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NEW INSIGHTS INTO WATER TRANSPORT AND EDEMA IN THE CENTRAL NERVOUS SYSTEM FROM PHENOTYPE ANALYSIS OF AQUAPORIN-4 NULL MICE

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Abstract—Aquaporin-4 (AQP4) is the major water channel in the CNS. Its expression at fluid-tissue barriers (blood–brain and brain–cerebrospinal fluid barriers) throughout the brain and spinal cord suggests a role in water transport under normal and pathological conditions. Phenotype studies of transgenic mice lacking AQP4 have provided evidence for a role of AQP4 in cerebral water balance and neural signal transduction. Primary cultures of astrocytes from AQP4-null mice have greatly reduced osmotic water permeability compared with wild-type astrocytes, indicating that AQP4 is the principal water channel in these cells. AQP4-null mice have reduced brain swelling and improved neurological outcome following water intoxication and focal cerebral ischemia, establishing a role of AQP4 in the development of cytotoxic (cellular) cerebral edema. In contrast, brain swelling and clinical outcome are worse in AQP4-null mice in models of vasogenic (fluid leak) edema caused by freeze-injury and brain tumor, probably due to impaired AQP4-dependent brain water clearance. AQP4-null mice also have markedly reduced acoustic brainstem response potentials and significantly increased seizure threshold in response to chemical convulsants, implicating AQP4 in modulation of neural signal transduction. Pharmacological modulation of AQP4 function may thus provide a novel therapeutic strategy for the treatment of stroke, tumor-associated edema, epilepsy, traumatic brain injury, and other disorders of the CNS associated with altered brain water balance.

Key words: brain injury, cytotoxic edema, epilepsy, stroke, vasogenic edema water channel.

Abnormalities in water balance play an important role in the pathophysiology of traumatic brain injury, stroke, and a variety of neurological disorders (Fishman, 1975). Cerebral edema, defined as an abnormal increase in brain water content, leads to an increase in intracranial pressure (ICP), potentially leading to brain ischemia, herniation and death. Despite its clinical importance, the molecular mechanisms of CNS water accumulation and clearance remain poorly understood, such that current treatments aimed at reducing edema and other disturbances in water balance have changed little since their introduction more than 80 years ago (Weed and McKibben, 1919). In particular, brain swelling is treated clinically primarily with hyperosmolar agents (e.g. mannitol, hypertonic saline) in the attempt to draw water out of brain tissue and decrease ICP; however, the precise effects of these agents on distinct water compartments within the brain are as yet unclear. Thus, better understanding of the mechanisms of brain water accumulation and clearance in distinct neurological disorders will help to tailor current therapies as well as to develop new therapeutic strategies.

The discovery of aquaporins (AQPs) has provided a molecular basis for understanding water transport in a number of tissues, including the nervous system (Manley et al., 2000; Agre et al., 2002; Badaut et al., 2002; Amiry-Moghaddam and Ottersen, 2003). The AQPs are a family of homologous water channel proteins (numbering at least 11 in mammals) that provide the major route for water movement across plasma membranes in a variety of cell types (Agre et al., 2002; Verkman, 2002). AQPs are small hydrophobic membrane proteins (approximately 30 kDa monomer) that assemble in homotetramers and facilitate bi-directional water transport across the plasma membrane in response to osmotic gradients created by solute movement. At least three AQPs are expressed in brain and spinal cord. AQP1 is expressed in the choroid plexus epithelium (Hasegawa et al., 1994; Speake et al., 2003), where it has been shown to facilitate CSF (cerebrospinal fluid) secretion (Oshio et al., 2004b). AQP9 is found in a subset of astrocyte processes that form the glia limitans (Badaut et al., 2001) and specialized ependymal cells in the brain and spinal cord (Oshio et al., 2004a). However, AQP4 is by far the most abundant water channel in the CNS (see below). This review is focused on the role of AQP4 in CNS water balance and what has been learned from phenotype analysis of AQP4-null mice.

AQP4: structure and function

AQP4, initially named MIWC (mercurial-insensitive water channel), was cloned from mouse lung (Hasegawa et al., 1994) and a second isoform with longer N-terminus was later identified in rat brain (Jung et al., 1994). Both isoforms (M1, 323 amino acids; and M23, 301 amino acids) are expressed in the brain but the smaller (M23) isoform is more abundant

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Abbreviations: AQP, aquaporin; BBB, blood–brain barrier; CSF, cerebrospinal fluid; ECS, extracellular space; FFEM, freeze-fracture electron micrograph; ICP, intracranial pressure; MCA, middle cerebral artery; OAP, orthogonal arrays of particles; PTZ, pentylenetetrazol; RNAi, RNA interference.

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(Neely et al., 1999). Similar to the other AQPs, AQP4 has six transmembrane domains (Shi et al., 1995), but is unique among the mammalian AQPs in that its water permeability is not inhibited by mercurials due to the absence of critical cysteine residues (Shi and Verkman, 1996). The COOH-terminus of AQP4 also contains a PDZ domain interaction sequence (Ser-Ser-Val) that appears to be required for proper subcellular protein targeting (Neely et al., 2001).

