Involvement of aquaporin-4 in astroglial cell migration and glial scar formation

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Summary

Aquaporin-4, the major water-selective channel in astroglia throughout the central nervous system, facilitates water movement into and out of the brain. Here, we identify a novel role for aquaporin-4 in astroglial cell migration, as occurs during glial scar formation. Astroglia cultured from the neocortex of aquaporin-4-null mice had similar morphology, proliferation and adhesion, but markedly impaired migration determined by Transwell migration efficiency (18±2 vs 58±4% of cells migrated towards 10% serum in 8 hours; $P<0.001$) and wound healing rate (4.6 vs 7.0 μm/hour speed of wound edge; $P<0.001$) compared with wild-type mice. Transwell migration was similarly impaired (25±4% migrated cells) in wild-type astroglia after ~90% reduction in aquaporin-4 protein expression by RNA inhibition. Aquaporin-4 was polarized to the leading edge of the plasma membrane in migrating wild-type astroglia, where rapid shape changes were seen by video microscopy. Astroglial cell migration was enhanced by a small extracellular osmotic gradient, suggesting that aquaporin-4 facilitates water influx across the leading edge of a migrating cell. In an in vivo model of reactive gliosis and astroglial cell migration produced by cortical stab injury, glial scar formation was remarkably impaired in aquaporin-4-null mice, with reduced migration of reactive astroglia towards the site of injury. Our findings provide evidence for the involvement of aquaporin-4 in astroglial cell migration, which occurs during glial scar formation in brain injury, stroke, tumor and focal abscess.

Key words: AQP4, Astrocyte, Chemotaxis, Reactive gliosis, Water channel

Introduction

The central nervous system (CNS) in mammals (Pekny and Nilsson, 2005), amphibia (Wujek and Reier, 1984), birds (Lurie and Rubel, 1994), reptiles (Romero-Aleman et al., 2004), fish (Nona et al., 1992) and insects (Smith et al., 1987) responds to trauma by producing a scar, which is primarily comprised of reactive astroglia. In mammals, the glial scar has beneficial as well as harmful properties: it allows prompt repair of the blood-brain barrier, inhibits the entry of inflammatory cells into damaged brain, and limits neuronal death (Bush et al., 1999; Faulkner et al., 2004), but also impairs axonal regeneration (McGraw et al., 2001) and incorporation of neuronal grafts (Kinouchi et al., 2003). Following focal CNS injury, a subset of astroglia or astroglial precursors throughout the brain undergo mitosis and morphological changes to yield reactive astroglia, which migrate towards the lesion and organize into a densely packed glial scar (Hampton et al., 2004; Rhodes et al., 2003; Wang et al., 2004; Zhou et al., 1986).

Reactive astroglia overexpress several proteins, including glial fibrillary acidic protein (GFAP), which is often used to identify them (Eng et al., 2000), and the water channel protein aquaporin-4 (AQP4) (Saadoun et al., 2002; Saadoun et al., 2003; Vizuete et al., 1999). AQP4, the main water channel of mammalian brain, is normally expressed in astroglia at the border between brain parenchyma and major fluid compartments (cerebrospinal fluid, blood). Phenotype data from AQP4-knockout mice have indicated that AQP4 facilitates water fluxes into and out of the brain parenchyma, and is involved in the pathophysiology of brain edema in stroke, water intoxication, brain tumor, focal cortical freeze injury, acute bacterial meningitis and brain abscess (Bloch et al., 2005; Manley et al., 2000; Papadopoulos et al., 2004; Papadopoulos and Verkman, 2005; Saadoun et al., 2002; Saadoun et al., 2003).

Here, we provide evidence for a new role of AQP4 in astroglia that is unrelated to its role in brain edema. This work was motivated by our recent finding that a different water-selective transporter, AQP1, facilitates tumor angiogenesis by accelerating endothelial cell migration (Saadoun et al., 2005). AQP4 knockout or knockdown in astroglial cell cultures was found to greatly impair their migration, probably by reducing membrane water fluxes that occur during cell migration. Experimental evidence for an in vivo role of AQP4-dependent astroglial cell migration was obtained from analysis of glial scar formation after stab injury. We propose that the AQP4

upregulation found in reactive astroglia after a variety of insults is an adaptive response that facilitates astroglial cell migration and glial scar formation. Our results also suggest the possibility of modulation of AQP4 expression/function as a novel strategy to enhance or disrupt glial scar formation for the treatment of a variety of CNS pathologies.

Materials and Methods

AQP4-null mice

AQP4-null mice were generated by targeted gene disruption (Ma et al., 1997). Weight-matched male mice in CD1 and C57BL/6 genetic backgrounds were used. Investigators were blinded to genotype information in all experiments. Protocols were approved by the University of California San Francisco Committee on Animal Research.

