

Increased Seizure Duration and Slowed Potassium Kinetics in Mice Lacking Aquaporin-4 Water Channels

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KEY WORDS

aquaporin; astrocyte; epilepsy; extracellular space; glial cell; potassium; seizure

ABSTRACT

The glial water channel aquaporin-4 (AQP4) has been hypothesized to modulate water and potassium fluxes associated with neuronal activity. In this study, we examined the seizure phenotype of AQP4 $-/-$ mice using *in vivo* electrical stimulation and electroencephalographic (EEG) recording. AQP4 $-/-$ mice were found to have dramatically prolonged stimulation-evoked seizures after hippocampal stimulation compared to wild-type controls (33 ± 2 s vs. 13 ± 2 s). In addition, AQP4 $-/-$ mice were found to have a higher seizure threshold (167 ± 17 μ A vs. 114 ± 10 μ A). To assess a potential effect of AQP4 on potassium kinetics, we used *in vivo* recording with potassium-sensitive microelectrodes after direct cortical stimulation. Although there was no significant difference in baseline or peak $[K^+]_o$, the rise time to peak $[K^+]_o$ ($t_{1/2}$, 2.3 ± 0.5 s) as well as the recovery to baseline $[K^+]_o$ ($t_{1/2}$, 15.6 ± 1.5 s) were slowed in AQP4 $-/-$ mice compared to WT mice ($t_{1/2}$, 0.5 ± 0.1 and 6.6 ± 0.7 s, respectively). These results implicate AQP4 in the expression and termination of seizure activity and support the hypothesis that AQP4 is coupled to potassium homeostasis *in vivo*. ©2006 Wiley-Liss, Inc.

INTRODUCTION

The aquaporins (AQPs) are membrane “water channels” found in cell types and tissues in which fluid transport is crucial. There is increasing evidence that water movement in the brain involves aquaporins (Amiry-Moghaddam and Ottersen, 2003; Manley et al., 2004). Aquaporin-4 (AQP4) is expressed ubiquitously by glial cells, especially at specialized membrane domains including astroglial endfeet in contact with blood vessels and astrocyte membranes that ensheath glutamatergic synapses (Nielsen et al., 1997; Nagelhus et al., 2004). Mice deficient in AQP4 (AQP4 $-/-$) have decreased accumulation of brain water (cerebral edema) after water intoxication and focal cerebral ischemia (Manley et al., 2000) and impaired clearance of brain water in models of vasogenic edema (Papadopoulos et al., 2004), suggesting a functional role for AQP4 in brain water transport. Similarly, mice deficient in dystrophin or α -syntrophin, in which there is mislocalization of the AQP4 protein (Frigeri et al., 2001; Neely et al., 2001; Vajda et al., 2002), also

demonstrate attenuated cerebral edema (Vajda et al., 2002; Amiry-Moghaddam et al., 2003a).

Glial cells are involved in important physiologic functions such as sequestration and/or redistribution of K^+ during neural activity, neurotransmitter cycling, and energy metabolism (Ransom et al., 2003). In view of the localization of AQP4 on astrocyte membranes, we hypothesized that AQP4 may participate in K^+ and H_2O clearance from the extracellular space (ECS). One possibility is that AQP4 could affect neural signal transduction primarily by water homeostasis and ECS physiology. Previous work has demonstrated that brain tissue excitability appears to be exquisitely sensitive to osmolarity and ECS size (Schwartzkroin et al., 1998). Alternatively, AQP4 and its known molecular partners could modulate extracellular ion homeostasis. In particular, the subcellular colocalization of AQP4 with the inwardly rectifying potassium channel $K_{ir}4.1$ (Connors et al., 2004; Nagelhus et al., 2004) has suggested that AQP4 may participate in the coupled influx of water and K^+ into astrocytes that occurs after neural activity (Walz, 1987; Amiry-Moghaddam and Ottersen, 2003; Manley et al., 2004).

In this study, we examined the effect of AQP4 deletion on neural signal transduction using *in vivo* electroencephalographic (EEG) recording after stimulation-evoked seizures. To determine the contribution of AQP4 to K^+ homeostasis *in vivo*, we also characterized potassium dynamics after direct cortical stimulation using ion-selective microelectrodes.