AQP4 expression in brain and spinal cord

AQP4 is abundantly expressed in the brain and spinal cord at putative sites of fluid transport (Hasegawa et al., 1994; Jung et al., 1994; Frigeri et al., 1995; Nielsen et al., 1997). AQP4 is expressed in the dense astrocyte cell processes that form the glia limitans, a structure that lines the pial and ependymal surfaces in contact with the CSF in the subarachnoid space and the ventricular system (Frigeri et al., 1995; Nielsen et al., 1997). Highly polarized AQP4 expression is found in astrocytic foot processes in direct contact with blood vessels in the brain and spinal cord (Frigeri et al., 1995; Nielsen et al., 1997). AQP4 is also expressed in osmoregulatory areas of the brain including the hypothalamic magnocellular nuclei where AQP4-rich glial lamellae are found in direct contact with the magnocellular neurons (Jung et al., 1994; Nielsen et al., 1997). In spinal cord, AQP4 is abundantly expressed in gray matter in the glial processes that are in direct contact with neuronal cell bodies and synapses (Frigeri et al., 1995; Oshio et al., 2004a). AQP4 is not expressed by neurons or meninges (Frigeri et al., 1995). High-resolution immunogold electron microscopy has confirmed these results and demonstrated that AQP4 is concentrated in glial membranes facing blood–brain and brain–CSF interfaces and in the basolateral membrane of ependymal cells (Nielsen et al., 1997). This expression pattern of AQP4 suggests that it is involved in the movement of water between blood and brain, and between brain and CSF compartments.

Orthogonal arrays: role of AQP4

AQP4 has also been identified as a structural component of membrane "orthogonal arrays." Membranes of astrocyte foot processes contain numerous arrays of intramembrane particles in freeze-fracture electron micrographs (FFEM). These particles, which are regular square arrays with a characteristic cobblestone pattern, are referred to as square arrays or orthogonal arrays of particles (OAPs; Landis and Reese, 1974). Subsequent studies have confirmed the presence of OAPs in human brain (Cuevas et al., 1985) and have shown that the number of OAPs is altered in a variety of neurological disorders (Wolburg, 1995). Based on the finding that AQP4 was present in the same cell types in which OAPs were identified, it was proposed that AQP4 is an OAP protein (Frigeri et al., 1995). Support for this hypothesis came from FFEM on stably transfected CHO cells expressing functional AQP4 in OAPs (Yang et al., 1996). Direct evidence that AQP4 is an OAP protein *in vivo* came from FFEM studies of brain, kidney and skeletal muscle from AQP4-null mice (Verbavatz et al., 1997). OAPs were identified in every sample

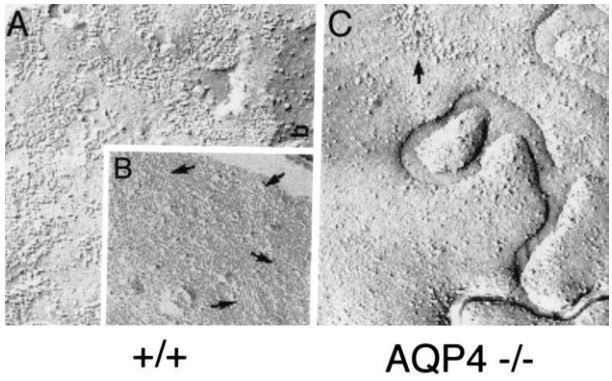

Fig. 1. FFEMs of brains from wild-type (+/+) and AQP4-null (−/−) mice. (A) OAPs on Pf (protoplasmic)-face electron micrograph of brain from wild-type (+/+) mice. (B) Immunogold labeling of OAPs with anti-AQP4 (arrows). (C) Pf (protoplasmic)-face electron micrograph of brain from AQP4-null (−/−) mice demonstrating lack of OAPs. Data from Verbavatz et al., 1997.

from wild-type and AQP4 heterozygous mice, and in no sample from AQP4-null mice (Fig. 1). Subsequent anti-AQP4 antibody labeling of OAPs in brain tissue confirmed that OAPs contain AQP4 (Rash et al., 1998). This group has also recently demonstrated that the interaction of the M1 and M23 isoforms of AQP4 regulates OAP size (Furman et al., 2003). The functional significance of this interaction remains to be elucidated.

Targeted AQP4 gene disruption in mice

To assess the role of AQP4 in the physiology of CNS water balance, we have focused on the generation and phenotypic evaluation of AQP4-null mice. Transgenic mice lacking AQP4 were generated by targeted gene disruption in embryonic stem cells (Ma et al., 1997). AQP4-null mice have no general behavioral, motor, sensory, or coordination deficits (Yang et al., 2000). Comparison of the brains from wild-type and AQP4-null mice revealed no gross anatomic differences by visual inspection, structural MRI imaging, or light microscopy. Examination of cerebral vascular anatomy indicated no differences in the size or distribution of the major cerebral arteries (Manley et al., 2000). Physiological parameters such as ICP, bulk intracranial compliance, heart rate, temperature, mean arterial pressure and blood gases are also unchanged (Manley et al., 2000; Papadopoulos et al., 2004a). While AQP4-null mice do manifest a mild defect in maximal urinary concentrating ability (Ma et al., 1997), there are no differences in serum sodium concentration and osmolality compared with wild-type mice, suggesting that AQP4 does not function as a regulator of serum osmolality as previously suggested (Jung et al., 1994). Given their normal anatomy, development, growth, and survival, the AQP4-null mice provide a valid model to examine to role of this water channel in cerebral water transport, edema, and physiology.