Astroglial cultures

Astroglia were cultured from neocortex of wild-type and AQP4-null neonatal mice as previously described (Solenov et al., 2004). Briefly, the cerebral hemispheres were isolated, minced with forceps and incubated in minimal essential medium (MEM) plus 0.25% trypsin and 0.01% DNase. Dissociated cells were centrifuged, resuspended in MEM containing 10% fetal bovine serum (FBS), seeded on poly-L-lysine-coated flasks and grown at 37°C in a 5% CO_2 incubator with a change of medium twice a week. At confluence (days 12-15), cultures were treated with 10 μM cytosine arabinoside for 48 hours to prevent proliferation of other cell types, and the medium was replaced with MEM containing 3% FBS and 0.15 mM dibutyryl cAMP to induce differentiation. Cultures were maintained for up to two more weeks with a change of medium twice a week.

RNA inhibition (RNAi) experiments

Custom SMART pool® RNAi duplexes (more than four sequences) for AQP4 and AQP9 were chemically synthesized by Dharmacon Research (Lafayette, CO). After 10 days in culture, primary astroglia were shaken at 200 rpm for 18 hours at 37°C to remove microglia. Astroglia were recovered in normal medium for 2 days and replated in six-well or 12-well plates. Double-stranded RNAs for RNAi (100 nM, AQP4 or AQP9) were mixed with Oligofectamine (Invitrogen, Carlsbad, CA), diluted 1:50 in Opti-MEM (Invitrogen), and incubated for 20 minutes at room temperature for complex formation. The mixture was added to the cells and AQP4 protein expression was determined by immunoblotting after 6 days.

Osmotic water permeability

Cell membrane water permeability was measured by a calcein-quenching method as described previously (Solenov et al., 2004). The time course of cytoplasmic calcein fluorescence was measured in response to cell swelling produced by the twofold dilution of the extracellular bathing solution with water.

Immunocytochemistry

Cultured astroglia were fixed in 10% formalin and incubated with 1:200 rabbit anti-AQP4 or 1:1000 rabbit anti-GFAP antibody (Chemicon, Temecula, CA), followed by FITC- or Texas-Red-conjugated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) at 1:80. Nuclei were counterstained blue with DAPI. In some experiments, the plasma membrane was stained with Alexa-Fluor-linked wheatgerm agglutinin (WGA, Molecular Probes, Carlsbad, CA).

Immunohistochemistry

Mice were anaesthetized using 2,2,2-tribromoethanol and perfusion-fixed with 5 ml of 10% formalin injected i.v. Brains were removed, fixed for another 24 hours in 10% formalin, and processed in paraffin. Coronal sections (7 μm) were cut, deparaffinized, rehydrated and immunolabeled using 1:200 rabbit anti-AQP4 or 1:1000 rabbit anti-GFAP antibody followed by anti-rabbit HRP-linked secondary antibody (1:1000). Immunolabeling was visualized in brown using DAB/H_2O_2. Distances of reactive astroglia from the wound edge were estimated using Image J (NIH freeware).

Western blotting

Astroglial cell cultures were trypsinized and plasma membrane proteins were extracted (Yang and Verkman, 1997), separated on a 4-12% SDS-PAGE minigel (3 μg protein/lane) and transferred onto a PVDF membrane. The membrane was exposed to rabbit anti-AQP4 antibody (1:1000) followed by HRP-linked anti-rabbit IgG antibody (1:1500) and visualized using enhanced chemiluminescence. AQP4 protein was quantified by scanning densitometry using as standards serial dilutions of protein from wild-type astrocytes.

Adhesion and proliferation assays

Confluent cultured astroglia were trypsinized and suspended in MEM with 3% FBS. After measurement of cell density using a hemocytometer, 2.8×10^4 cells per cm^2 were plated in 24-well plates coated with poly-L-lysine. The medium was exchanged 4 hours after plating. Adhesion was defined as the percentage of plated cells remaining immediately after medium exchange. To assess proliferation, the number of cells per well was determined every 2 days by trypsinization and cell counting.

In vitro migration

Migration was assayed using a modified Boyden chamber (Corning Costar, Fisher Scientific, Pittsburgh, PA) containing a polycarbonate membrane filter (6.5 mm diameter, 8 μm pore size) coated with poly-L-lysine (Saadoun et al., 2005). The upper chamber contained cells in DMEM plus 1% FBS, and the lower chamber contained DMEM plus 10% FBS (chemoattractant) or 1% FBS (control). Cells were incubated for 10 hours at 37°C in 5% CO_2/95% air. Non-migrated cells were scraped off the upper surface of the membrane with a cotton swab. Migrated cells remaining on the bottom surface were counted after staining with Coomassie Blue.

In some experiments, the assay was performed in the presence of an osmotic gradient produced by addition of raffinose to the top or bottom chambers. Because pilot experiments showed that the osmotic gradient dissipates by 50% in 2 hours (not shown), the medium in both chambers was exchanged hourly to restore the initial osmotic gradient.

