor was also abolished by knocking down AQP4. In addition, blocking of AQP4 with 10 μM tetraethylammonium inhibited osmotically induced cell swelling and stimulation of glial cell proliferation by basic fibroblast growth factor. These results demonstrate a clear-cut involvement of AQP4 in the regulation of proliferation and implicate that modulation of AQP4 could be used therapeutically in the treatment of neurodegenerative diseases as well as in the regulation of reactive astrogliosis by preventing or reducing the glia scar formation, thus improving regeneration following ischemia or other trauma.

Introduction

Astrocytes are found ubiquitously in the developing and in the mature central nervous system (CNS) and play an important role in the regulation of brain function. Their proliferation occurs during ontogeny and adolescence, and in the adult CNS in various regions (Nixdorf-Bergweiler et al., 1994; Markham et al., 2007). In the case of brain injury caused by trauma, ischemia, inflammation or neurodegenerative diseases, neural stem cells that are present throughout adulthood will proliferate and differentiate into new neurons and/or glia (reviewed by Kernie et al., 2001; Mazurova et al., 2006; Zhu & Dahlström, 2007). There is evidence that these adult neural stem cells exhibit properties associated with glia both in vivo (Doetsch et al., 1999; Seri et al., 2001; Garcia et al., 2004) and in vitro (Laywell et al., 2000; Imura et al., 2003; Morshead et al., 2003), and express glial fibrillary acidic protein (GFAP), a marker for differentiated astrocytes (Eng et al., 2000). Interestingly, proliferation of GFAP-positive cells in the brain is not confined to a response to injury but also seems to occur under normal physiological conditions in the hippocampus (Rietze et al., 2000) and the striatum (Mao & Wang, 2001). The relevance of the proliferation of astrocytes in the uninjured mature brain is still far from being defined, although implications of changes in the number of astrocytes in the gyrus dentatus of the hippocampus in memory and learning functions are discussed (reviewed by Kitabatake et al., 2007; Bruel-Jungerman et al., 2007). There is also evidence that changes in cell numbers of GFAP-positive cells are involved in the neuropathology of major depression and bipolar disorders (Rajkowska & Miguel-Hidalgo, 2007) as well as in neurodegenerative diseases such as Morbus Parkinson or Huntington’s disease (Mazurova et al., 2006).

A variety of factors that regulate the proliferation and/or the differentiation of astrocytes and progenitor cells have been described. The cellular mechanisms underlying the astrocytic proliferation are still not well understood. The neurotransmitter dopamine not only represents an important developmental signal for the striatum but also stimulates proliferation of progenitor cells in the subventricular zone...
of the adult brain (Van Kampen et al., 2004). In the present study we provide evidence that dopamine regulates the proliferation of striatal astrocytes in culture and that these dopaminergic effects on proliferation are mediated by the water channel protein aquaporin 4 (AQP4). Aquaporins (AQPs) are water channel proteins that play important roles in the regulation of water homeostasis in physiological and pathological conditions (Agre & Kozono, 2003). The primary AQPs in the mammalian brain are AQP1, exclusively expressed in choroid plexus cells, and AQP4, localized predominantly in the endfeet and in the perivascular membrane of astrocytes, where it is involved in the regulation of water movement between brain parenchyma and vascular space (Yool, 2007). AQP4 is expressed in two isoforms corresponding to alternative initiations of transcription at M1 or M23.

Furthermore, by either knocking down AQP4 in striatal glial cultures through siRNA or by blocking AQP4 with low micromolar concentrations of tetraethylammonium (TEA), we present unambiguous evidence for an important role of AQP4 in the regulation of proliferation of astrocytes.

Materials and methods

Animals and cell culturing

Pregnant Balb/c mice were obtained from Harlan–Winkelmann GmbH, Germany, and were kept in an in-house animal facility until parturition. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) regarding the care and use of animals for experimental procedures. Primary glial cultures were prepared from newborn mouse pups (Ivanova et al., 2001). In brief, the animals were killed by decapitation and the striatum was excised and dissociated as described by Ivanova et al. (2001). These cultures consisted of ~95% astrocytes and only few oligodendrocytes (precursors), and were virtually free of neurons and microglial cells. The cells were plated into 100-mm culture dishes coated with poly-DL-ornithine in MEM supplemented with 20% fetal calf serum (FCS). Cells were allowed to grow until reaching confluency (usually after 3 days). Then, culture dishes were shaken and the medium was taken off to remove oligodendrocytes and microglia. Cells then were slightly trypsinized, scraped off, replated (first passage), grown again to reach confluency, and replated again (second passage). After the second passage, cells were resuspended for 1 day in serum-free neurobasal medium (NBM; Gibco Life Technologies, Eggenstein, Germany) for synchronisation and then were used in experiments.