Alternative existing mouse models have also been used to study the role of AQP4, including the dystrophin null *mdx*-β*geo* transgenic mouse and the α-syntrophin null mouse (Frigeri et al., 2001; Vajda et al., 2002; Amiry-Moghaddam et

al., 2003a). Several studies have demonstrated that proper membrane localization of AQP4 in the brain requires the dystrophin-associated protein complex and α-syntrophin (Frigeri et al., 2001; Neely et al., 2001). Both the dystophin null *mdx*-β*geo* transgenic mouse and the α-syntrophin null mouse have normal total levels of AQP4 protein but significant reduction of AQP4 in the astrocytic foot processes surrounding capillaries and at the glia limitans (Vajda et al., 2002; Amiry-Moghaddam et al., 2003a). While studies with these mice have confirmed and extended our original water intoxication and stroke studies, the *mdx* and α-syntrophin mice display baseline morphological abnormalities, including swollen astrocyte foot processes (Amiry-Moghaddam et al., 2003a), increased blood–brain barrier (BBB) permeability, and altered molecular composition of endothelial cell tight junctions (Frigeri et al., 2001; Nico et al., 2003), which may account for some of the phenotypic differences compared with the AQP4-null mice (see below).

Role of AQP4 in astrocyte water permeability

Water permeability was first assessed using brain vesicle fractions from AQP4-null mice and wild-type mice (Ma et al., 1997). Vesicle shrinkage in response to an osmotic gradient measured by stopped-flow light scattering demonstrated high water permeability in a mixed population of AQP4-enriched brain vesicles from wild-type mice, which was absent in vesicles from AQP4-null mice, suggesting that AQP4 provides a principal molecular pathway for water permeability in the brain.

In order to define the role of AQP4 in astrocyte water permeability, we recently established and characterized primary astrocyte cultures from wild-type and AQP4-null mice (Fig 2A). There are no differences in the cellular morphology or growth characteristics of astrocyte cultures from brains of wild-type vs. AQP4-null mice. To follow acute changes in astrocyte volume after osmotic challenge, we adapted and validated a calcein quenching method (Hamann et al., 2002) to measure osmotic water permeability in astrocytes (Fig. 2B; Solenov et al., 2004). Representative data for the kinetics of astrocyte cell swelling in response to solution exchange between isotonic and hypotonic saline are shown in Fig. 2C (left). The kinetics of cell swelling and shrinking was remarkably slowed in AQP4-null astrocytes (Fig. 2C, right). Deduced osmotic water permeability (P_f) was reduced 7.1-fold in astrocytes from AQP4-deficient mice compared with wild-type mice. These studies thus establish that AQP4 provides the principal route for water transport in astrocytes.

In other studies, RNA interference (RNAi) has been used to reduce AQP4 expression in primary cultures of astrocytes from wild-type mice (Nicchia et al., 2003). While RNAi treatment resulted in approximately 50% decrease in apparent water permeability, it also significantly altered cell morphology and inhibited cell growth by 68%, raising questions about RNAi toxicity. In contrast with these results, we found no alteration in astrocyte morphology or growth characteristics in primary astrocyte cultures from AQP4-null mice (unpublished data). It should be noted that the polarized expression of AQP4 is lost in primary cultures, raising concerns for some models in which polarized AQP4 expression might be necessary for physiologic function (Nicchia et al., 2000). It will be useful to develop co-culture systems with other cell types such as endothelia and neurons to determine whether polarized expression of AQP4 can be re-established *in vitro*.

Role of AQP4 in cytotoxic brain edema

Based on the expression of AQP4 at blood–brain and brain–CSF interfaces, we tested the hypothesis that AQP4 plays a role in the early accumulation of brain water *in vivo* in response to two established neurological insults: acute water intoxication and ischemic stroke.

AQP4 deletion improves outcome and reduces brain swelling after water intoxication

Our initial studies evaluating the effect of AQP4 deletion on the development of cerebral edema used a classic model of water intoxication. In this model, rapid i.p. water infusion causes serum hyponatremia that creates an osmotic gradient driving water entry into the brain. The added water produces cytotoxic edema without disruption of the BBB. We postulated that if water transport across the BBB is dependent on AQP4, mice lacking AQP4 would have decreased edema and improved outcome (Manley et al., 2000). As shown in Figure 3A, the survival of AQP4-null mice after water intoxication was significantly improved. While the initial neurological status of the AQP4-null and wild-type mice was similar, over time the wild-type mice became uncoordinated with rapid progression to paralysis whereas AQP4-null mice remained mildly lethargic. Within 60 min, only 8% of the wild-type mice compared with 76% of the AQP4-null mice were alive (Fig. 3A). Using a gravimetric method to measure and compare brain tissue water content in wild-type and AQP4-null mice, we confirmed that deletion of AQP4 reduced water accumulation in brain tissue following water intoxication, presumably by reducing water transport from the intravascular space to the intracellular space (Manley et al., 2000).

To further investigate cellular mechanisms of the protective effect of AQP4 deletion following water intoxication, a transmission electron microscopy method was used to examine swelling of astrocytic foot processes. Wild-type mice had more widespread pericapillary astrocytic foot process swelling as compared with AQP4-null mice (Fig. 3B). Average foot process area of wild-type and AQP4-null mice was similar at baseline, but increased substantially after water intoxication (Fig. 3C). The threefold reduction in astrocytic foot process swelling of in the AQP4-null mice supports the conclusion that AQP4 participates in water transport from the intravascular to the intracellular space.