In vitro wound healing

Cells were cultured as confluent monolayers, synchronized in 1% fetal bovine serum for 24 hours, and wounded by removing a ~1 mm strip of cells across the well with a standard 200 μl pipette tip (Saadoun et al., 2005). The wounded monolayers were washed twice to remove non-adherent cells. Phase-contrast light micrographs were taken immediately after cell removal, and at 24 and 48 hours for analysis using Image J. Wound healing was quantified as the average linear speed of the wound edges over 48 hours. Phase-contrast micrographs of the wound edges were also taken 6 hours post-wounding and the leading edge of individual migrating astroglia was outlined. Irregularity (ruffling) of the leading edge was quantified as the fractal dimension of the outline, calculated using the 'box-counting method' (Image J) as previously described (Smith et al., 1996). The fractal

dimension is a number between 1 and 2, which increases with the extent of ruffling.

In vivo stab

Anesthetized mice were mounted on a stereotactic frame. After incising the skin, a 5-mm-long stab was performed through the skull in the sagittal plane, 1 mm lateral to the sagittal suture, 2 mm deep from the cerebral cortex. The blade was removed and the skin was sutured closed.

Statistics

Results were analyzed using the two-tailed Student's *t*-test, Student-Newman-Keuls test or linear regression.

Results

Characterization of cultured astroglia

Confluent astroglial cells cultured from wild-type and AQP4-null mice had similar appearance by phase-contrast microscopy and similar expression of the astrocytic marker GFAP (Fig. 1A, left and middle panels). More than 95% of cells stained for GFAP, and >90% of wild-type astroglia expressed AQP4 protein with no detectable AQP4 expression in the AQP4-null astroglia (right panels). In confluent astroglia, GFAP was located in intracellular fibers, but AQP4 was diffusely expressed in the plasma membrane, as found previously (Nicchia et al., 2000; Solenov et al., 2004). AQP4 expression appears diffuse because the plasma membranes of cultured astroglia overlap considerably. Consistent with their lack of AQP4 expression, the AQP4-null astroglia have a sevenfold-reduced osmotic water permeability compared with wild-type astroglia (Solenov et al., 2004). The cultured AQP4-null and wild-type astroglia had comparable growth and proliferation as assessed by serial cell counting (Fig. 1B), and adherence as assessed by adhesion to poly-L-lysine (Fig. 1C).

Astroglial cell migration in vitro

Cell migration was measured using a modified Boyden Transwell™ assay. The fraction of cells that migrated across 8 μm diameter pores over 10 hours was determined by counting stained cells on Transwell filters before and after scraping off non-migrated cells on the upper filter surface (Fig. 2A, left). There was remarkably impaired migration of AQP4-null compared with wild-type astroglia (18±2% vs 58±4% migrated cells after 8 hours, $P<0.001$) towards 10% FBS (Fig. 2A, right). Differences in cell size cannot account for the differences in migration because wild-type and AQP4-null astroglia have similar sizes (respective cell surface areas, 1261±333 vs 1291±316 μm^2, $P=0.9$; area of cell projections, 861±219 vs 803±184 μm^2, $P=0.8$; mean±s.e.m.) as measured using Image

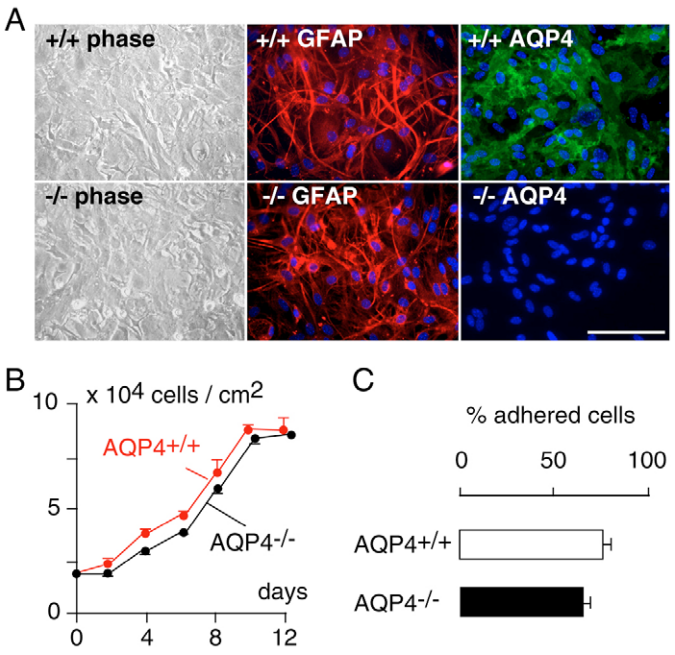

Fig. 1. Characterization of cultured astroglia. (A) Confluent wild type (+/+) and AQP4-null (–/–) astroglial cell cultures showing similar morphology by phase-contrast microscopy (left panel) and similar GFAP expression (in red, middle panel). Right panel shows AQP4 immunofluorescence (green). Nuclei are counterstained blue using DAPI. (B) Proliferation of wild-type ($AQP4^{+/+}$) and AQP4-null ($AQP4^{-/-}$) astroglia. Cells were counted (eight wells per time point) on alternate days after plating 2.8×10^4 astroglia/cm^2. (C) Percentage of astroglia adhering to poly-L-lysine at 4 hours after plating (ten wells per genotype). B and C show mean±s.e.m.; differences were not significant. Bar, 200 μm.