MATERIALS AND METHODS

All animal procedures were approved by the UCSF Committee on Animal Research.

D.K. Binder and X. Yao contributed equally to this work.

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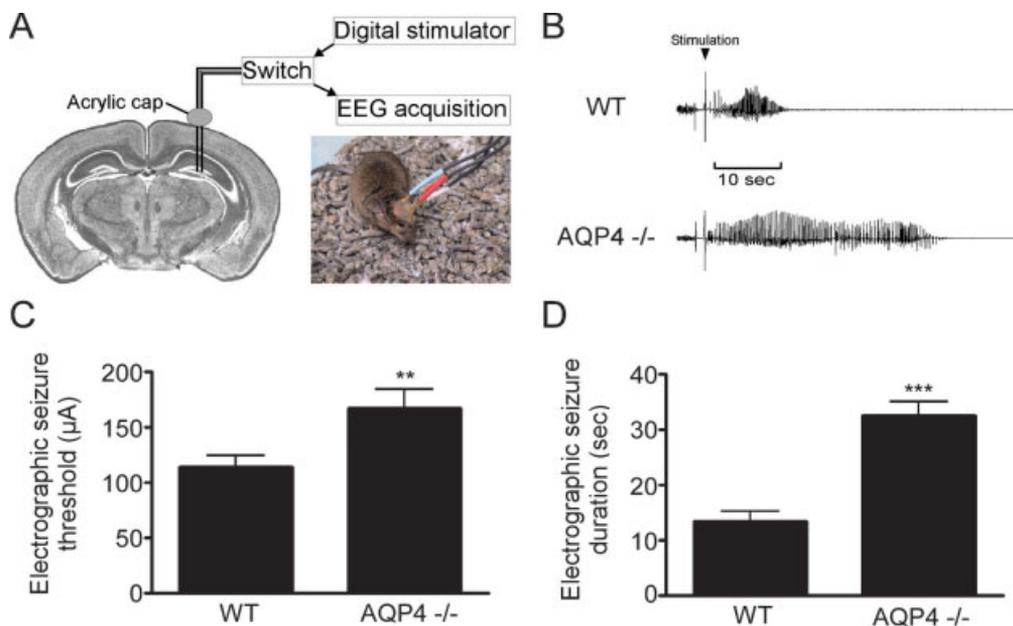


Fig. 1. Electrographic seizure threshold and duration in WT vs. AQP4 $-/-$ mice. **A:** Bipolar electrodes implanted in the right hippocampus were connected to a stimulator and digital EEG acquisition system. Mice were awake and behaving normally at the onset of stimulation (inset). **B:** Representative electroencephalograms from WT and AQP4 $-/-$ mice. Baseline EEG before stimulation is similar (left). Hippocampal stimulation-induced electrographic seizures are shown for a WT mouse (top) and an AQP4 $-/-$ mouse (bottom). The WT mouse had an 11-s seizure, whereas the AQP4 $-/-$ mouse had a much longer seizure

(37 s). Behavioral arrest was observed in both animals during the seizure. Post-ictal depression is evident on the EEG in both cases. **C:** Electrographic seizure threshold (μA) (mean \pm SE) in WT vs. AQP4 $-/-$ mice. AQP4 $-/-$ mice had a higher electrographic seizure threshold than that of wild-type controls. **D:** Electrographic seizure duration (s) (mean \pm SE) after hippocampal stimulation in WT vs. AQP4 $-/-$ mice. AQP4 $-/-$ mice had remarkably longer stimulation-evoked seizures compared to wild-type controls.

AQP4 $-/-$ Mice

AQP4 $-/-$ mice were generated as previously described (Ma et al., 1997). These mice lack detectable AQP4 protein and phenotypically have normal growth, development, survival, and neuromuscular function.

Electrode Implantation

Adult male (2-month-old) AQP4 $-/-$ mice ($n = 8$) and wild-type (WT) littermates ($n = 10$) were anesthetized with 2,2,2-tribromoethanol (125 mg/kg, administered intraperitoneally) and placed in a mouse stereotaxic frame. Bipolar stainless steel wire electrodes were implanted in the right dorsal hippocampus (bregma as reference: 2.0 mm posterior; 1.5 mm lateral; 1.8 mm below dura) (Franklin and Paxinos, 1997). Electrodes were secured to the skull with dental acrylic and anchor screws.