A similar study was subsequently performed in dystrophin null (*mdx*) transgenic mice that have reduced AQP4 expression in astrocyte foot processes (Vajda et al., 2002). Following water intoxication, wild-type mice began to deteriorate neurologically 30 min after water intoxication whereas the dystrophin-null mice had a delayed response, deteriorating after more than 50 min (Vajda et al., 2002). However, in contrast to our study demonstrating a survival benefit of AQP4 deletion, all animals in both groups died. These results

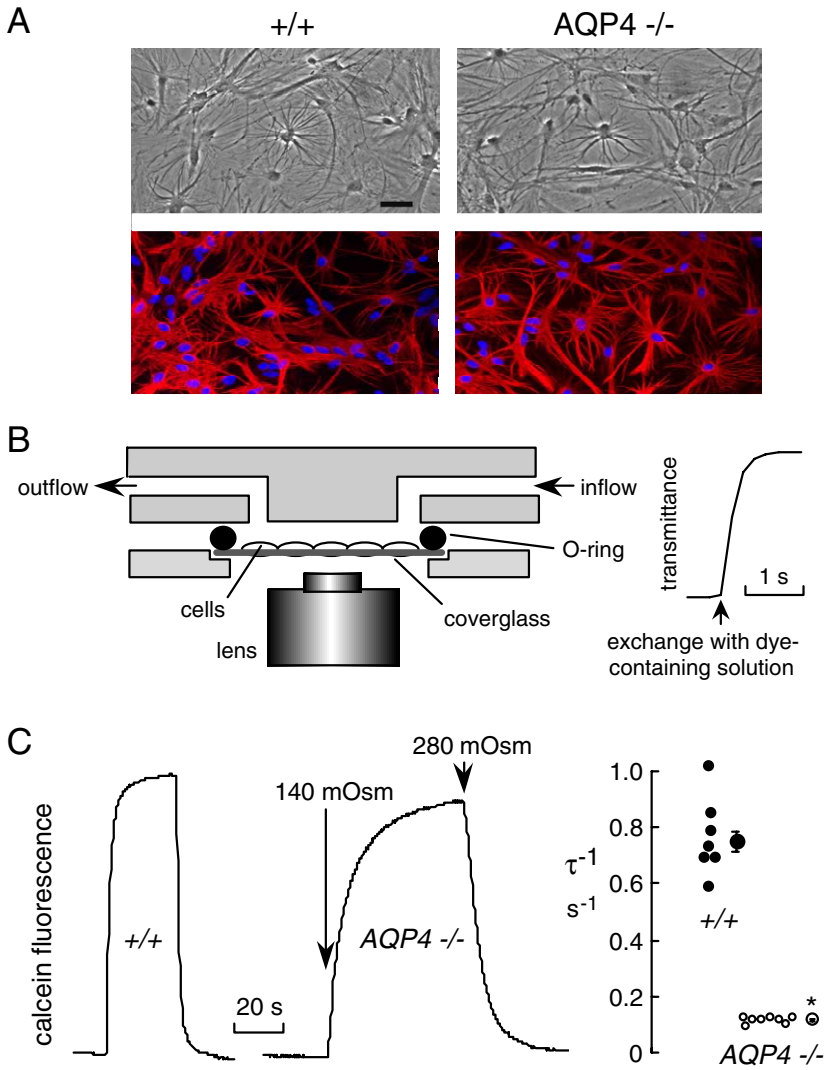

Fig. 2. Water permeability in primary cultured astrocytes from wild-type and AQP4-null mice. (A) Phase-contrast micrographs of wild-type (+/+) and AQP4-null (−/−) astrocytes (upper panels) and astrocytes with GFAP staining (red) and nuclear staining (blue). (B) Calcein-quenching method for measuring osmotic water permeability. Perfusion chamber showing cultured cells on a glass coverslip with rapid superfusion with solutions (see Solenov et al., 2004 for details). (C) Representative signal changes for measurement of reversible cell swelling in response to an osmotic gradient in astrocytes from wild-type (+/+) and AQP4-null (−/−) mice (left). Swelling rates in different astrocyte cell cultures (right). Values are means S.E. * $P<0.0001$. Data from Solenov et al., 2004.

demonstrate that dystrophin null mice (lacking AQP4 in the astrocytic foot processes) and the AQP4-null mice (completely lacking AQP4) have a delayed response to water intoxication. The differences in survival between the dystrophin null mice and the AQP4-null mice may be in part related to baseline morphological alterations found in the dystrophin null mice such as increased BBB permeability (Nico et al., 2003), which may enhance the transport of water from the intravascular space to the brain thereby increasing cerebral edema and worsening outcome.

