AQP4 knockout impairs proliferation, migration and neuronal differentiation of adult neural stem cells

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Summary
Aquaporin-4 (AQP4), a key molecule for maintaining water and ion homeostasis in the central nervous system, is expressed in adult neural stem cells (ANSCs) as well as astrocytes. However, little is known about the functions of AQP4 in the ANSCs in vitro. Here we show that AQP4 knockout inhibits the proliferation, survival, migration and neuronal differentiation of ANSCs derived from the subventricular zone of adult mice. Flow cytometric cell cycle analysis revealed that AQP4 knockout increased the basal apoptosis and induced a G2-M arrest in ANSCs. Using Fluo-3 Ca²⁺ imaging, we show that AQP4 knockout alters the spontaneous Ca²⁺ oscillations by frequency enhancement and amplitude suppression, and suppresses KCN-induced Ca²⁺ influx. AQP4 knockout downregulated the expression of connexin43 and the L-type voltage-gated Ca²⁺ channel CaV1.2 subtype in ANSCs. Together, these findings suggest that AQP4 plays a crucial role in regulating the proliferation, migration and differentiation of ANSCs, and this function of AQP4 is probably mediated by its action on intracellular Ca²⁺ dynamics.

Key words: Aquaporin-4, Adult neural stem cells, Calcium oscillations, Connexin43, L-type calcium channel

Introduction
Aquaporins are a family of water channel proteins that provide a major pathway for osmotically driven water transport through cell membranes. So far, 13 aquaporin isoforms (AQP0-AQP12) have been identified in mammalian species (Verkman, 2005). AQP4, the predominant isoform in adult brain, is primarily expressed at the border between brain parenchyma and major fluid compartments, including astrocyte foot processes and glia limitans, as well as ependymal cells and subependymal astrocytes (Venero et al., 2001). The bidirectional water channel AQP4 has an important role in water homeostasis in the brain. It probably helps in the redistribution and absorption of edema fluid, because disruption of AQP4 is found to contribute to the pathophysiology of brain edema (Zador et al., 2005). AQP4 knockout markedly reduced brain swelling in mouse models of cytotoxic brain edema, whereas it significantly worsened outcome in mouse models of vasogenic brain edema (Papadopoulos and Verkman, 2007). Thus, AQP4 appears to facilitate water movement into astroglia in cytotoxic edema, as well as water movement out of the brain in vasogenic edema. An intriguing discovery is the detection of AQP4 autoantibodies in the sera of human patients with neuromyelitis optica (NMO; Devic’s syndrome), which is a demyelinating condition (Lemon et al., 2005). This finding has led to the development of an objective diagnostic test for NMO. AQP4 knockout increased seizure threshold and duration (Binder et al., 2004; Binder et al., 2006), suggesting that astroglial AQP4 modulates neuronal excitability by regulating osmotic and ionic environments surrounding neurons.

In addition to providing structural and trophic supports for neurons, astrocytes are known to modulate the local environment around neural stem cells (Doetsch, 2003). Moreover, astrocytes also have an important role in supporting adult neurogenesis through the secretion of neurotrophic factors (Song et al., 2002). Since AQP4 is predominantly expressed in astrocytes, we hypothesize that astrocytic functions in adult neurogenesis may depend on the function of AQP4. This hypothesis is based on the following observations. First, AQP4 has a vital role in the regulation of astrocytic function, including local ion and pH homeostasis and neurotransmission regulation. For example, AQP4 knockout alters the basal level of amino acids (Fan et al., 2005), slows the cellular K⁺ release and uptake in the brain (Padmawar et al., 2005) and downregulates glutamate uptake and glutamate transporter-1 (GLT-1) expression in astrocytes (Zeng et al., 2007). These microenvironmenal changes induced by AQP4 knockout are potential factors influencing adult neurogenesis. Second, AQP4 knockout strongly inhibits the formation of glial-derived neurotrophic factor (GDNF) in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated Parkinson disease (PD) mice (Fan et al., 2008). GDNF is a member of the TGFβ superfamily, signals via the tyrosine kinase receptor c-Ret and the GDNF receptors GFRA. It augments survival and differentiation of TH-positive neurons in neural progenitor cells in vitro (Sun et al., 2004) and promotes adult neurogenesis in vivo (Chen et al., 2007). Third, AQP4 is extensively expressed in the brain regions where adult neurogenesis is found (Venero et al., 2001). In the normal brain, they are the subventricular zone (SVZ) in the lateral wall of the lateral ventricle and the subgranular zone (SGZ) of the hippocampus (Ming and Song, 2005). In the damaged brain, adult neurogenesis is also found in the neocortex, striatum, amygdala and substantia nigra (Gould, 2007) where AQP4 has a high level of expression. Finally, it is reported that AQP4 is the main subtype of aquaporin in adult neural stem cells (ANSCs) (Cavazzin et al., 2006; La Porta et al., 2006; Schwartz et al., 2005).