AQP4 knockdown with siRNA

RNA duplexes of 21 nucleotides with a sequence identical to mouse AQP4 (AQP4 siRNA: 5'-UCAAUUAACUGGAGCCAGUU-3') were chemically synthesized by MWG Biotech, Ebersberg, Germany. These siRNA have been shown to effectively knock down AQP4 in human, mouse and rat astrocytes (Nicioha et al., 2003). For control purpose the cells were treated with transfection reagent reagent only and with transfection reagent plus scrambled RNA.

For optimal transfection conditions 1-week-old cultured striatal astrocytes were seeded the day before transfection at 30% confluency (50,000 cells/cm²) using Dulbeccos’s modified Eagle’s medium (DMEM) with 10% FCS. Subsequent transfection was carried out using INTERFERin (Polyplus transfection Inc., New York, USA) according to the manufacturer’s instructions. For each experiment, specific silencing was confirmed by RT-PCR and Western blot analysis.

Treatment of cells

Cultures were used for experiments 2 days after the second passage. They were treated daily with dopamine (DA) at a concentration of 100 μM in the presence of glutathione. We used this apparently high dose of DA as studies on the DA concentration in wet brain tissue revealed 10 μM to be physiological (Jobe et al., 1982) and as it is reasonable to assume that even higher concentrations occur around varicosities. In order to analyze the receptor subtype which might mediate the effects of DA on proliferation and/or AQP4 expression, cultures were simultaneously treated with DA and DA antagonists specific for receptors of the dopamine D1 (SCH23390, 1.0 μM; Tocris, UK) or D2 (sulpiride, 1.0 μM, Tocris) receptor family and the alpha2-adrenergic receptor antagonist yohimbine (1.0 μM; Sigma, Germany).

In order to analyze the time-dependency of the effects of DA on proliferation, cultures were exposed for 48 and 72 h. Proliferation of the cells was stimulated by treatment with basic fibroblast growth factor (bFGF; 10 μg/mL) for 48 and 72 h. To study the effect of AQP4 on the proliferation, some cultures were treated simultaneously with bFGF (10 μg/mL) and siRNA duplexes (1 or 5 nM) or with bFGF (10 μg/mL) and TEA (10 μM) for 48 and 72 h.

RT-PCR

Total RNA was isolated from cultures usingpeqGOLD RNApure (Peqlab, Erlangen, Germany) according to the manufacturer’s instructions. Reverse transcription was performed using the Light Cycler system (Roche Molecular Biochemicals, Germany) and applying the ready-to-use ‘hot start’ reaction mix from QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany). PCR reactions were carried out in a reaction mixture consisting of 2 μL cDNA, 6 μL RNase-free water, 10 μL ‘hot start’ reaction mix and 1 μL of each primer (10 pmol). Reactions were conducted in glass capillaries (Roche Molecular Biochemicals) in the Light Cycler system, subjected to a 15 min initial ‘hot start’ activation of the Taq DNA polymerase at 95°C, followed by 45 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 20 s and elongation at 72°C for 20 s. External standard curves were generated by amplification of 10-fold dilutions of purified PCR products of target genes. Samples were analyzed in the log-linear phase where amplification efficiency is constant. The cycle numbers of the log-linear phase were plotted against the logarithm of the concentration of template DNA. The concentrations of target genes were calculated by comparing the cycle numbers of the log-linear phase of the samples with the cycle numbers of the external standards. Final data were expressed as the ratio between the amounts of each transcript of interest versus the amount of hypoxanthine phosphoribosyltransferase (HPRT) transcript (housekeeping gene). Melting curves were analyzed to determine the specificity of the PCR reaction. Forward and reverse primers were as follows: 5'GCA TCG CTA AGT CCG TCT TCT TCT AC-3' and 5'CCA ATC CTC CAA CCA CAC TG-3' (antisense). These primers yielded a 133-bp product.