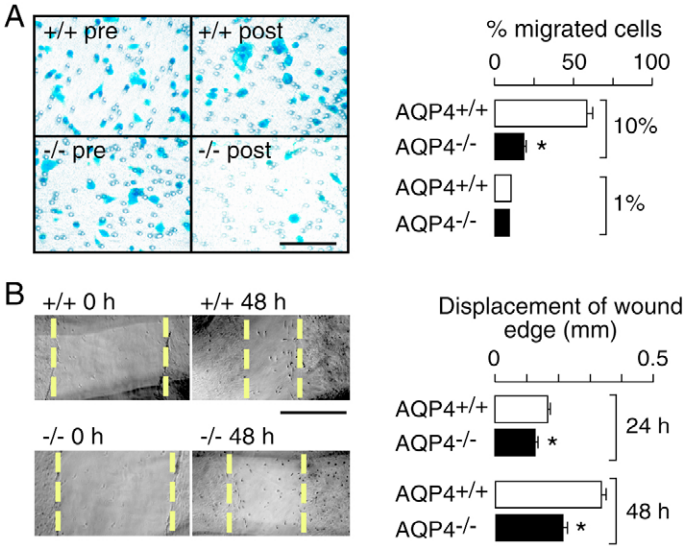

Fig. 2. Slowed migration of AQP4-deficient astroglia in vitro. (A) Representative images (left) of Transwell migration assay using 10% FBS as chemoattractant, showing wild-type astroglia (+/+) and AQP4-null astroglia (–/–) before (pre) and after (post) scraping the non-migrated cells. Cells were stained with Coomassie Blue. Data summary (right) of migration experiments towards 10% FBS (15 wild-type vs. 13 AQP4-null Transwells; *$P<0.001$) or 1% FBS (1 wild-type vs. 1 AQP4-null Transwell). (B) Phase-contrast micrographs (left) showing astroglial cultures immediately after (0 h) and 48 hours after scratch. Dashed yellow lines indicate wound edges. Summary (right) of astroglial migration data at 24 and 48 hours after scratch (24 wild-type vs. 24 AQP4-null astroglial cultures per time point). Data are mean±s.e.m. Differences are significant at 24 hours (*$P<0.01$) and 48 hours (*$P<0.001$). Bar, 100 μm (A); 400 μm (B).

J from light micrographs of poly-L-lysine-adhered astroglial cells. Background Transwell migration (in the absence of a serum gradient) was 9-10%; with no difference between wild-type and AQP4-null astroglia.

As a separate assessment of astroglial cell migration, a coverslip wound-healing assay was done in which the rate of wound closure (initial width ~1 mm) in confluent cell monolayers was measured. The wounds healed linearly over 48 hours for wild-type and AQP4-null astroglia (r^2>0.98), but the speed of the wound edge, as deduced from the displacement data in Fig. 2B, was significantly slowed in the AQP4-null cultures (7.0 vs 4.6 mm/hour; P<0.001).

RNAi studies were done to confirm that the reduced migration in AQP4-null astroglia is the primary effect of AQP4 deficiency. Treatment conditions (concentration and time) were established to strongly reduce AQP4 protein expression without toxic effects. Under these conditions AQP4 RNAi treatment did not produce a noticeable effect on cell morphology (Fig. 3A), but reduced AQP4 protein expression by ~94% in wild-type astroglia (Fig. 3B). AQP4 protein expression was not significantly reduced by treatment with AQP9 RNAi or oligofectamine alone. Plasma membrane osmotic water permeability was measured by a calcein fluorescence-quenching method. As reported previously (Solenov et al., 2004), water permeability was much lower in AQP4-deficient cells (Fig. 3C). Treatment with RNAi (under the same conditions as in immunoblotting) reduced water permeability substantially in the wild-type astrocytes, but had no effect on water permeability in the AQP4-deficient astrocytes.