The main purpose of the present study was to test the hypothesis that AQP4 played a critical role in regulating the fundamental properties of ANSCs and to delineate the mechanisms underlying its action. Under osmotic stress, water flux through AQP4 is bidirectional and driven solely by osmotic gradients. AQP4
knockdown or knockout slowed the kinetics of astrocyte volume changes after hypo-osmotic challenge (Nicchia et al., 2000; Solenov et al., 2004). Meanwhile, hypotonic conditions induced intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]) increases in different cell types (Pasantes-Morales et al., 2000). Therefore, we hypothesized that AQ4P might influence adult neurogenesis by modulating Ca\(^{2+}\) signaling in ANSCs. Ca\(^{2+}\) is a universal ionic second messenger that regulates a great number of diverse cellular processes including cell proliferation, motility, differentiation, development, learning and memory (Berridge et al., 2003; Berridge et al., 2000). Spontaneous Ca\(^{2+}\) oscillations are observed in some cell types during proliferation or differentiation (Parri et al., 2001; Sauer et al., 1998; Scemes et al., 2003). It is thought that Ca\(^{2+}\) oscillations can change the threshold for the activation of Ca\(^{2+}\)-dependent transcription factors and prevent the toxic effects of a sustained increase in [Ca\(^{2+}\)] (Hu et al., 1999; Thellung et al., 2000). Ca\(^{2+}\) oscillations reduce in frequency and amplitude, reducing the threshold for activation of Ca\(^{2+}\)-dependent transcription factors, suggesting that this signaling system has a high level of specificity for cellular functions (Dolmetsch et al., 1997; Tomida et al., 2003; Uhlen et al., 2006).

In the present study, we investigated the proliferation, migration and differentiation of Aqp4\(^{+/+}\) and Aqp4\(^{-/-}\) ANSCs in vitro to determine whether AQ4P regulates the fundamental properties of ANSCs. Spontaneous Ca\(^{2+}\) oscillations and KCl-depolarization-induced Ca\(^{2+}\) transients were also investigated using Fluo-3 Ca\(^{2+}\) imaging.

**Results**

**AQ4P knockout impaired ANSC proliferation and self-renewal in vitro**

Fig. 1A shows the number and diameter of neurospheres derived from Aqp4\(^{+/+}\) and Aqp4\(^{-/-}\) ANSCs observed under a microscope. The adult Aqp4\(^{-/-}\) SVZ cultures generated fewer neurospheres that were smaller in diameter than those derived from Aqp4\(^{+/+}\) mice. To further determine the effect of AQ4P knockout on self-renewal of ANSCs, secondary and tertiary neurosphere formation efficiencies were assayed. Aqp4\(^{+/+}\) ANSCs exhibited a decreased capacity to generate neurospheres following serial subcloning, suggesting an impaired self-renewal (P<0.05 vs Aqp4\(^{+/+}\) genotype) (Fig. 1A-C). Furthermore, AQ4P knockout resulted in a significantly reduced proliferation of ANSCs (Aqp4\(^{+/+}\), 22±1.3%; Aqp4\(^{-/-}\), 8.5±0.8%; n=4, P<0.05) (Fig. 1D,E) as shown by lower BrdU incorporation into cultured ANSCs.