Seizure Stimulation

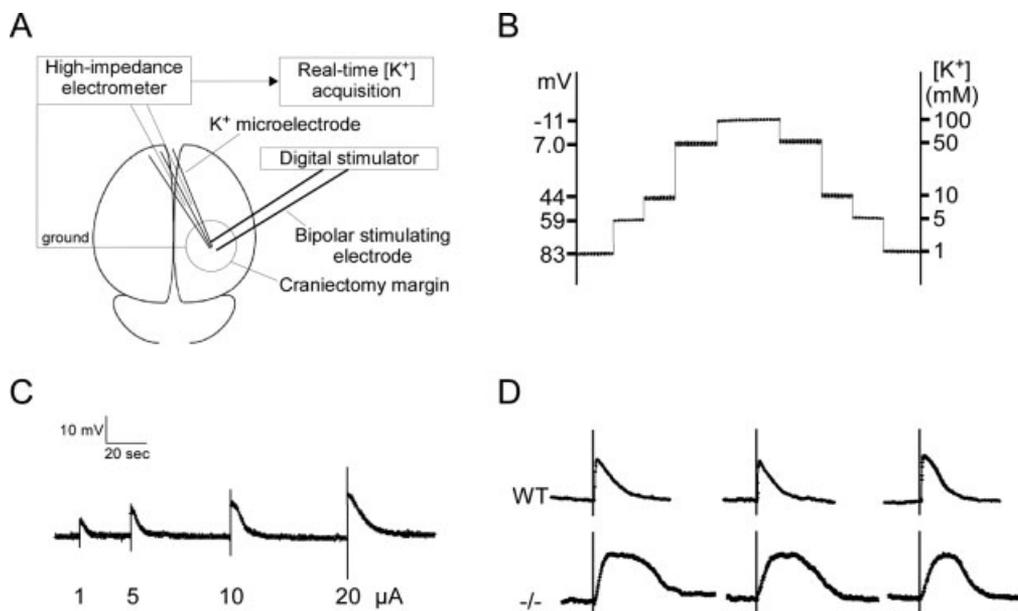
Electrical stimulations were given to assess electrographic seizure threshold and duration (Fig. 1A). Each stimulation consisted of a 60-Hz, 1-s train of 1-ms biphasic rectangular pulses. To determine electrographic seizure threshold (EST), stimulation intensity was increased by 10- μA increments starting at 20 μA . EST was recorded as the threshold at which at least 3 s of hippocampal

afterdischarge (seizure) was recorded. Electrographic seizure duration (ESD) was recorded as the total duration of electrographic seizure at the EST. Behavioral characteristics of the seizure (such as immobility) were also recorded. All EEG data were recorded digitally (Biopac Inc., Goleta, CA) and analyzed by an observer blinded to genotype. Power spectra of baseline EEGs were derived as the square of the fast-Fourier transform of a 30-s waveform of baseline EEG and averaged within group.

K⁺-Sensitive Microelectrodes

[K⁺]_o was measured using double-barreled potassium-sensitive microelectrodes (Sick et al., 1999). Potassium ion exchanger (WPI IE190, WPI, Sarasota, FL) was introduced into the tip of one barrel of a double-barreled micropipette and the electrode was backfilled with 100 mM KCl. The other barrel (150 mM NaCl) served as a reference electrode for subtraction of cortical DC potential shifts by means of a high input impedance differential electrometer (WPI FD223, WPI). Electrodes were calibrated in potassium standard solutions (1, 5, 10, 50, and 100 mM). Voltage data were converted into potassium ion concentration using least-squares nonlinear regression. Electrode tip diameters were about 10 μm . Electrodes were not used for this study unless there was at least a 35-mV change between 1 and 10 mM potassium standards.

Fig. 2. In vivo K^+ measurements in WT and AQP4 $-/-$ mice. **A:** Double-barreled potassium-selective microelectrodes were introduced through a small craniectomy and connected to a high-impedance electrometer for real-time $[K^+]$ acquisition. A bipolar electrode connected to a digital stimulator was positioned to provide direct current stimulation of the cortex at varying intensities. **B:** Sample calibration curve for a K^+ -sensitive microelectrode placed in serial K^+ standard solutions. **C:** Representative family of K^+ curves in WT mouse after stimulation intensities of 1, 5, 10, and 20 μA . **D:** Representative 20- μA stimulation curves from three different WT mice (top) and three different AQP4 $-/-$ mice (bottom). Calibration in C also applies to D.