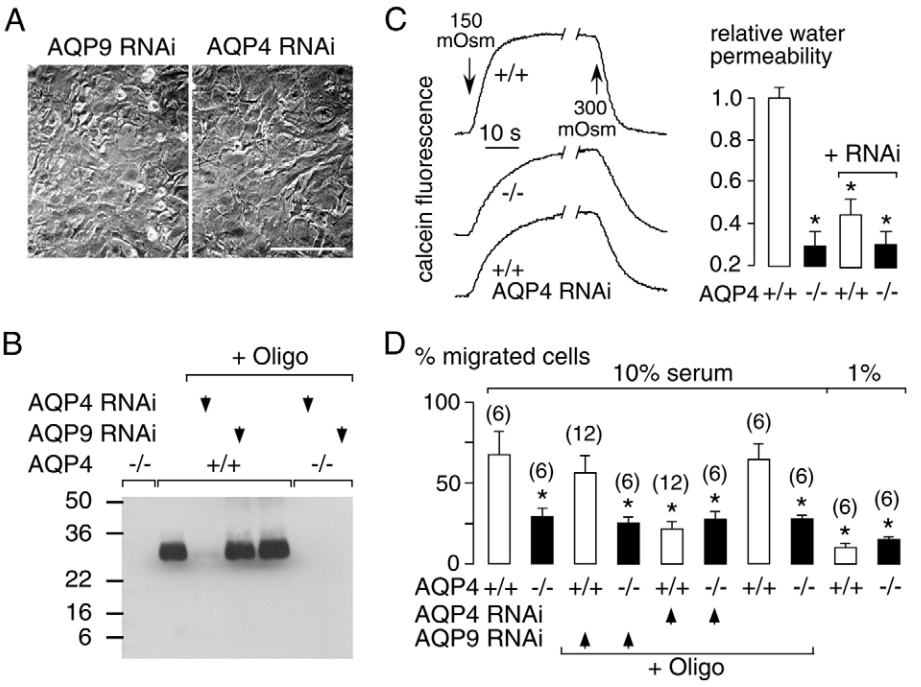

Fig. 3. Reduced AQP4 expression, water permeability and migration in AQP4 RNAi-treated wild-type astroglia. (A) Phase-contrast micrographs of wild-type astroglial cell cultures after exposure to AQP9 (control) RNAi (left) or AQP4 RNAi (right). (B) Immunoblot of AQP4 expression in wild-type astroglia (+/+) and AQP4-null astroglia (–/–) without pre-treatment (blank), or with pre-treatment (arrow) with AQP4 RNAi, AQP9 RNAi or oligofectamine (+ Oligo). Positions of molecular size markers in kDa are indicated. (C) Osmotic water permeability of astroglial cells with and without AQP4 RNAi treatment. Changes in calcein fluorescence signal (left) in response to hypo-osmolar medium (150 mOsm) and return to iso-osmolarity (300 mOsm). Summary (right) of relative rates of water transport (mean±s.e.m.; n=3-4 cultures/condition; *P<0.01). (D) Summary of Transwell migration data for 1% or 10% serum, labeled as in B. Data are the mean±s.e.m. The number of Transwells used for migration is shown over each data bar in parentheses. *P<0.05 compared with migration of untreated wild-type astroglial cells. Bar, 200 μm.

The cell migration data from Transwell assays are summarized in Fig. 3D. AQP4 knockdown using RNAi significantly impaired astroglial cell migration to approximately that of AQP4-null cells, whereas treatment with AQP9 siRNA under the same conditions did not impair migration. RNAi treatment did not influence the migration of AQP4-null astroglia, or the background migration (toward 1% FBS) of AQP4-RNAi compared with AQP9-RNAi-treated astroglia.

Mechanistic studies

Membrane proteins that facilitate cell migration are sometimes polarized to the leading edge of the plasma membrane (Huttenlocher, 2005; Klein et al., 2000). AQP4 has the potential to polarize because in normal brain AQP4 is expressed primarily in astroglial foot processes (Nielsen et al., 1997). We investigated whether AQP4 polarizes in migrating astroglial cells and found increased AQP4 expression at the leading edge of migrating astroglia (Fig. 4A). The increased green AQP4 immunostaining at the leading edge was not due to folding of the plasma membrane evidenced by the lack of increased staining of the leading edge with the plasma membrane marker wheatgerm agglutinin (WGA).

Cell migration is associated with marked changes in cell shape, including the rapid formation and retraction of plasma membrane protrusions such as lamellipodia and filopodia (Small et al., 2002; Svitkina and Borisy, 1999). Differences in water fluxes at the front end of migrating wild-type and AQP4-null astroglial cells are expected to influence the shape of the leading edge. We therefore compared the shape of migrating wild-type and AQP4-null astroglial cells, by computing the fractal dimension of the leading edge (Fig. 4B). The fractal dimension was significantly (P<0.005) higher in the wild type (1.203±0.017, mean±s.e.m.; n=12) than in AQP4-null cells (1.133±0.008; n=12), indicating more prominent plasma membrane protrusions in the front end of the wild-type cells.