**AQ4P knockout increases basal apoptosis and induces G2-M arrest in ANSCs**

The cell cycle characteristics of Aqp4\(^{+/+}\) and Aqp4\(^{-/-}\) ANSCs were examined by using flow cytometric analysis of propidium iodide fluorescence. Frequency histograms of the DNA content revealed no significant differences in the proportion of G0-G1 phase and S phase between Aqp4\(^{+/+}\) and Aqp4\(^{-/-}\) ANSCs (Fig. 2A). However, the proportion of cells in G2-M phase was increased in Aqp4\(^{-/-}\) ANSCs (6.7±0.3%) compared with the Aqp4\(^{+/+}\) cells (5.2±0.3%, n=3, P<0.05 vs Aqp4\(^{+/+}\)). AQ4P knockout also significantly increased the basal apoptosis of ANSCs (Aqp4\(^{+/+}\), 0.9±0.3%; Aqp4\(^{-/-}\), 4.0±0.2%; n=3, P<0.05) (Fig. 2B), suggesting that AQ4P is essential for the survival of ANSCs.

**AQ4P knockout disrupts migration of ANSCs**

An in vitro migration assay was used to determine whether AQ4P knockout could alter the mobility of ANSCs. Neurospheres with similar diameters were initially selected (Aqp4\(^{+/+}\), 211.4±8.3 μm; n=19 neurospheres; Aqp4\(^{-/-}\), 206.9±7.1 μm; n=18 neurospheres from three independent experiments; P=0.68) and plated on coated coverslips. Neurospheres cultured on coverslips migrated outwards radially after adhesion (Fig. 3A). As shown in Fig. 3B, AQ4P knockout significantly attenuated radial migration of ANSCs out of neurospheres compared with the Aqp4\(^{+/+}\) control (Aqp4\(^{-/-}\), 357.3±17.5 μm; n=18 neurospheres; Aqp4\(^{+/+}\), 701.2±23.6 μm; n=19 neurospheres, P<0.001).

**AQ4P knockout decreases neuronal differentiation of ANSCs**

Dissociated ANSCs were differentiated for 7 days to determine their ability to generate multiple neural cell lineages. As shown in Fig. 4A, both genotypic ANSCs could generate neurons (TUJ1), astrocytes (GFAP) and oligodendrocytes (GalC) in vitro. To investigate the role of AQ4P in neuronal and astroglial differentiation of ANSCs, the percentage of TUJ1\(^+\) and GFAP\(^+\) cells was quantified by normalizing total TUJ1\(^+\) or GFAP\(^+\) cells to the total number of cell nuclei labeled with Hoechst 33342. AQ4P knockout significantly decreased the neuronal differentiation of ANSCs compared with the Aqp4\(^{+/+}\) control (Aqp4\(^{+/+}\), 14.9±2.3%; Aqp4\(^{-/-}\), 7.6±1.3%; n=3, P<0.05) (Fig. 4B,C). The proportion of GFAP\(^+\) cells was not significantly different between these two genotypes (Aqp4\(^{+/+}\), 70.5±2.2%; Aqp4\(^{-/-}\), 75.5±4.1%; n=3, P=0.35) (Fig. 4D).
AQP4 knockout alters Ca2+ oscillations and suppresses depolarization-induced Ca2+ transient in ANSCs

After 24 hours in culture, the cells that had emigrated out of the remaining neurospheres expressed nestin, an intermediate filament protein of undifferentiated neural cells (Fig. 5A). Therefore, these cells were classed ANSCs. In the Aqp4+/+ cell population, more than 95% of Hoechst-33342-labeled cells were also labeled with both AQP4 and nestin (Fig. 5A). After 24 hours in culture, 80% (151 of 189) of Aqp4+/+ ANSCs displayed spontaneous Ca2+ activity. The spontaneous intracellular Ca2+ oscillations observed in ANSCs exhibited variable durations (0.46±0.03; n=40 cells) was significantly lower than that of AQP4–/– ANSCs population. (B) There was no significant difference in the proportion of cells at S phase between Aqp4+/+ and Aqp4–/– ANSC populations. The proportion of G2-M phase cells was increased in Aqp4–/– ANSCs compared with the wild type (*P<0.05, n=3). Moreover, AQP4 knockout significantly increased the basal apoptosis of ANSCs (*P<0.05, n=3).