AQP4 deletion reduces edema and improves outcome in a focal ischemia model

The protective effect of AQP4 deletion in a cytotoxic edema model prompted us to examine the role of AQP4 in a clinically relevant model of stroke, which results primarily in cytotoxic edema with some vasogenic edema. The permanent middle cerebral artery (MCA) occlusion model was chosen because of its similarity to ischemic hemispheric stroke in humans (Hossman, 1998). MCA occlusion generates a reproducible injury resulting in well-defined neurologic and neuropathologic outcomes (Kondo et al., 1997). Neurological evaluation of mice after permanent MCA occlusion demonstrated that wild-type mice had a higher mortality rate and significantly greater neurological deficit at 24 h compared with AQP4-null mice (Manley et al., 2000). Figure 4A shows histological findings in representative wild-type and AQP4-null mice at 24 h after permanent MCA occlusion. In wild-type mice, significant hemispheric enlargement and midline shift resulted from cerebral edema; this hemispheric enlargement was sig-

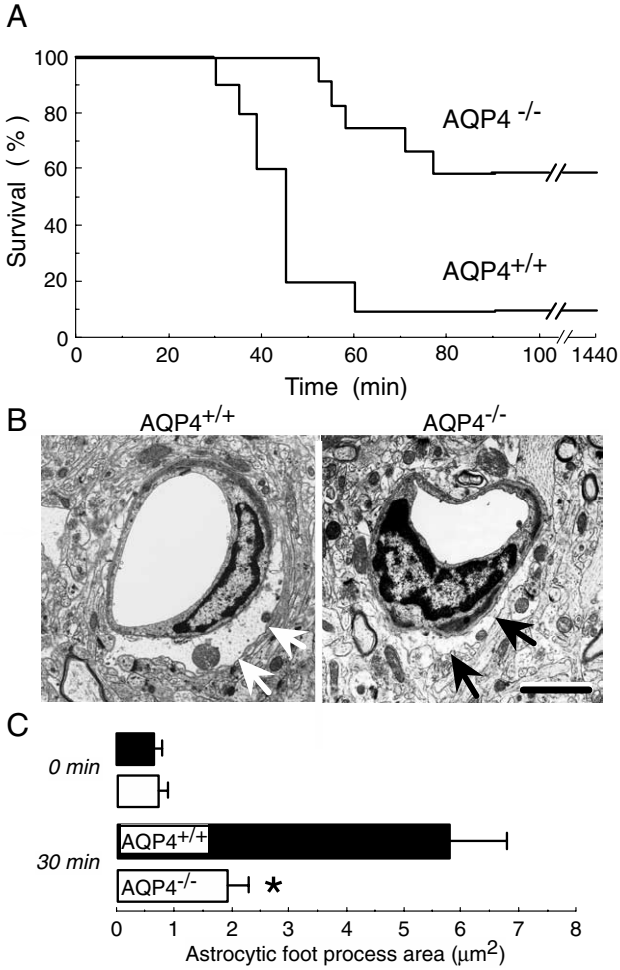

Fig. 3. Improved survival and reduced edema in AQP4-null mice following acute water intoxication. (A) Mice received i.p. injection of distilled water (20% body weight) containing DDAVP. Neurological deficit scores were determined and time of death was recorded. Mice were followed for 24 h (1440 min). The percentage of surviving wild-type and AQP4-null mice is shown for each time point. (B) Transmission electron micrograph showing edematous cerebral cortex at 30 min. Note the swollen astrocytic foot process in brain from wild-type (white arrows) and AQP4-null (black arrows) mice. (C) Quantitation of pericapillary astrocyte foot process area. There was significantly less (* $P<0.005$) swelling of the astrocyte foot processes in the AQP4-null mice following 30 min of water intoxication. (Data from Manley et al., 2000).

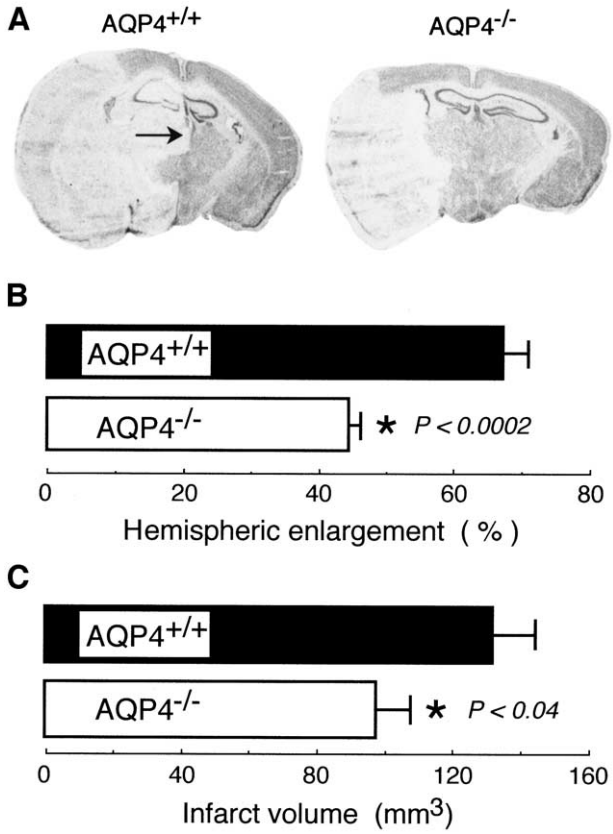

Fig. 4. Reduced brain edema in AQP4-null mice following ischemic stroke. (A) Representative low magnification image of Cresyl Violet-stained brain at 24 h after permanent MCA occlusion. Note the increased swelling and hemispheric enlargement in the wild-type mouse brain section. (B) Hemispheric enlargement, determined by quantitative image analysis, was significantly lower in the AQP4-null mice ($P<0.0002$). (C) Infarct volume, was also significantly lower in the AQP4-null mice ($P<0.04$). (Data from Manley et al., 2000).

nificantly reduced in AQP4-null mice (Fig. 4B). The volume of infarct was also decreased in AQP4-null mice (Fig. 4C). A similar protective effect was recently described for α-syntrophin null mice following transient cerebral ischemia (Amiry-Moghaddam et al., 2003a). Thus, AQP4 provides a principal pathway for water influx in models of cytotoxic edema, providing proof of principle evidence for the utility of AQP4 inhibitors in reducing stroke-associated edema.