We hypothesized that AQP4-facilitated astroglial cell migration probably involves increased plasma membrane osmotic water permeability, which enhances water transport into the cell at its leading edge (where membrane protrusions

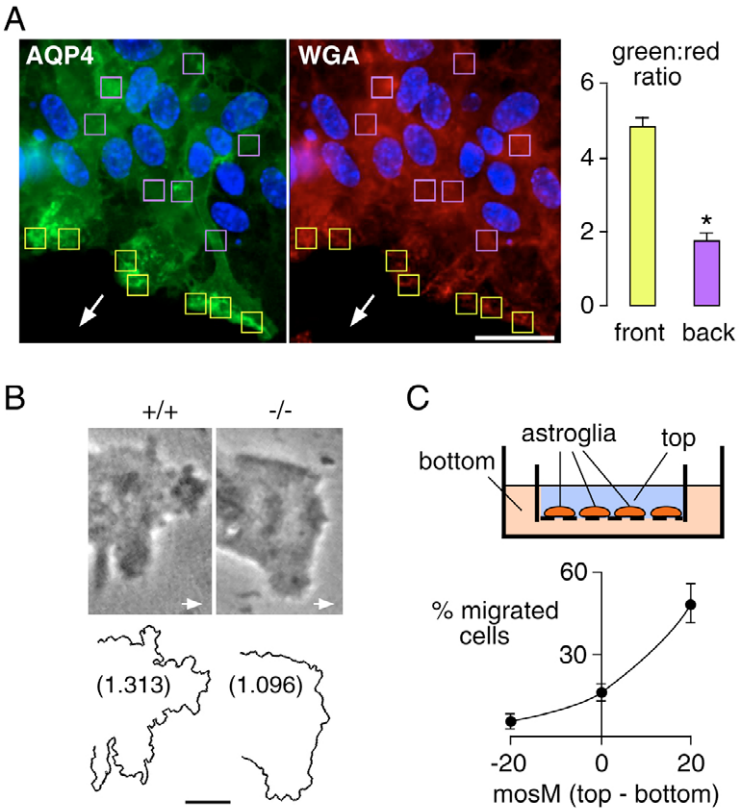

Fig. 4. Evidence for AQP4-faciliated water transport at the leading edge of migrating cells. (A) Cultured astroglia at the edge of a wound (left) immunostained for AQP4 (green) and the plasma membrane marker WGA (red), with nuclei counterstained with DAPI. Arrow indicates direction of migration. Green:red fluorescence ratios (right) are shown for each of the corresponding pairs of squares shown on the left. Squares were placed at the leading edge (yellow, front) and away from the wound (purple, back). (B) Phase-contrast micrographs (top) and outline (bottom) of the leading end of a migrating wild-type and AQP4-null astroglial cell. Arrows indicate direction of migration. (Numbers are fractal dimensions.) (C) Experimental set-up (top) used for studying the effect of extracellular osmotic gradient on cell migration. Percentage of astroglia that migrated to the bottom surface of the Transwell within 10 hours (bottom), as a function of the difference in osmolality (top chamber minus bottom chamber). Data are mean±s.e.m.; *P<0.005 front vs back. Bar, 25 μm (A); 10 μm (B).

form). To test this hypothesis, we carried out the Transwell assay in wild-type astroglia, as in Fig. 2A, except that raffinose (20 mM) was added to the upper or lower chambers to create small osmotic gradients from cell back-to-front or front-to-back, resulting in osmotic water transport into or out of the front of the cell, respectively. Fig. 4C shows accelerated cell migration when water flow was induced from cell front-to-back (opposite to the direction of migration), supporting the conclusion that migration involves water movement into the cell at its front edge.

Reactive gliosis in vivo

To determine whether the AQP4-dependent astroglial cell migration in culture models might occur in the brain in vivo, we studied reactive gliosis and glial scar formation using a well-established cortical-stab-injury model (Bush et al., 1999; Hampton et al., 2004; Rhodes et al., 2003; Wang et al., 2004). The stab-injury mouse model, unlike other brain-injury models (tumor, infection, trauma), is not associated with significant brain swelling, which could confound interpretation of results. Intracranial pressure was normal in two wild-type and two AQP4-null mice, measured at day 1 and day 3 after the stab injury.

In the in vivo studies, reactive astroglia were identified by their multiple processes and strong GFAP immunoreactivity (Fig. 5A). Three days after the stab injury, reactive astroglia were detected throughout the brain, including the contralateral hemisphere, in both wild-type and AQP4-null mice; however, reactive astroglia were notably absent from the region immediately adjacent to the stab (Fig. 5B). At day 7 following the stab injury, the front of reactive gliosis reached the edge of the stab wound in all mice (three wild-type and three AQP4-null mice) (Fig. 5C). We compared the speed of migration of reactive astroglia in wild-type and AQP4-null mice by measuring the average distance of the gliotic front from the edge of the stab wound at day 3. The representative GFAP-immunolabeled sections of mouse brain (Fig. 5D,E) and the summarized data (Fig. 5F) show that at day 3 the margin of the