AQP4 knockout decreases expression of Cav1.2 and connexin43

KCl depolarization-induced Ca2+ influx depends on the opening of voltage-gated Ca2+ channels. The transcription of two main voltage-gated Ca2+ channels, L-type (Cav1.2 and Cav1.3) and T-type (Cav3.1, Cav3.2 and Cav3.3), in both genotypic ANSCs were screened by reverse transcription polymerase chain reaction (RT-PCR). The transient receptor potential channel 1 (TRPC1), a non-voltage-gated Ca2+ channel was also examined. As shown in Fig. 6A, both Aqp4+/+ and Aqp4–/– ANSCs expressed mRNA encoding Cav1.2 (668 bp), Cav3.1 (578 bp) and TRPC1 (372 bp). By contrast, mRNAs of genes encoding Cav1.3 (326 bp), Cav3.2 (298 bp) and Cav3.3 (404 bp) were not detectable. In Aqp4+/+ ANSCs, Cav1.2 and Cav3.1 mRNA was significantly decreased when normalized by comparison with mRNA from the housekeeping gene GAPDH (Fig. 6B). However, no significant difference was found in the transcription of TRPC1 between Aqp4+/+ and Aqp4–/– ANSCs. Western blot analysis showed that AQP4 knockout significantly inhibited the production of Cav1.2 protein to 50.7% of that in Aqp4+/+ ANSCs (P<0.05, n=3). Cav3.1 was not detectable in either Aqp4+/+ or Aqp4–/– ANSCs.

Connexin43 (Cx43), one isoform of the connexin family, is the major connexin expressed in neural stem cells and has a vital role in cellular communication and cell proliferation or differentiation (Scemes et al., 2003). As shown in Fig. 6D, AQP4 knockout also significantly inhibited the expression of Cx43 in ANSCs to 39% of that in the Aqp4+/+ control (P<0.05, n=3).

Discussion

AQP4 is one of the predominant aquaporins in ANSCs as well as in astrocytes in the brain (Cavazzin et al., 2006). In the present study, we showed that AQP4 knockout inhibited proliferation, migration and neuronal differentiation of ANSCs in vitro. Ca2+ imaging analysis revealed that AQP4 knockout enhanced the frequency and suppressed the amplitude of spontaneous Ca2+ oscillation and inhibited the Ca2+ transient induced by high concentration KCl depolarization in ANSCs. Furthermore, AQP4...
knockout caused significant downregulation of the expression of connexin43 and Cav1.2 in ANSCs. These findings suggest that AQP4 might regulate fundamental properties of ANSCs through the Ca\(^{2+}\)-related signaling pathway.

The spatial and temporal pattern of Ca\(^{2+}\) influx is crucial in the regulation of several cellular processes (Clapham, 2007; Schuster et al., 2002). The stable increase in [Ca\(^{2+}\)]\(_i\) and oscillations of [Ca\(^{2+}\)]\(_i\) are a nearly universal mode of signaling in both excitable and non-excitatory cells (Dupont et al., 2007). In neural stem cells, spontaneous Ca\(^{2+}\) oscillations occur without stimulation by agonists (Scemes et al., 2003). AQP4 knockout altered the rhythm of spontaneous Ca\(^{2+}\) oscillations by frequency enhancement and amplitude suppression, which might inhibit activation of some Ca\(^{2+}\)-dependent transcription factors (Kupzig et al., 2005; Lipskaia and Lompre, 2004). For example, nuclear factor of activated T cells (NFAT) is a well documented transcription factor regulated by Ca\(^{2+}\) oscillations (Kawano et al., 2006; Lipskaia and Lompre, 2004). For example, nuclear factor of activated T cells (NFAT) is a well documented transcription factor regulated by Ca\(^{2+}\) oscillations (Kupzig et al., 2005; Lipskaia and Lompre, 2004). For example, nuclear factor of activated T cells (NFAT) is a well documented transcription factor regulated by Ca\(^{2+}\) oscillations (Kawano et al., 2006; Lipskaia and Lompre, 2004). For example, nuclear factor of activated T cells (NFAT) is a well documented transcription factor regulated by Ca\(^{2+}\) oscillations (Kawano et al., 2006; Lipskaia and Lompre, 2004).

Connexins, a family of proteins that form gap junctions, allow cells to share small molecules (<1 kDa), such as metabolites and ions (Nakase and Naus, 2004). Functional gap junction channels are formed by docking and opening of two hemichannels in contacting membranes of adjacent cells. It is well recognized that gap junctional intercellular communication has an important role in the control of cell growth and differentiation in different cell types and tissues (Cheng et al., 2004; Duval et al., 2002; Saez et al., 2003). Cx43, an isoform of connexins, is abundantly expressed in both undifferentiated neural stem cells or neurospheres (Scemes et al., 2003). It was demonstrated that Cx43 knockout inhibited proliferation and migration of neural progenitors in vitro (Cheng et al., 2004; Scemes et al., 2003). In the present study, we show that AQP4 knockout resulted in a significant downregulation of Cx43 expression in ANSCs. Therefore, it is reasonable that the downregulation of Cx43 might contribute to the inhibition of proliferation and migration in Aqp4\(^{-/-}\) ANSCs.