K^+ Measurements In Vivo

Male AQP4 $-/-$ mice ($n = 5$) and WT littermates ($n = 5$) were anesthetized as above. A burr hole craniectomy was made over the right frontal lobe and potassium-sensitive microelectrodes were inserted into the cortex to 400–500 μm . Great care was taken to avoid damage either during the craniectomy or during electrode insertion because this was accompanied by a rise in $[K^+]_o$. Local cortical stimulation was accomplished by a bipolar stimulating electrode connected to a digital stimulator (Biopac Inc.). The tips of the bipolar stimulating electrode (separation, ~ 1 mm) were positioned on the cortical surface on either side of the microelectrode tip (Sick et al., 1999) (Fig. 2A). Increased $[K^+]_o$ was induced by applying 2-s trains (20 Hz) of rectangular constant-current pulses (0.5-ms duration) at varying current amplitudes (1, 5, 10, and 20 μA) to the cortical surface. Baseline measurements, as well as each stimulation-evoked K^+ measurement, were repeated three times for each amplitude in a particular animal. $[K^+]_o$ was allowed to return completely to baseline between successive stimulations.

Half-times ($t_{1/2}$) for potassium increase to peak and recovery to baseline were obtained by exponential regressions separately for K^+ rise to peak and for decay to baseline.

Western Blot Analysis

Whole brain tissue from WT or AQP4 $-/-$ mice ($n = 3$ each) was homogenized in 250 mM sucrose, 10 mM Tris-HCl, pH 7.4, containing 20 $\mu g/ml$ PMSF and centrifuged at 10,000g for 10 min. Supernatants were resolved on either 12 or 5–20% polyacrylamide gels, electrotransferred to membranes (Amersham), blocked with 3% albumin for 1 h, incubated with AQP4 antibody (1:1000, Chemicon), $K_{i,r}4.1$ antibody (1:400, Alomone), and GAPDH

mouse monoclonal antibody (1:2000, Chemicon), then developed with ECL (Amersham).

Statistics

Numerical values are given as means \pm SE and statistical analysis was performed with Student's t -test, with statistical significance determined at $P = 0.05$.

RESULTS

Electrographic Seizure Threshold and Duration

AQP4 $-/-$ mice and WT littermates were implanted with indwelling electrodes into the right dorsal hippocampus and allowed to recover from surgery for at least 3 days. Mice were awake and freely moving at the onset of stimulation (Fig. 1A). Baseline EEG in WT and AQP4 $-/-$ mice was indistinguishable (Fig. 1B), and there was no difference in baseline EEG power spectra (data not shown). Representative examples of hippocampal stimulation-evoked seizures for WT vs. AQP4 $-/-$ mice are shown in Fig. 1B. AQP4 $-/-$ mice had a higher mean electrographic seizure threshold ($167 \pm 17 \mu A$) than that of WT controls ($114 \pm 10 \mu A$; $P < 0.01$) (Fig. 1C). AQP4 $-/-$ mice were found to have remarkably more prolonged stimulation-evoked seizures (33 ± 2 s) compared to WT controls (13 ± 2 s) (Fig. 1D). The only change in behavior during seizures (in both genotypes) was postural immobility (Grade 1 in the Racine classification; Racine, 1972).

In Vivo K^+ Measurements

To account for the prolonged seizures in AQP4 $-/-$ mice, we hypothesized that the absence of AQP4 might be