In addition, conventional RT-PCR was performed with oligonucleotide primers specific for AQP4.4 (207 bp) 5'TGG TGG CTT TCA AAG GAG TCT GTG 3' and 5'CTG ATG TGG CCA AAG CAC TGC AC-3'; for AQP4.M23 (288 bp) 5'GGA AGG CTA GGT TGG TGA CTT C-3' and 5'CTG ATG TGG CCA AAG CAC TGC AC-3'; for AQP4.M1 (253 bp) 5'ATG AGT AGG CAG AGA GCT GAC CCA GCG A-3' and 5'ACC ATG GTA GCA ATG CTT AGT CC-3' according to Genbank accession number AF.219992. PCR was performed with 34 cycles (1 min denaturation at 95°C, followed by 1 min annealing at 62°C (HPRT, AQP4.M1 and AQP4.M23) or 60°C (AQP4.4),
respectively, and 2 min elongation at 72°C. The PCR products were analyzed following standard electrophoretical separation (1.5% ethidiumbromide-stained agarose gels). The size of each PCR product was estimated by using O'GeneRuler™ 50 bp ladder (Fermentas, Germany). For internal standard and to exclude genomic contamination HPRT expression was determined with PCR using the following sense and antisense oligonucleotides: nt 576–594 (sense) and nt 805–824 (antisense) according to Genbank accession number J00423. As expected, only the 249-bp control band but not the 1100-bp genomic band was seen, demonstrating that the amplified RT-PCR products were only derived from the respective mRNA. Gels were scanned and the optical density was measured with an image processing program (NIH IMAGE J software). Relative quantification was performed determining ratios of target genes and the housekeeping reference gene HPRT. Statistical analysis was conducted using Student’s t-test for unpaired data.

Western blot
For purification of the membrane fraction cells were washed twice with ice-cold phosphate-buffered saline (PBS) and scrapped off in homogenization buffer (sodium phosphate, pH 7.0, 7.5 mM; sucrose, 0.25 M; EDTA, 5 mM; and EGTA, 5 mM) containing Pefabloc SC-Protease Inhibitor (Carl Roth, Karlsruhe, Germany), transferred to 1.5-mL Eppendorf tubes, and spun at 1000 g for 10 min at 4°C; the obtained supernatant was respun at 20 000 g for 30 min at 4°C. The membrane fractions yielded in the pellet were solubilized according to Neely et al. (1999). Protein concentrations were measured by using bicinchoninic acid (BCA Protein Assay Kit; Pierce, Rockford, IL, USA). A methanol–chloroform precipitation was carried out and the pellet was resuspended in sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) sample buffer (SDS, 3%; Tris, pH 6.8, 10 mM; glycerol, 60%; bromphenolblue, 0.01%; and dithiothreitol, 0.1 M) adjusted to concentrations of 2 μg/μL protein. Twenty micrograms of each sample was separated on 12.5% SDS–polyacrylamid–4.0 M urea gel, and electrotransferred onto a nitrocellulose membrane (GenScript Corp., Piscataway, NJ, USA). Western blot detection was made with a One Step Western Blot Kit (GenScript Corp.) using a rabbit anti-AQP4 (Chemicon International, Hampshire, UK) affinity-purified polyclonal antibody diluted 1:500. The bands of interest from three blots were scanned and the optical density was measured with an image processing program (NIH IMAGE J software). Statistical analysis was conducted using Student’s t-test for unpaired data.

Immunocytochemistry
Primary cultures of striatal mouse astrocytes were washed twice with ice-cold PBS supplemented with 1 mM MgCl₂ and 0.1 mM CaCl₂. Cells were fixed for 30 min in 2% paraformaldehyde in PBS, permeabilized with 50 mM NH₄Cl in PBS for 15 min (20–24°C) and washed with 0.1% bovine serum albumin and 0.3% Triton X-100 in PBS. Blocking was carried out for 30 min at room temperature in goat serum dilution buffer (GSDB; goat serum, 16%; Triton X-100, 0.3%; and NaCl, 0.3 M; in PBS). Cells were incubated overnight at 4°C with rabbit anti-AQP4 (Chemicon International; catalogue number: AB3594) affinity-purified polyclonal antibody diluted 1:200 in GSDB. The next day, astrocytes were incubated with Alexa fluor 546-conjugated goat antirabbit antibody (Molecular Probes–Invitrogen, Carlsbad, Ca, USA; catalogue number: A11010) diluted 1:1000 in GSDB for 45 min at room temperature. Cells were visually examined with an Axioplan 2 imaging Pol (Carl Zeiss, Göttingen, Germany). Controls for immunocytochemistry were performed by omitting the primary antisera.