Our study shows that AQP4 knockout altered spontaneous Ca\(^{2+}\) oscillations in ANSCs and inhibited the KCl-induced Ca\(^{2+}\) transient. Depolarization-induced Ca\(^{2+}\) influx depends on the opening of voltage-gated Ca\(^{2+}\) channels (Lacinova, 2005). Consistently, AQP4 knockout also resulted in a significant downregulation of the Cav1.2 subunit of L-type Ca\(^{2+}\) channel at both mRNA and protein levels. The Cav1.2 channel is a high-voltage-activated Ca\(^{2+}\) channel. In mature neurons, this channel opens primarily in response to the depolarization provided by excitatory synaptic inputs (Weisskopf et al., 1999). Measurements of resting potential indicate that neural stem cells maintain resting potentials in the –55 mV range (Wang et al., 2003). Since neural stem cells tend to be more depolarized than mature quiescent neurons, Cav1.2 channels might partially open without depolarization stimulus (Wang et al., 2003; Westerlund et al., 2003). Blockade of L-type Ca\(^{2+}\) channels inhibits neurogenesis both in vivo and in vitro (D’Ascenzo et al., 2006; Deisseroth et al., 2006).
AQP4 regulates functions of ANSCs

2004; Luo et al., 2005; Wang et al., 2005). Nifedipine, a L-type Ca\textsuperscript{2+} channel blocker, inhibits the proliferation of neural stem cells under the resting, non-stimulated condition (Deisseroth et al., 2004). Thus, our finding that downregulation of Cav1.2 by knockout of AQP4 provides additional evidence for the functional involvement of L-type Ca\textsuperscript{2+} channel in the proliferation inhibition of ANSCs in vitro. In the cell cycle, Ca\textsuperscript{2+} regulates progression through several checkpoints, including the G1-S-phase transition, G2-M-phase transition and the exit of mitosis (Kahl and Means, 2003; Takuwa et al., 1995; Whitaker and Patel, 1990). Especially in G2-M-phase cells, Ca\textsuperscript{2+} influx via L-type channels promotes progression through mitosis (Ramsdell, 1991; Uehara et al., 1992). Accordingly, G2-M-phase arrest in Aqp4\textsuperscript{−/−} ANSCs was consistent with the finding of Cav1.2 downregulation. Furthermore, the L-type Ca\textsuperscript{2+} channel is essential for cell survival. Blockage or deletion of the L-type Ca\textsuperscript{2+} channel increases apoptosis in a variety of cells (Benoff et al., 2005; Florio et al., 1998; Galli et al., 1995; Marshall et al., 2003; Thellung et al., 2000). Therefore, the increased basal apoptosis of Aqp4\textsuperscript{−/−} ANSCs might be associated with the downregulation of Cav1.2.

Intracellular Ca\textsuperscript{2+} spikes trigger cell differentiation as well as proliferation. Alterations in the intracellular Ca\textsuperscript{2+} activities could induce embryonic stem cells to differentiate into neuronal phenotypes (Ulrich and Majumder, 2006). In the nervous systems, the pathways underlying Ca\textsuperscript{2+} influx involve voltage-dependent and transmitter-activated channels (Arundine and Tymianski, 2003). Neural stem cells possess functional L-type voltage-dependent Ca\textsuperscript{2+} channels, which are strongly correlated with neuronal differentiation (D’Ascenzo et al., 2006). In cultured neural stem cells, blockade of L-type Ca\textsuperscript{2+} channel abolishes the enhancement of neuronal differentiation by different stimuli (D’Ascenzo et al., 2006; Liu et al., 2007; Piacentini et al., 2008). Moreover, blockade of L-type Ca\textsuperscript{2+} channel also inhibits neuronal differentiation even in resting, nonstimulated conditions (Deisseroth et al., 2004). Ca\textsuperscript{2+} influx through the L-type Ca\textsuperscript{2+} channel inhibits expression of the glial fate genes Hes1 and Id2 and increases expression of NeuroD, a positive regulator of neuronal differentiation (Deisseroth et al., 2004). Thus, the reduced neuronal differentiation of Aqp4\textsuperscript{−/−} ANSCs could be a result of the downregulated expression of Cav1.2. Furthermore, our study showed that AQP4 knockout failed to alter glial differentiation. These findings suggest that AQP4 preferentially regulates the neuronal differentiation rather than glial differentiation.