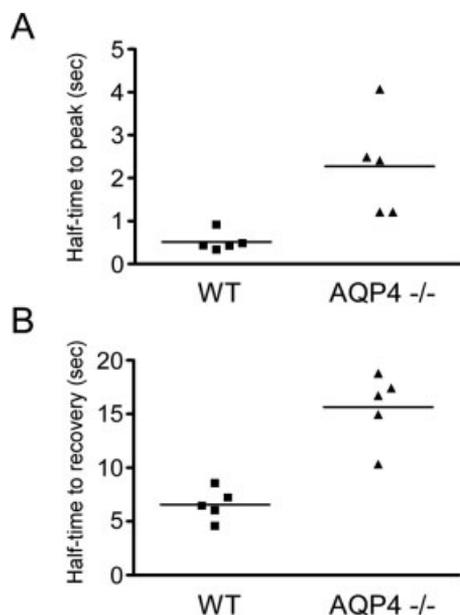


Fig. 3. Half-times for potassium rise to peak and recovery to baseline. **A:** Half-time to peak in WT and AQP4^{-/-} mice. **B:** Half-time to recovery in WT and AQP4^{-/-} mice. Each symbol denotes derived value from one mouse.

associated with impaired K⁺ reuptake. Residual extracellular K⁺ would contribute to ongoing seizure activity and inhibit seizure termination (see Discussion). To address this possibility, we examined K⁺ dynamics in vivo in AQP4^{-/-} mice.

Potassium measurements were made from intact cortex with double-barreled K⁺-sensitive microelectrodes (Fig. 2A). Before and after each in vivo measurement, K⁺ calibration curves were measured (Fig. 2B). Stimulation produced an amplitude-dependent rise in [K⁺]_o that rapidly returned to baseline over a few seconds. Figure 2C demonstrates representative time courses of [K⁺]_o after stimulation intensities of 1, 5, 10, and 20 μ A in a WT mouse. These curves were reproduced at least three times in each animal at each stimulation intensity with similar results. Higher stimulation intensities (100 and 500 μ A) were associated with a much greater rise in [K⁺]_o to 30–50 mM with a very slow recovery to baseline, characteristic of spreading depression (data not shown) (Vyskocil et al., 1972; Lian and Stringer, 2004).

The kinetics of potassium rise and decay were quite different in AQP4^{-/-} mice. Figure 2D demonstrates representative K⁺ curves (at 20 μ A stimulation intensity) from three WT mice and three AQP4^{-/-} mice. Qualitative inspection of the curves reveals a slower rise and decay time in AQP4^{-/-} mice.

To quantify K⁺ kinetics, we derived half-times ($t_{1/2}$) for rise to peak and decay to baseline. Because the higher stimulation intensities were associated with spreading depression-like events, we analyzed rise and decay kinetics in the family of curves with intermediate stimulation intensity (20 μ A). After stimulation, the $t_{1/2}$ to peak was significantly delayed in AQP4^{-/-} mice (2.3 ± 0.5 s

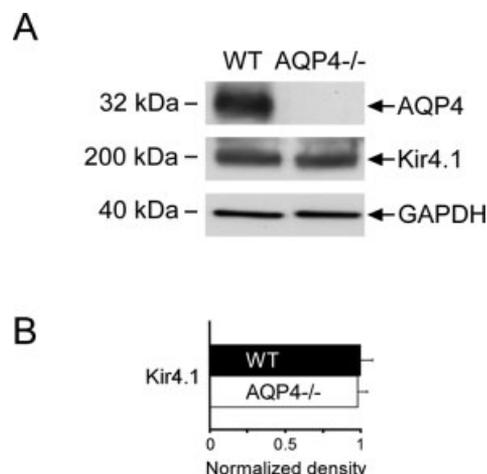


Fig. 4. Western blot analysis of Kir_{4.1} protein expression in WT vs. AQP4^{-/-} mice. **A:** As expected, no AQP4 protein was detected in AQP4^{-/-} mice (top). Kir_{4.1} expression was unaltered in AQP4^{-/-} mice (middle). GAPDH served as an internal control (bottom). **B:** Densitometric analysis demonstrated no difference in Kir_{4.1} protein expression in WT vs. AQP4^{-/-} mice.