**Proliferation assay**
Proliferation was measured using either a CyQUANT® NF Cell Proliferation Assay Kit (Molecular Probes) or by [3H]-thymidine incorporation. Glial cells were cultivated as described above. With the second splitting, cells were counted and a total of 1.25 x 10⁶, 2.5 x 10⁶, 5 x 10⁶ and 1 x 10⁷ cells per well was seeded in 96-well round-bottomed microtiter plates in MEM supplemented with 10% FCS, corresponding to cell densities ranging from 3906.25 to 31 250 cells/cm². The latter is about one-fifth of the density at which the cells were grown for the experiments to determine the effects of dopamine on the expression of AQP4. The higher cell density in the AQP4-expression experiments were necessary in order to obtain sufficient amounts of protein.

To study the effect of DA proliferation was measured by estimating the number of cells grown in the different culture conditions using the CyQuant cell proliferation assay kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Briefly, medium was taken off and cells were detached and suspended in 100 μL MEM. After centrifuging, the pellet was resuspended in 200 μL of CyQUANT GR dye/cell-lysis buffer and transferred to a microplate, and fluorescence was measured using a microplate reader set up with the following filter set: excitation 480 nm, emission 520 nm. A standard curve was made with a bacteriophage lambda DNA included in the kit and was used to calibrate the assay for use at different times or on different days. Variation in the signal intensity of the standard curve is directly related to variation that will be observed for assay cells on different days. To evaluate the effect of AQP4 knockdown, proliferation of astrocytes was measured using the thymidine incorporation assay. After 72 h of treatment cells were ‘pulsed’ with 1 μCi/mL [³H]-TdR (Hartmann Analytic, Braunschweig, Germany) per well for an additional 16 h. DNA was collected on glass fiber filters (Wallac, Turku, Finland) and [³H]-TdR incorporation was measured in a scintillation counter (1450 Microbeta Plus; Wallac). The experiments were performed in duplicate. Assay data comparing [³H]-thymidine incorporation to the CyQUANT assay demonstrated that the CyQUANT GR reagent can be substituted for [³H]-thymidine incorporation. Both assays revealed results with similar cell number and proliferation rates in control and dopamine-treated conditions.

Fluorescence-activated cell sorting
Annexin V conjugate (Molecular Probes) was used for apoptosis detection. A positive control was prepared incubating the cells with 300 nM of staurosporine 24 h before the assay. After the incubation with dopamine or staurosporine, astroglial cells were harvested with trypsin with 0.1% EDTA. After washing with PBS, cells were centrifuged and resuspended in 100 μL of annexin-binding buffer and incubated with 2.5 μL of the annexin conjugate for 10 min. Thereafter, the stained cells were analyzed using a fluorescence-activated cell sorting scan flow cytometer. The number of annexin-positive cells was counted and expressed as the number of cells shifted after fluorescence labeling from the polygonal gate R1 to the polygonal gate R2. To distinguish necrotic cells propidium iodide was used as a marker of dead cells and the polygonal gate R3 was defined for this population of cells.
Water permeability measurement

Water permeability measurement was carried out according to Noell et al. (2007). Astrocytes were plated onto poly-DL-ornithine-coated coverslips and incubated with a fluorescence dye (5 μM calcine; Molecular Probes, Invitrogen, Karlsruhe, Germany) in DMEM with 10% FCS for 30 min at 37°C in a 5% CO2 atmosphere. Calcine-loaded cells were mounted in a closed perfusion chamber (POC-Chamber; LaCon, Ulm, Germany) on the stage of a Zeiss 510 inverted laser scanning microscope (LSM). Perfusion solutions were pumped with a tubing pump (IPC high-precision multi-channel pump; Ismatec, Wertheim, Germany) and switched using three-way valves. The perfusion rate (350 μL/s) was chosen to be fast enough to reduce mixing and exchange times (100 s) and slow enough not to deform cells during perfusion. To establish a baseline HEPES–Hanks’s solution (CaCl2 · 2H2O, MgSO4 · 7 H2O, MgCl2 · 6H2O, NaCl, KCl, HEPES, l-glutamine, d-glucose; 300 mOsm) was used for isotonic perfusion. Hypotonic solution preparation was by dilution of HEPES–Hanks solution with distilled water (200 mOsm). Perfusion time of each solution was 3 min. In the blocking experiments, both perfusion solutions contained 10 μM TEA (Sigma-Aldrich, Germany). The water permeability measurements were carried out at 37°C. Images were recorded using a 63× long-distance water-immersion objective. Fluorescence was excited by the argon laser line emitting light at a wavelength of 488 nm and excited fluorescence was detected using a bandpass filter at 515–525 nm. Laser intensity was kept as low as possible to avoid cell damage and bleaching during the experiment. The measurements were made using the confocal microscope software of the Zeiss LSM (ZEISS LSM510, microscope software, Release 3.2; Zeiss, Oberkochen Germany). Confocal images were recorded in an automated time series at an interval of 10 s; each confocal scan set took 4–5 s. Cells were recorded in a line scan through the z-axis of the selected cells. The images were analysed off-line by setting at least three regions of interest (ROIs) inside the cytoplasmic area of several (up to three) cells and the mean fluorescence intensity within the ROIs was determined. The 3-min isosmotic phases before the hypo-osmolar stimulation of each single cell were used to determine the bleaching curve for which all data were corrected off-line. The corrected fluorescence measurements were plotted as ΔF/Δt0, where F0 is the averaged initial fluorescence intensity and ΔF the difference between the measured fluorescence and the initial fluorescence.