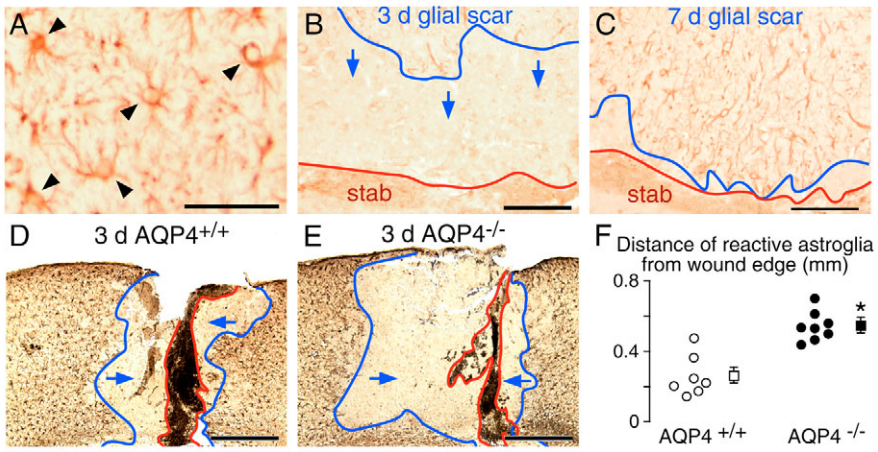

Fig. 5. Astroglial cell migration after stab injury in vivo. (A) Reactive astroglia (arrowheads) stain strongly for GFAP (brown) and have multiple processes. (B-C) Stab injury produces a front of reactive astroglia (blue line) that migrates towards (arrows) the margin of injured brain (red line). Representative data for wild-type mice at 3 days (B) and 7 days (C) after stab injury. (D-E) Astroglial front (blue line) and margin of stab injury (red line) in wild-type (D) and AQP4-null (E) mouse at 3 days. Arrows indicate direction of migration of the reactive astroglial front. (F) Average distance of reactive astroglial front from the edge of the stab wound at 3 days after stab injury. Data are mean±s.e.m. *P<0.001. Bar, 50 μm (A); 100 μm (B-C); 400 μm (D-E).

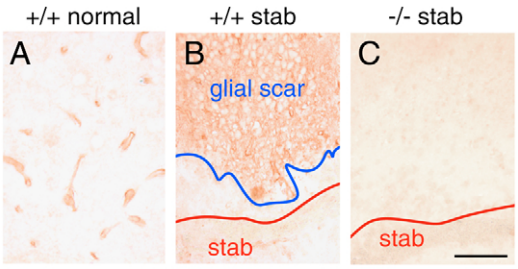

Fig. 6. AQP4 immunostaining of astroglia in vivo. (A) Control (uninjured) wild-type (+/+) mouse brain parenchyma, showing AQP4 expression in pericapillary astroglial foot processes. (B) Stab-injured wild-type mouse brain showing strong AQP4 immunoreactivity in the astroglial scar. (C) Absence of AQP4 expression in stab-injured AQP4-null (–/–) mouse brain. The blue line demarcates the edge of the glial scar; red lines indicate the margin of injured brain. Each micrograph is representative of three mice. Bar, 100 μm.

glial scar was significantly ($P<0.001$) farther from the wound edge in the AQP4-null mice. These findings are consistent with a slower migration of AQP4-null reactive astroglia towards the stab lesion.

Because several studies have reported that insults to the brain increase AQP4 expression in astroglia (Bloch et al., 2005; Papadopoulos and Verkman, 2005; Saadoun et al., 2002; Saadoun et al., 2003; Vizuete et al., 1999), AQP4 protein expression was studied in stab-injured brains, reasoning that reactive astroglia may upregulate AQP4 expression to facilitate their migration towards the site of injury. In brain parenchyma of normal wild-type mice, AQP4 protein was largely restricted to pericapillary astroglial foot processes (Fig. 6A) as reported previously (Nielsen et al., 1997). However, in stab-injured wild-type mouse brain, AQP4 protein expression was strongly upregulated within the glial scar (Fig. 6B). No AQP4 protein was detected in the brains of AQP4-null mice (Fig. 6C).

Discussion

Migration of reactive astroglia is an important component of glial scar formation around damaged brain. In vitro data (obtained using two different assays) and in vivo experiments provided evidence for AQP4-facilitated migration of reactive astroglia towards the site of injury. However, AQP4 deletion did not affect other astroglial functions, such as adhesion or proliferation. These findings extend our recent discovery that AQP1 deletion reduces the migration speed of mouse aortic endothelial cells (in vitro and in vivo), and that transfection with functional AQP1 or AQP4 accelerates the migration of cultured Chinese hamster ovary fibroblasts and Fisher rat thyroid epithelial cells (Saadoun et al., 2005). AQP1 was also reported recently to be involved in the formation of plasma membrane protrusions in cholangiocytes (Chen et al., 2005). Taken together, these observations suggest that the ability of water channels to accelerate cell migration may be a general phenomenon in mammalian cells. In astroglial cells, AQP4 may also be important in other processes that involve cell migration, such as the infiltration of astroglial tumor cells into surrounding brain. This might explain the strong upregulation of AQP4 in malignant human astroglia in vivo (Saadoun et al., 2002; Saadoun et al., 2003; Vizuete et al., 1999).