It has been demonstrated that aquaporins facilitate cell migration (Papadopoulos et al., 2007). AQP-dependent cell migration has been
found in a variety of cell types in vitro and in vivo (Papadopoulos et al., 2007). Based on our results and evidence from other reports, we propose the following potential mechanism whereby AQP4 regulates the migration of ANSCs. At the initial stage of cell migration, AQP4 might facilitate Ca\(^{2+}\) influx (Faux and Parnavelas, 2007), which directly regulates actin depolymerization (Disanza et al., 2005; Yoneda et al., 2000). Ion influx increases cytoplasmic osmolality followed by water influx via AQP4 at the front end of the migrating cell (Papadopoulos et al., 2007). Water influx increases local hydrostatic pressure causing cell membrane expansion, which forms a protrusion subsequently (Papadopoulos et al., 2007). At the final stage, actin re-polymerizes to stabilize the emerging protrusion (Papadopoulos et al., 2007). AQP4 knockout could slow the migration of astrocytes in vivo and in vitro (Auguste et al., 2007; Saadoun et al., 2005). Similarly, blockade of L-type Ca\(^{2+}\) channel also inhibits migration of different cells in vitro (Ruiz-Torres et al., 2003; Yang and Huang, 2005) and impairs glia migration in vivo (Lohr et al., 2005). Therefore, downregulation of Cav1.2 induced by AQP4 knockout in ANSCs might involve the inhibition of migration in vitro.

In conclusion, our study demonstrates that AQP4 might be involved in the proliferation, survival, migration and neuronal differentiation of ANSCs by regulating intracellular Ca\(^{2+}\) dynamics in ANSCs, including spontaneous Ca\(^{2+}\) oscillations and Ca\(^{2+}\) transients.

Materials and Methods

Generation of Aqp4\(^{-/-}\) mice

AQP4-knockout mice were generated as previously described (Fan et al., 2005). Mice were kept under environmentally controlled conditions (ambient temperature, 22±1°C; humidity, 40%) on a 12 hour light-dark cycle with food and water ad libitum. All experiments were approved by IACUC (Institutional Animal Care and Use Committee) of Nanjing Medical University. All efforts were made to minimize animal suffering and to reduce the number of animals used for the experiments.

NSC culture and neurosphere assay

Primary cultures of ANSCs were established as described (Ferron et al., 2007; Gritti et al., 1999). Briefly, brains of 2- to 3-month-old male CD1 mice were microdissected to obtain the rostral periventricular region upon coronal sectioning. Tissues were finely minced and dissociated with papain enzymatic solution by incubation at 37°C for 30 minutes. After centrifugation, cells were carefully dissociated by passaging in fire-polished Pasteur pipettes and resuspended in serum-free medium consisting of DMEM/F12 (1:1) medium (Gibco), supplemented with 2% B27 (Gibco), and 20 ng/ml of EGF and 20 ng/ml of bFGF (Peprotech). The primary cell culture technique used for this study was the neurosphere assay (Reynolds and Weiss, 1992; Seaberg and van der Kooy, 2002). Cell viability was assessed using trypan blue exclusion (0.4%, Sigma) and was >85%. Primary tissue was plated at 10,000 cells per well in uncoated 24-well culture plates. The total number and size of spheres that formed in each well was counted after 7 days in vitro (DIV). Self-renewal was also assayed by dissociating neurospheres in bulk and reculturing the cells at a constant density of 10,000 cells per well. The number and size of secondary or tertiary spheres were determined after 7 DIV. Neurospheres were counted under an optical microscope, with a minimum cutoff of 40 μm in diameter. The diameter of randomly chosen neurospheres was determined using Image-Pro Plus software.