Statistics

To investigate the effects of DA and norepinephrine (NE) on AQP4 mRNA levels, 3–10 independent culture experiments were performed. Every culture condition was done in triplicate. Optical densities (ODs) of amplification products are given as relative ODs normalized to the corresponding HPRT values. Differences between experimental groups were tested using the Mann–Whitney rank-sum test or Student’s t-test if normality and variance tests were passed.

Protein levels were analyzed by measuring optical densities from the bands of interest from three independent blots. Statistical analysis was conducted with Student’s t-test.

Proliferation either measured by thymidine incorporation (three independent experiments) or by CyQUANT (3–6 independent experiments) was evaluated using Mann–Whitney rank-sum test or Student’s t-test if normality and variance tests were passed. All mean values in the text and graphs are ± SEM.

For the statistical analysis of the swelling experiments (Fig. 5) three independent experiments were used for each experiment with TEA and for the experiments with the siRNA. For each single TEA experiment cells were initially swollen under control conditions, followed by swelling in the presence of 10 μM TEA and finally under control conditions again. For the siRNA experiments cells were exposed to hypotonic solution and compared to the controls from the TEA experiments. Statistical analysis was performed by applying the moving average method for each graph in order to determine the minima of the ΔF/Δt0 time course. Differences between the minima were then analyzed using the Kruskal–Wallis one-way analysis of ranks followed by Dunn’s method.

Results

Effects of dopamine on the proliferation of striatal glia cells in vitro

Although it is well established that catecholamines are involved in the regulation of the migration of a variety of cells comprising GABA-ergic neurons (Crandall et al., 2007) and gonadotropin releasing hormone neurons (Pronina et al., 2003) as well as lymphocytes (Spiegel et al., 2004), findings concerning the regulation of proliferation by DA are contradictory with respect to the cells investigated (Ohtani et al., 2003; Van Kampen & Robertson, 2005).

Therefore we determined the effect of DA on the cell numbers after 72 h in striatal glial cultures that were seeded initially with a total number of cells ranging from 1.25 × 103 to 1 × 104 cells per well. We observed a decreased proliferation in DA-treated cultures compared to control cultures. The effect of DA was inversely proportional to the density of the cells, with DA giving rise to a decrease of ~15% in cultures with 1 × 104 cells per well and ~65% in cultures with 1.25 × 103 cells per well (Fig. 1A).

As high concentrations of DA are known to induce apoptosis in some experimental approaches (Simantov et al., 1996; Barzilai et al., 2000), we performed an annexin-binding assay in order to exclude that the decrease in cell number observed here was due to an increased cell death. As can be seen from the representative fluorescence-activated cell sorting analysis shown in Fig. 1B, there was no higher apoptosis rate and no increase in the number of dead cells in DA-treated cultures compared to controls, indicating that the reduction in proliferation by DA was not brought about by an increase in apoptosis, presumably because of the presence of glutathione as it has been shown that DA in combination with glutathione exerts a neuroprotective effect (Pardo et al., 1995; Emdadul Haque et al., 2003). This result was obtained in a total of three experiments and the statistical analysis using a paired t-test between controls and DA-treated cells showed that the two populations were not statistically significantly different (P = 0.33).