Role of AQP4 in vasogenic brain edema

As expected for all AQPs, AQP4 permits bidirectional water transport (Meinild et al., 1998; Amiry-Moghaddam et al., 2003a); consequently, AQP4 may not only provide the major pathway for water entry into the brain, but also for water exit. A role for AQP4 in facilitating the removal of excess brain water has recently been demonstrated by our group (Papadopoulos et al., 2004a). After continuous intracerebral fluid infusion, AQP4-null mice developed higher ICP and brain water content, compared with wild-type controls (Fig. 5). Isotonic fluid was infused into the brain parenchyma with continuous ICP recording (Fig. 5A). At 60 min there was a significantly greater change in ICP in AQP4-null mice (Fig. 5B). Also, the water content of the infused hemisphere after a 60 min infusion was significantly elevated in AQP4-null mice, whereas the water content of the non-infused contralateral hemisphere was not different (Fig. 5C). In a well-established freeze-injury model of vasogenic brain edema (Chan et al., 1991; Oury et al., 1993), it was also shown that AQP4 deficient mice had worse clinical outcome, higher ICP, and greater brain water content than controls. The role of AQP4 in tumor-associated vasogenic brain edema is discussed further in this issue of *Neuroscience* (Papadopoulos et al., 2004b). Experimental data showing worse clinical outcome and ICP in AQP4 null mice after brain tumor implantation sup-

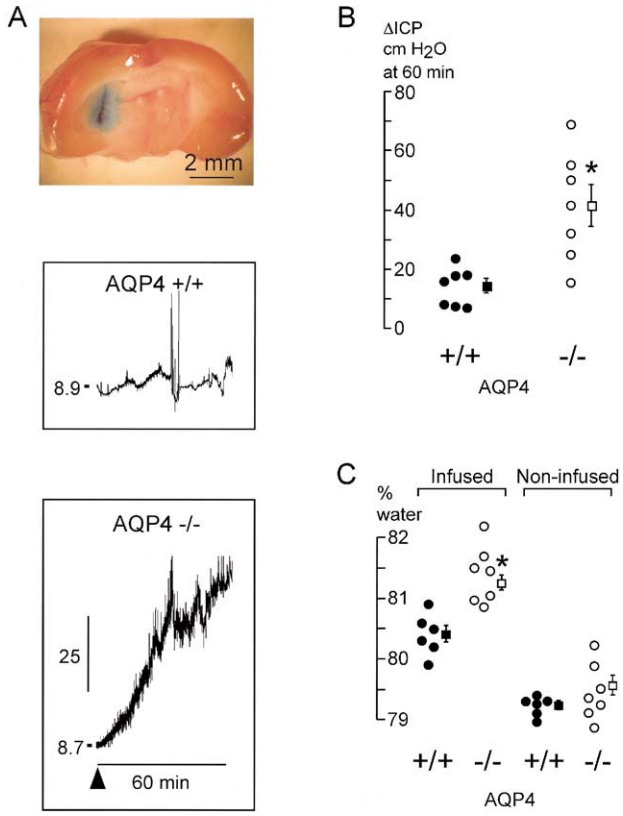

Fig. 5. AQP4 facilitates the elimination of fluid infused into the brain parenchyma. (A) Coronal section of mouse brain after infusing isotonic fluid (containing blue dye) into the brain parenchyma (top panel). Representative traces from wild-type and AQP4-null mice show the effect of intraparenchymal fluid infusion (0.5 μl/min) on ICP (Bottom panel). (B) The AQP4-null mice develop higher ICP over 60 min because they are unable to efficiently eliminate the excess fluid load. (C) Brain water content is also more markedly increased in the infused hemisphere of AQP4-null mice. Circles represent individual mice; boxes are mean±S.E.M. * $P<0.05$. Data from Papadopoulos et al., 2004a.

ports the conclusion that AQP4 facilitates removal of excess brain water in vasogenic edema.

These experiments suggest that in vasogenic brain edema, water enters the brain parenchyma independent of AQP4, but exits the brain through AQP4. This finding is intriguing because excess fluid in vasogenic brain edema enters the *extracellular* space of the brain parenchyma, but seems to exit the brain through a *transcellular* pathway.

There are three main barriers across which edema fluid can be eliminated from the brain: ependyma, glia limitans and BBB. All three barriers express AQP4 protein, but their relative contributions to the clearance of vasogenic brain edema fluid are unclear. It has been suggested that when ICP is low, vasogenic brain edema is primarily eliminated through the ependyma into the ventricular CSF, but when ICP is high, excess fluid leaves the brain parenchyma through the BBB (Reulen et al., 1977; Marmarou et al., 1994). However, these studies traced the flow of dyes and/or albumin, as markers of vasogenic edema fluid. Since AQP4 is impermeable to these markers, it is likely that the pathways through which they are cleared from the brain are different from the pathways of water reabsorption. The border between brain parenchyma and the CSF space around cerebral blood vessels (known as the Virchow-Robin space) may provide another route for the movement of substances between brain and blood (Abbott, 2004). At present, it is not known whether Virchow-Robin spaces provide a significant pathway for water flow between CSF and brain parenchyma.