vs. 0.5 ± 0.1 s; $P < 0.01$) (Fig. 3A). In addition, the $t_{1/2}$ to recovery was significantly delayed in AQP4^{-/-} mice (15.6 ± 1.5 s vs. 6.6 ± 0.7 s; $P < 0.001$) (Fig. 3B). Analysis of the K⁺ kinetics from lower-intensity (10- μ A) stimulation similarly revealed delayed K⁺ rise ($t_{1/2}$ to peak, 2.4 ± 0.2 s vs. 0.9 ± 0.1 s; $P < 0.01$) and K⁺ recovery ($t_{1/2}$ to recovery, 14.5 ± 1.1 s vs. 7.0 ± 0.3 s; $P < 0.001$) in AQP4^{-/-} mice compared to WT controls. At the lowest stimulation intensities (1 and 5 μ A) a significant difference was still observed (5 μ A: $t_{1/2}$ to peak, 1.6 ± 0.1 s vs. 0.7 ± 0.1 s; $t_{1/2}$ to recovery, 10.5 ± 1.4 s vs. 4.8 ± 0.5 s; 1 μ A: $t_{1/2}$ to peak, 1.1 ± 0.1 s vs. 0.17 ± 0.03 s; $t_{1/2}$ to recovery, 3.4 ± 0.4 s vs. 1.8 ± 0.8 s; all $P < 0.01$, AQP4^{-/-} vs. WT).

To compare baseline and peak potassium ion concentrations, we converted the voltage data into potassium ion concentration. Baseline [K⁺]_o (mM) did not differ between WT (3.6 ± 0.3) and AQP4^{-/-} (4.0 ± 0.4) mice. Peak [K⁺]_o (mM) also did not differ significantly between WT (11.2 ± 0.7) and AQP4^{-/-} (10.4 ± 0.7) mice.

To determine whether there was an alteration in expression of the glial inwardly rectifying K⁺ channel Kir_{4.1} in AQP4^{-/-} mice, we performed Western blot analysis.

As expected, AQP4 immunoreactivity was absent from the AQP4^{-/-} mice (Fig. 4A). Western blot indicated no alteration in expression of Kir_{4.1} in AQP4^{-/-} mice compared to WT mice (Fig. 4A). Densitometric analysis of Western blot results showed no difference in Kir_{4.1} protein expression (Fig. 4B).

DISCUSSION

We used in vivo electrophysiological techniques to address the role of AQP4 in modulation of seizure activity and in K⁺ homeostasis, which produced several novel findings. First, AQP4^{-/-} mice have an increased electro-

graphic seizure threshold. Second, AQP4 $-/-$ mice have a remarkably prolonged electrographic seizure duration in response to hippocampal stimulation. Third, baseline $[K^+]_o$ and stimulation-evoked peak $[K^+]_o$ do not differ between AQP4 $-/-$ and WT mice. Fourth, the kinetics of $[K^+]_o$ after cortical stimulation are appreciably slowed in AQP4 $-/-$ mice, with a delay in both rise to peak and decay to baseline. These results establish a novel physiological role for glial AQP4 in modulating neuronal excitability and K^+ regulation in vivo.

Together with our previous results using pentylene-tetrazol (PTZ) (Binder et al., 2004a), these results demonstrate increased seizure threshold in AQP4 $-/-$ mice. Why should the absence of AQP4 increase seizure threshold? One possibility is altered ECS volume. Relative cellular and ECS volumes have been demonstrated to play an important role in propensity to epileptic seizures. Decreasing ECS volume produces hyperexcitability and enhanced epileptiform activity (Dudek et al., 1990; Roper et al., 1992; Chebabo et al., 1995; Pan and Stringer, 1996). Conversely, hyperosmolar medium attenuates epileptiform activity (Traynelis and Dingledine, 1989; Dudek et al., 1990; Pan and Stringer, 1996). These experimental data parallel extensive clinical experience indicating that hyposmolar states such as hyponatremia lower seizure threshold, whereas hyperosmolar states elevate seizure threshold (Andrew et al., 1989).

Because seizure propensity is sensitive to ECS volume, we hypothesized that AQP4 deletion increased ECS volume fraction and thus reduced seizure susceptibility. We recently found evidence for an expanded ECS in AQP4 $-/-$ mice using in situ cortical fluorescence recovery after photobleaching of extracellular fluorescein-dextran (Binder et al., 2004b). Thus, more intense stimuli (higher current intensities in our current study and higher PTZ doses in our previous study; Binder et al., 2004a) may be required to overcome the expanded ECS of AQP4 $-/-$ mice to initiate a seizure. Consistent with this hypothesis, it appears that the ECS constricts just before seizure onset (Binder et al., 2004b). This raises the interesting possibility that ECS constriction may be necessary for seizure onset. If so, a larger baseline ECS and/or slowed depolarization-induced ECS constriction after seizure-inducing stimuli would limit seizure susceptibility in AQP4 $-/-$ mice.