Effects of dopamine on the expression of the water channel protein AQP4

Although ischemia, diabetes, osmolality, protein kinase C activation, cytokines and steroid hormones have been shown to modulate AQP4 expression, it is not known whether DA influences the expression of AQP4 mRNA and protein, although an effect of DA on the water permeability of AQP4 has been described (Zelenina et al., 2002). Therefore we investigated the expression of AQP4 in striatal cultures (1.5 × 103 cells/cm2) in the absence or presence of DA. DA down-regulated AQP4 mRNA expression (Fig. 2A, black and white bar; n = 10) as well as protein level (Fig. 2B; n = 3, P = 0.04) and this effect was not blocked successfully by either the D1 receptor antagonist SCH23390 or by the D2 receptor antagonist sulpiride (Fig. 2A, hatched bars; n = 10). The alpha2-adrenoceptor antagonist yohimbine effectively blocked the effects of DA on the expression of...
AQP4 (Fig. 2A, black bar, right; \( n = 10 \)). The down-regulation of AQP4 mRNA expression was restricted to the M23 isoform (Fig. 2C; \( n = 3 \), \( P = 0.03 \)) whereas the M1 isoform (Fig. 2D; \( n = 3 \), \( P = 0.1 \)) was not affected.

Effects of norepinephrine on the proliferation of striatal glia cells in vitro

As we observed that the alpha2-adrenoceptor antagonist yohimbine effectively blocked the effects of DA on the expression of AQP4 (Fig. 2A and B), suggesting that DA might exert its effect via alpha2-adrenoceptors, we performed additional proliferation experiments with NE. As expected, treatment of glial cultures seeded with a total number of \( 1 \times 10^4 \) cells per well with 100 \( \mu \)M NE reduced the proliferation (Fig. 3) similarly to what had been observed with DA (Fig. 1A). Simultaneous treatment of the culture with NE and the alpha(2)-adrenoceptor antagonist yohimbine abolished the effect of NE on the proliferation (Fig. 3).

The impact of AQP4 on the proliferation of striatal glial cells

In order to study the influence of AQP4 on the proliferation of striatal glial cells we first wanted to demonstrate that we were able to either knock down the expression of AQP4 by means of siRNA gene silencing techniques (see Fig. 4) or to functionally block AQP4 with TEA (see Fig. 5).

The siRNA reduced AQP4 mRNA (Fig. 4A) as well as protein (Fig. 4B) by \(~85\%\) after 48 h. This down-regulation of AQP4 can also be visualized in striatal glia cell cultures (Fig. 4C). Under control culture conditions (left) a clear staining for AQP4 (red) was observed; this was strongly reduced in identical cultures treated 48 h with siRNA (right). Figure 4C also shows that under control conditions the number of cells in culture was much higher than the cell number in the culture treated with siRNA, although an identical number of cells was originally plated out. This indicated to us a possible link between proliferation and AQP4.

To suppress AQP4 function, i.e. reduce water permeance, we used TEA as a blocker as suggested by Detmers et al. (2006). The authors showed that TEA in concentrations \(< 20 \mu \)M effectively blocked AQP4 water permeability. The sensitivity of other ion channels, in particular of potassium channels (Kv1.1 and Kv3.1) and in the region of 1 mm for other ion channels. Therefore, possible effects of TEA on these ion channels can be neglected.

We therefore measured water permeability of striatal astrocytes in culture in the absence and presence of TEA in response to a change in osmolarity. Cells were loaded with calcein and exposed to hypoosmotic solution (200 mOsm), and the change in relative fluorescence intensity was monitored using confocal microscopy as described by...
Zelenina & Brismar (2000). They successfully demonstrated that measuring swelling- and shrinkage-related changes in fluorescence intensity of intracellularly trapped membrane-impermeant fluorescent dyes was a valid method of measuring changes in cell volume in heterogeneous cell cultures such as astrocytic cultures. Swelling experiments are shown in Fig. 5. The cells under control conditions lost fluorescence by ~20% when exposed to hypo-osmotic solution (200 mOsm). This increase in cell volume was completely reversible upon changing to iso-osmotic (300 mOsm) solution. In the presence of a hypotonic solution containing 10 μM TEA hardly any increase in cell volume could be measured (Fig. 5, left). After TEA washout the cells showed an increase in cell volume in response to a hypotonic stimulus compared to before TEA treatment. The right part of Fig. 5 shows swelling experiments using cells treated with siRNA for AQP4. Gene silencing of AQP4 revealed a reduction in cell swelling in response to a hypotonic challenge similar to pharmacologically blocking AQP4 with TEA (compare right side of Fig. 5). Taken together, treatment with 10 μM TEA or knocking down AQP4 with siRNA significantly lowered cell swelling in response to hypotonic solutions compared to control (P < 0.05).

As we were able to show that we could either knock down AQP4 expression (Fig. 4) or block AQP4 function (Fig. 5) we wanted to investigate the role of AQP4 in proliferation of striatal glial cells. Proliferation was stimulated by treatment of the cultures with bFGF, yielding an increase in proliferation of 150% on average (Fig. 6A). This increase was abolished in cultures in which AQP4 had been knocked down by means of siRNA techniques. In addition, blocking AQP4 with TEA (10 μM) resulted in a similar disappearance of the stimulative effect of bFGF on the proliferation.