Role of AQP4 in neural signal transduction and ion homeostasis

Astrocytes are involved in many important physiologic functions, such as sequestration and/or redistribution of K^+ during neural activity, neurotransmitter cycling (including synthesis of GABA and glutamate precursors as well as removal of glutamate and GABA at synapses), provision of energy substrates to neurons, and regulation of synaptogenesis (Ransom et al., 2003). In view of the important role of astrocytes in neural function, we hypothesized that astrocytic AQP4 may be critical for brain water and ion homeostasis by facilitating K^+ and H_2O clearance

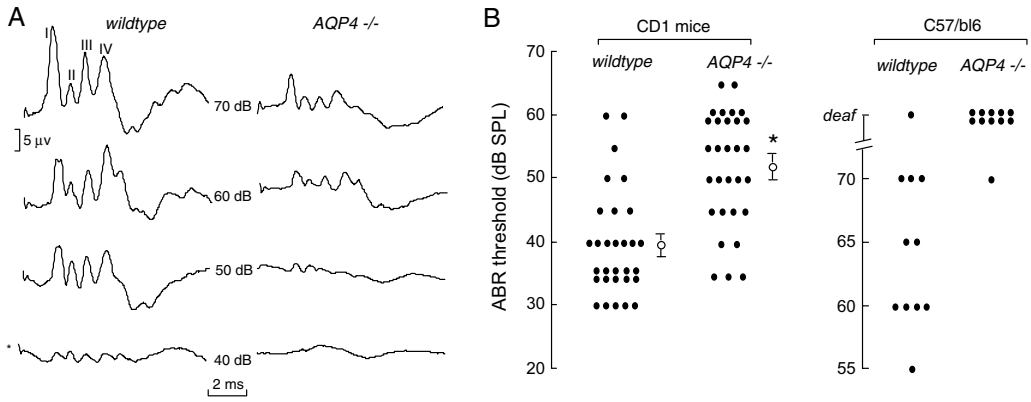

Fig. 6. Impaired hearing in AQP4-null mice. (A) Representative auditory brainstem response (ABR) waveforms measured in wild-type and AQP4-null mice measured in response to stimuli of indicated intensities. * Denotes ABR threshold. (B) Summary of ABR thresholds measured in CD1 (left) and C57/b16 (right) mice. Filled circles represent individual mice; open circles are mean±S.E.M. * $P<0.001$. Data from Li and Verkman, 2001.

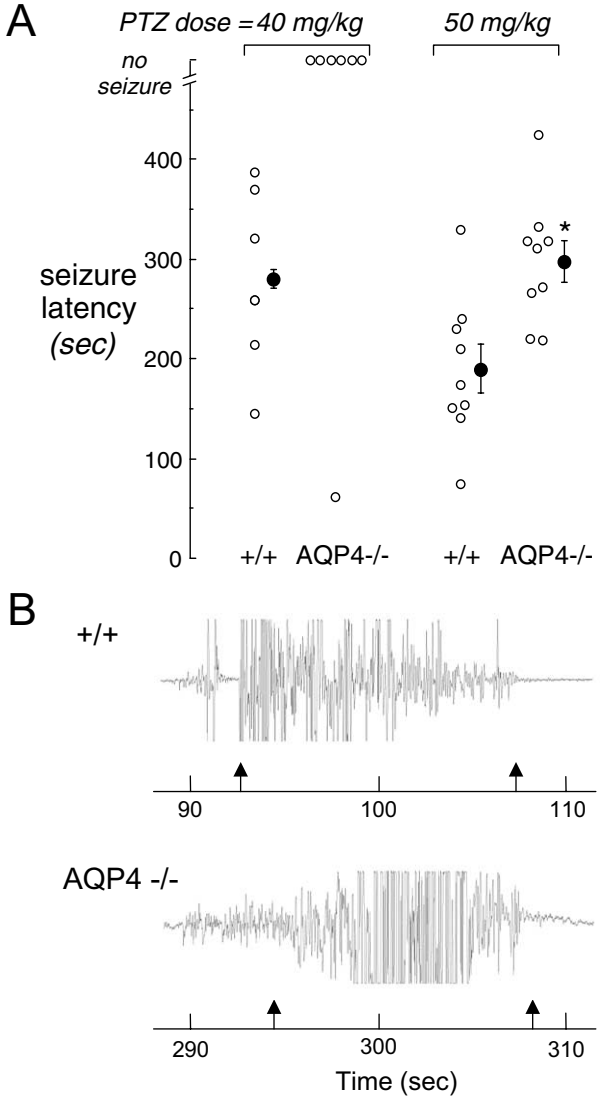

Fig. 7. Reduced seizure susceptibility of AQP4-null mice. (A) Latency to generalized seizure in wild-type (+/+) and AQP4-null (−/−) mice. Open circles represent individual mice; filled circles are mean±S.E.M. * $P<0.05$. (B) Representative electrographic recordings. Generalized seizures in a wild-type mouse beginning 92 s after injection and in AQP4-null mouse beginning 295 s after injection are shown. Arrows denote onset and end of observed behavioral seizure activity. Data from Binder et al., 2004.

and/or recycling during rapid neural activity. In support of this possibility is the co-localization of AQP4 with the inwardly rectifying potassium channel, Kir4.1 (Nagelhus et al., 1998; Dalloz et al., 2003), which is thought to mediate spatial K^+ buffering by astrocytes (Kofuji et al., 2000; Horio, 2001). Therefore, we studied AQP4-null mice in several *in vivo* models in the attempt to identify phenotypic alterations in neural signal transduction.