More dramatic than the effect on seizure threshold was the nearly threefold increase in seizure duration in AQP4 $-/-$ animals. We hypothesized that the absence of AQP4 was associated with impaired K^+ reuptake. Impaired K^+ clearance from the ECS after the intense neuronal activity accompanying the seizure would lead to prolonged depolarization of neurons and inhibit seizure termination (Rutecki et al., 1985; Yaari et al., 1986; Traynelis and Dingledine, 1988; Steinhäuser and Seifert, 2002). During rapid neuronal firing, $[K^+]_o$ increases from about 3 to 10–12 mM, and potassium released by active neurons is thought to be primarily taken up by glial cells (Heinemann and Lux, 1977; Sykova, 1997; Xiong and Stringer, 1999; Somjen, 2002). Such K^+ reuptake into glial cells could be AQP4-dependent because water influx coupled to

K^+ influx is thought to underlie activity-induced glial cell swelling (Walz, 1987, 1992). In support of this possibility is the subcellular co-localization of AQP4 with the inwardly rectifying potassium channel $K_{ir}4.1$ (Connors et al., 2004; Nagelhus et al., 2004). $K_{ir}4.1$ is thought to contribute to K^+ reuptake and spatial K^+ buffering by glial cells because pharmacological or genetic inactivation of $K_{ir}4.1$ leads to impairment of extracellular K^+ regulation (Ballanyi et al., 1987; Kofuji and Newman, 2004).

Our K^+ data support the hypothesis that there was a deficit in extracellular K^+ clearance in AQP4 $-/-$ mice. Both baseline $[K^+]_o$ and the “Lux–Heinemann ceiling” level of activity-induced physiologic $[K^+]_o$ elevation (~12 mM) (Heinemann and Lux, 1977; Somjen, 2002) were not altered in AQP4 deficiency, indicating that basic K^+ homeostasis was intact. However, there was a distinction in kinetics, with a considerable delay in rise and decay time. A similar delay in K^+ kinetics has been observed after cortical spreading depression in AQP4 $-/-$ mice using a novel fluorescent K^+ sensor (Padmawar et al., 2005). Slowed $[K^+]_o$ rise time is consistent with increased ECS volume fraction in AQP4 $-/-$ mice (Binder et al., 2004b). Slowed $[K^+]_o$ decay is likely the result of impaired K^+ reuptake into AQP4 $-/-$ astrocytes. To investigate this possibility, in vitro studies of K^+ influx into cultured AQP4 $-/-$ astrocytes will be required. We found no alteration in expression of $K_{ir}4.1$ protein in AQP4 $-/-$ mice, but its subcellular distribution and function remain to be characterized.

Mice deficient in α -syntrophin, an adapter protein in the dystrophin-containing protein complex (Neely et al., 2001), demonstrate altered subcellular distribution of AQP4, and afferent stimulation of hippocampal slices from α -syntrophin-deficient mice demonstrated a deficit in extracellular K^+ clearance after evoked neuronal activity (Amiry-Moghaddam et al., 2003b). Together with our findings, these data suggest that AQP4 and its molecular partners together constitute a multifunctional “unit” responsible for clearance of K^+ and/or H_2O after neural activity.

In summary, our results implicate the glial water channel AQP4 in direct modulation of brain excitability, and add to a growing body of evidence implicating nonsynaptic mechanisms in seizure physiology (Schwartzkroin et al., 1998). Our potassium data demonstrate a mechanistic link between AQP4 and K^+ homeostasis in vivo. Further investigation of this mechanism will require measurements of water and potassium fluxes using simultaneous monitoring of ECS and K^+ together with pharmacological and genetic dissection of the contribution of specific ion channels to K^+ reuptake in glial cells. Interestingly, the expression and subcellular localization of AQP4 have recently been shown to be altered in sclerotic hippocampi obtained from patients with medically intractable temporal lobe epilepsy (Lee et al., 2004; Eid et al., 2005). Thus, further understanding of the glial modulation of ECS ion and water homeostasis may lead to new understanding of epileptogenic tissue as well as novel concepts and targets for anticonvulsant drug development.

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