Striatal glial cultures that had not been stimulated by bFGF but had been incubated with siRNA for AQP4 for 48 or 72 h revealed a decrease in the basal proliferation of ~40–50% of the control cultures (Fig. 6B). This reduction was similar to the reduction in proliferation observed under DA treatment (Fig. 1A). Treatment with 10 μM TEA also reduced basal proliferation (Fig. 6B).

To more directly show that reducing AQP4 levels could explain the decrease in proliferation induced by DA, we measured proliferation in cells treated with siRNA for AQP4 in the absence and presence of DA (Fig. 6B, right). The experiments show that decreasing AQP4 levels...
reduced proliferation to the same degree in the absence and presence of DA, indicating that reducing AQP4 levels prevented further effects of DA on proliferation.

Discussion

The results presented here show a reduction in proliferation of striatal glial cells \textit{in vitro} following application of DA, presumably via alpha(2)-adrenergic receptors. In addition, we demonstrated a down-regulation by DA of the expression of AQP4 in striatal glial cells \textit{in vitro}, also providing pharmacological evidence for an interaction of DA with the alpha(2)-adrenergic receptor. These findings are in agreement with Cornil \textit{et al.} (2002, 2008) who showed that DA can bind to alpha(2)-adrenergic receptors in different areas of the bird brain, mediating its effects via this receptor without being converted to NE. The reported binding of DA to alpha(2)-adrenergic receptors was with a 10- to 28-fold lower affinity than for NE. This is in line with our observation that the alpha(2)-adrenergic receptor-dependent reduction in the proliferation of striatal astrocytes was \~15\% with dopamine and 25\% with norepinephrine in cultures with high densities of cells.

![Figure 3: Effect of NE on the proliferation of striatal glial cells.](image1)

![Figure 4: Effect of AQP4 siRNA gene silencing after 48 h.](image2)
Effects of DA

Modulation of proliferation by DA has been described in various studies. However, findings are contradictory with respect to the cell type investigated: dopaminergic nigrostriatal projections up-regulated the proliferation of neural precursor cells in the adult subventricular zone of mice (Baker et al., 2004) and primates (Freundlieb et al., 2006), and dopaminergic innervation decreased proliferation of rat pituitary melanotrope cells during ontogeny. As several authors assume that neurodegenerative diseases are linked to changes in the proliferation of astrocytes, our observation of a down-regulation of proliferation of astrocytes by DA confirm and extend these assumptions at least for those neurodegenerative diseases correlated with perturbations of the dopaminergic transmission such as Parkinson’s disease. We hypothesize that under normal physiological conditions the dopaminergic nigrostriatal projections might tonically inhibit the proliferation of striatal astrocytes.

Fig. 5. Astrocyte swelling induced by hypotonic solutions. Cells (n = 6) were loaded with calcein and the change in fluorescence at different times of a single cell was measured upon application and washout of a hypotonic solution (200 mOsm). Left, average swelling of three cells before (○), after (●) and during (△) application of 10 μM TEA in the hypotonic solution; right, average swelling in control cells (●) and in three cells treated with siRNA for AQP4 (○). During hypotonic conditions: C vs. TEA, P < 0.05; C vs. siRNA, P < 0.05. The apparent delay from the solution exchange to the onset of the fluorescence intensity change is due to the dead volume of the perfusion system and corresponds to ~100 s.

Fig. 6. Effect of AQP4 siRNA gene silencing on basal and induced proliferation of striatal cells. (A) Cells were treated for 72 h with bFGF alone or in combination with either 5 nM siRNA or 10 μM TEA. (B) Basal proliferation was determined in striatal glial cultures that were treated with 1 nM siRNA or 5 nM siRNA specific for AQP4 or with 10 μM TEA over periods of 48 or 72 h, and with 5 nM siRNA in combination with 100 μM DA for 72 h. *P < 0.05; **P < 0.01; ***P < 0.005. Solid bars, 5000 cells per well; hatched and empty bars, 10 000 cells per well.
In brain development a tonic influence of DA on the cell cycle has been reported (Ohtani et al., 2003). D1-like receptor activation specifically reduces G1- to S-phase entry in neuroepithelial cells during ontogeny. Given these cell cycle-arresting effects of DA one might speculate that in Parkinson’s disease the loss of dopaminergic projections might lead to an increase in proliferation of astrocytes due to the abolition of the tonic inhibition by DA. This hypothesis needs to be corroborated by in vivo lesion studies where proliferation of astrocytes and cell cycle in the striatum are studied after 6-OH-DA treatment. In addition, the expression of AQP4 in the lesioned striatum needs to be investigated, especially as Vizuete et al. (1999) reported in the substantia nigra an increase in AQP4 mRNA following 6-OH-DA lesion.