Role of AQP4 in sensory signal transduction

Based on the expression of AQP4 in supportive epithelial cells adjacent to (electrically excitable) hair cells in rat inner ear (Takumi et al., 1998), we investigated the involvement of AQP4 in auditory signal transduction (Li and Verkman, 2001). Using mice deficient in selected AQPs as controls, AQP4 was localized in supporting epithelial cells (Hensen's and Claudius cells) in organ of Corti. In 4–5 week old mice in a CD1 genetic background, ABR thresholds in response to a click stimulus were increased by >20 db in AQP4 null mice compared with wild-type mice (Fig. 6A). In a C57/bl6 background, nearly all AQP4-null mice were deaf whereas ABRs could be elicited in wild-type controls (Fig. 6B). Light microscopy showed no differences in inner ear morphology of wild-type vs. AQP4-null mice. Similar results were subsequently reported by Mhatre et al. (2002). These results provide evidence that AQP water channels play a role in hearing. We proposed that AQP4 facilitates rapid osmotic equilibration in epithelial cells in the organ of Corti which are subject to large K^+ fluxes during mechanoelectric signal transduction (Johnstone et al., 1989).

In the retina, AQP4 is expressed in retinal Müller cells in close proximity to bipolar cells (Nagelhus et al., 1998). Mice lacking AQP4 had mildly reduced b-wave activity in electroretinograms (Li and Verkman, 2001). Again, this phenotype may be related to altered retinal H_2O transport or K^+ spatial buffering.

Altered seizure threshold in AQP4-null mice

Relative cellular and extracellular space (ECS) volume has been demonstrated to play an important role in propensity to epileptic seizures. Decreasing ECS volume by hypotonic exposure produces hyperexcitability and enhanced epileptiform activity (Dudek et al., 1990; McBain et al., 1990; Roper et al., 1992; Chebabo et al., 1995; Pan and Stringer, 1996), whereas hyperosmolar medium attenuates epileptiform activity (Traynelis and Dingledine, 1989; Dudek et al., 1990; Pan and Stringer, 1996). Furosemide, a chloride cotransport inhibitor that blocks seizure-induced cell swelling, inhibits epileptiform activity *in vitro* and *in vivo* (Hochman et al., 1995; Stringer and Pan, 1997). These experimental data parallel extensive clinical experience indicating that hypo-osmolar states such as hyponatremia lower seizure threshold while hyperosmolar states elevate seizure threshold (Andrew et al., 1989).

Because of the importance of cell swelling in determining ECS volume, we hypothesized that AQP4-dependent water transport in astrocytes may modulate intrinsic brain excitability. To examine this possibility, seizure susceptibility was measured in AQP4-null mice using the convulsant pentylenetetrazol (PTZ, a $GABA_A$ antagonist) (Binder et al., 2004). At 40 mg/kg PTZ (i.p.), all wild-type mice exhibited seizure activity, whereas six of seven AQP4-null mice did not exhibit seizure activity (Fig. 7A). At 50 mg/kg PTZ, both groups exhibited seizure activity; however, the latency to generalized (tonic–clonic) seizures was lower in wild-type mice. In separate studies, *in vivo* EEG characterization of seizures induced by electrical stimulation in the hippocampus indicated greater electrographic seizure threshold and remarkably longer seizure duration in AQP4-null mice compared with wild-type mice (unpublished data).

Although these studies suggest that AQP4 modulates brain excitability, as well as the propagation and termination

of seizure activity, they do not establish a mechanism for the above phenotype. Since seizure propensity is sensitive to ECS volume, AQP4 deletion may alter ECS volume or composition at baseline and/or following neural activity. A larger ECS volume fraction prior to seizure-inducing stimuli and/or a blunted reduction in ECS volume during neural activity via abrogation of water influx through glial AQP4 would be expected to limit excitability and synchrony.

In a recent study, Amiry-Moghaddam et al. (2003b) studied hippocampal slices from α-syntrophin-deficient mice and found a deficit in extracellular K^+ clearance following evoked neuronal activity. In addition, using a hyperthermia model of seizure induction, they found more of the α-syntrophin-deficient mice had more severe seizures than wild-type mice. These data are consistent with the idea that AQP4 and its molecular partners (e.g. Kir4.1, α-syntrophin, dystrophin) together compose a multifunctional 'unit' responsible for clearance of K^+ and/or H_2O following neural activity.

CONCLUSIONS

The phenotype of the AQP4-null mice provides evidence for AQP4 involvement in brain water balance and neural signal transduction. Our studies indicate that AQP4 plays a key role in osmotically-driven water transport and the development of cytotoxic brain edema. AQP4 also participates in the absorption of excess brain water and the resolution of vasogenic brain edema. AQP4 appears also to be crucial for brain water and ion homeostasis during rapid neural activity; however, the precise mechanisms must still be elucidated. The relevance of these observations to human clinical diseases such as traumatic brain injury and epilepsy remains to be established. AQP4 inhibitors and activators will be required to establish the potential benefits of modulating AQP4 function. Once identified, these drugs might slow the accumulation or enhance the clearance of brain water, thereby reducing the morbidity and death associated with many common neurological disorders.

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