There are at present no selective AQP4 channel blockers for study of AQP4 function. Therefore, we used AQP4-null cells and RNA inhibition to investigate the role of AQP4 in astroglial cell migration. The AQP4 (but not the AQP9) siRNA treatment as used here strongly inhibited AQP4 protein expression and reduced plasma membrane water permeability, without altering cell morphology. This finding contradicts a previous report that AQP4 inhibition with siRNA-altered astroglial cell morphology and growth (Nicchia et al., 2003). In the earlier study, the upregulation of stress genes in all siRNA-treated cells, including the controls, suggested that the astroglia were damaged under the experimental conditions. Our siRNA data support the conclusion that the reduced migration of astroglia cultured from AQP4-null mice was not due to compensatory changes in these cells. The fact that a structurally different aquaporin (AQP1) can also accelerate cell migration (Saadoun et al., 2005) suggests that the water-transporting function of aquaporins is important in cell migration.

The importance of water fluxes across the plasma membrane in causing localized swelling of lamellipodia has been discussed extensively in the early literature on cell migration (Condeelis et al., 1990; Oster and Perelson, 1987). However, the idea that these water fluxes are primarily mediated by aquaporins is new (Saadoun et al., 2005). Theoretical calculations show that transmembrane water fluxes induced by small extracellular osmotic gradients can generate enough force to propel vesicles (Anderson, 1983; Zinemanas and Nir, 1995) and even cells (Jaeger et al., 1999) through extracellular solutions. During this process, termed osmophoresis, the vesicles/cells move towards hypo-osmolality as water enters into the front end and leaves from the rear end of the cell. To determine whether water fluxes across the plasma membrane play a role in astroglial cell migration, we altered the osmolalities of both chambers in the Transwell assay (Fig. 4C). This maneuver produces a steep osmotic gradient across the pore length (10 μm), which is maintained by hourly exchange of media in both chambers. During migration through the pore, the front end of astroglia becomes exposed to the osmolality of the bottom chamber while the rear end is exposed to the osmolality of the top chamber. In agreement with previous findings (Anderson, 1983; Jaeger et al., 1999; Zinemanas and Nir, 1995), our experiments showed that the speed of astroglial cell migration can be altered by a small osmotic gradient in the extracellular medium, resulting in accelerated astroglia migration towards hypo-osmolality.

We suggest that AQP4 accelerates astroglial cell migration by increasing plasma membrane water permeability, which in turn increases the transmembrane water fluxes that take place during cell movement. This hypothesis could explain the observation (Fig. 4) that AQP4 deletion slows the rapid changes in cell shape that take place at the leading end of migrating astroglia. It has been suggested that the generation of osmoles produced by rapid actin depolymerization drives the entry of water into the leading end of migrating cells (Oster and Perelson, 1987), possibly in concert with transmembrane ionic movements (Huttenlocher, 2005; Klein et al., 2000). This osmotic influx of water across the plasma membrane expands

the leading end of the cell, which is followed by actin polymerization to stabilize the membrane protrusion (Oster and Perelson, 1987).

Unlike normal brain, where AQP4 expression is polarized to astroglial foot processes and the basolateral plasma membranes of ependymal cells (Nielsen et al., 1997), in confluent cultured astroglia AQP4 is expressed throughout the plasma membrane (Fig. 1). An interesting observation is that in migrating cultured astroglia, AQP4 polarizes to the leading edge of the plasma membrane (Fig. 4), where rapid transmembrane water movement occurs. This is consistent with reports that several ion channels and ion transporters, which play a role in cell migration, also polarize to the front end of migrating cells (Huttenlocher, 2005; Klein et al., 2000). These ion movements might contribute to the osmotic gradient that drives water influx during cell movement. The molecular mechanisms responsible for AQP4 polarization in migrating astroglia are not known, but might involve interactions between AQP4, the α-syntrophin complex and the actin cytoskeleton.

In agreement with prior reports (Bloch et al., 2005; Papadopoulos and Verkman, 2005; Saadoun et al., 2002; Saadoun et al., 2003; Vizuete et al., 1999), we found upregulation of AQP4 within the glial scar in response to brain injury in vivo (Fig. 6). Increased AQP4 expression, in addition to its polarization to the leading edge, may further augment the contribution of AQP4 to cell migration. The glial scar, where AQP4 expression is increased, consists primarily of reactive astroglia, but also contains activated microglia (Fawcett and Asher, 1999; Rhodes et al., 2003). Interestingly, activated microglia have recently been reported to express AQP4 (Tomas-Camardiel et al., 2004), which may serve to increase their migration speed during glial scar formation.

In conclusion, we have discovered a new function for AQP4 in the brain: the enhancement of astroglial cell migration. This property of AQP4 appears unrelated to the well-established role of AQP4 in brain edema formation (Manley et al., 2000) and absorption (Papadopoulos et al., 2004), and suggests novel therapeutic possibilities. AQP4 inhibitors might reduce glial scarring, which could enhance neuronal regeneration after injury and improve the efficiency of incorporation of neural grafts into CNS tissue. AQP4 inhibitors might also limit the spread of the AQP4-overexpressing, malignant astroglial cells into the surrounding brain.

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