We also demonstrated in the present study a down-regulation of the mRNA of the M23 isoform of AQP4 by DA. Such a differential regulation of the M23 isoform expression has also been shown by other investigators (Zelenin et al., 2000) during development. The function of the isoforms M1 and M23 or M23x is not fully understood yet and the situation is getting even more complicated, as four new isoforms (AQP4b, AQP4d, AQP4e, AQP4f) have been described by Moe et al. (2008) recently. Interestingly, some of these isoforms do not transport water and their function remains speculative. According to the previous state of knowledge AQP4 water channels exist as heterotetramers of M1 and M23 splice variants that form orthogonal arrays of particles (OAPs) within the membrane. Different cellular expression levels of the M1 and M23 isoforms could regulate the organization of these OAPs and by this the amount of the water flux through these channels (Silverstein et al., 2004). This again might have effects on proliferation.

DA affected the proliferation of astrocytes in a cell-density-dependent fashion: the higher the cell density the smaller the effect of DA. This is in accordance with the well-known fact that in vitro astrocytes proliferate as long as they grow in low densities. When they reach confluency, they stop proliferating due to contact inhibition. Therefore, in our experiments, the cultures with the initially highest cell numbers revealed only moderate effects of DA on the cell number and the proliferation.

**Influence of AQP4 on the proliferation of striatal glial cells**

As we demonstrated that DA reduced proliferation of striatal astrocytes and the expression of AQP4 in these cells, we hypothesize that the water channel AQP4 might play a role in the regulation of proliferation of striatal astrocytes in vitro.

AQP4s represent a family of transmembrane water channel proteins that play a major role in transcellular and transepithelial water movement. Recent studies have identified some new and unanticipated roles of AQP4s, such as the regulation of migration, proliferation and glycerol metabolism in different cell types (Saadoun et al., 2005; Hu & Verkman, 2006; Levin & Verkman, 2006; Auguste et al., 2007; McCoy & Sontheimer, 2007; Papadopoulos et al., 2008). Expression of AQP4 in a variety of human tumors have been reported and there is growing evidence that AQP1 facilitates the proliferation of some tumor cells (Hoque et al., 2006). Expression of AQP3 in a variety of cell types has also been linked to proliferation, for example corneal re-epithelialization after injury (Levin & Verkman, 2006) and epithelial proliferation during wound healing (Hara-Chikuma & Verkman, 2008), and the proliferation of enterocytes in the gastrointestinal tract depends on functional AQP3 expression (Thiagarajah et al., 2007). Although involvement of AQP4 in cell migration has been demonstrated unequivocally, findings about the role of this water channel in proliferation are few and contradictory. Whereas Saadoun et al. (2005) reported no change in the proliferation of astrocytes cultured from transgenic mice lacking AQP4, Nicchia et al. (2003) found a nearly 70% reduction in the cell number of cultivated astrocytes after siRNA treatment with RNA duplexes specific for AQP4. Only the latter is in accordance with our findings, presumably due to the use of the identical method leading to similar results.

As we were primarily interested in the effects of AQP4 on proliferation and not vice versa we have not shown in our studies that an increase in proliferation was accompanied with an increase in AQP4 expression. Reports from the literature, however, point towards such a relationship: highly proliferating astrocytes, i.e. gliomas (Warth et al., 2004, 2007; McCoy & Sontheimer, 2007), have increased expression of AQP4.

**Conclusion**

Our results indicate a clear involvement of AQP4 in the regulation of proliferation of striatal astrocytes. These findings imply that modulation of AQP4 could be used therapeutically in the treatment of neurodegenerative diseases, in stem cell therapy and in the regulation of reactive astrogliosis by preventing or reducing glial scar formation thus improving regeneration following ischemia or other trauma.

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**Abbreviations**

AQP, aquaporin; AQP4, aquaporin 4; bFGF, basic fibroblast growth factor; CNS, central nervous system; DA, dopamine; FCS, fetal calf serum; HPRT, hypoxanthine phosphoribosyl-transferase; NE, norepinephrine; TEA, tetraethylammonium.

**References**