Cell proliferation assays

Proliferation was determined using 5-bromodeoxyuridine (BrdU) incorporation (Ferron et al., 2007). Briefly, neurospheres were collected and gently mechanically dissociated. Dissociated cells were plated on 24-well culture plates coated with poly-
L-ornithine (10 mg/ml) and laminin (5 μg/ml) and cultured for 48 hours. Cells were incubated for exactly 30 minutes with 10 μM BrdU, and then fixed and washed. DNA was denatured by treating the cells for 30 minutes with 2 N HCl at 37°C. Cells were extensively washed and blocked with 5% bovine serum albumin (40 minutes), and the primary mouse anti-BrdU antibody (1:15,000, Chemicon) was applied and incubated for 1 hour at 37°C followed by goat anti-mouse FITC antibody (1:200, Chemicon). The cells were mounted with anti-fade medium (DakoCytomation) and Hoechst 33342 (Sigma).

Cell cycle analysis
Dissociated ANSCs were fixed overnight in 70% ethanol in PBS, resuspended in PBS and treated with 25 μg/ml ribonuclease A (Sigma) for 1 hour at room temperature, followed by staining with 50 μg/ml propidium iodide (PI, Sigma). Argon-ion laser excitation (488 nm) was used to measure PI fluorescence with a 620 nm band-pass filter. Proliferating ANSCs were analyzed by flow cytometry (FACS Calibur).

Adult neural stem cell migration assay
To evaluate the contribution of AQP4 during migration of ANSCs, floating Aqp4+/+ and Aqp4–/– neureospheres of similar diameter were selected and plated on coated 24-well culture plates containing DMEM-F12 supplemented with 2% B27 but without bFGF and EGF for 48 hours. Migration was quantified as the mean difference between the leading edge of radially migrating cells and the original neureosphere diameter.

Adult neural stem cell differentiation
Dissociated ANSCs from neureospheres were seeded on coated coverslips at a density of 10,000 cells/ml in DMEM-F12, containing 1% fetal bovine serum (BSA, Gibco). After 7 days of differentiation, cells were probed with antibodies against neuron-specific beta-3 tubulin (TUJ1; 1:500, Chemicon), GFAP (1:4000, ABCam), galactocerebroside (GalC; 1:200, Chemicon). The percentages of TUJ1+ and GFAP+ cells were quantified by normalizing total TUJ1+ or GFAP+ cells to the total number of cell nuclei labeled with Hoechst 33342 (approximately 500 cells counted per well, n ≥ 3 dissections).

Assay of spontaneous Ca2+ oscillations and KCl-induced Ca2+ transient for cultured ANSCs
Neureospheres were plated on coated coverslips in DMEM-F12 with mitogens for 24 hours. Spontaneous Ca2+ oscillations were measured in cells outgrowing from adherent neureospheres loaded for 30 minutes at 37°C with fluo-3 AM (5 μM; Molecular Probes). Intracellular Ca2+ measurements were performed on cells bathed in imaging PBS three times and incubated in another cocktail solution containing goat anti-rabbit (1:3000, Chemicon), goat anti-mouse FITC (1:200; Chemicon), and goat anti-rat TRITC (1:200; Chemicon) antibodies. The cells were extensively washed and blocked with 5% bovine serum albumin (40 minutes), incubated for exactly 60 minutes with 10 μM BrdU, and then fixed and washed. The expression of each gene was normalized to the amount of GAPDH in order to calculate relative levels of mRNA.

Western blotting
Total cellular proteins of neureospheres were extracted with homogenization buffer. Equivalent amounts of extracted proteins (50 μg) were resolved on 12% for (cav1.2 3%) or 7% for (cav3.1 3%) SDS-PAGE and electrolubilized to PVDF membrane (Amersham Biosciences). After blocking the background staining with 5% skimmed milk in PBS, the membranes were incubated in a mouse anti-con-nexiniv43 antibody (1:500; Chemicon), rabbit anti-Cav1.2 (1:300; Chemicon) and rabbit anti-Cav3.1 (1:200; Chemoon). Antibody against β-actin (1:1000; Sigma) was used as an internal control for protein normalization. Immunoreactive proteins were detected using HRP-conjugated secondary antibodies and an ECL kit (Amersham Biosciences) according to the manufacturer’s instructions. The membranes were scanned and analyzed in an Omega 16ie Chemiluminescence Imaging System (Ultra-Lum, Claremont, CA).

Statistics
All values are expressed as the mean ± s.e.m. Differences between means were analyzed using Student’s t-test. P < 0.05 was defined as significant.

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