Aquaporin-4 Knockout Exacerbates Corticosterone-Induced Depression by Inhibiting Astrocyte Function and Hippocampal Neurogenesis

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SUMMARY

Aims: The predominant expression of aquaporin-4 (AQP4) in the brain implies that this water channel may be involved in a range of brain disorders. This study was designed to investigate the role of AQP4 in the pathogenesis of depression, and related possible biological mechanism. **Methods and Results:** Wild-type (AQP4^{+/+}) and AQP4 knockout (AQP4^{-/-}) mice were given daily subcutaneous injections of corticosterone (20 mg/kg) for consecutive 21 days. Forced swimming test (FST) and tail suspension test (TST) showed longer immobility times in corticosterone-treated AQP4^{-/-} genotype, indicating AQP4 knockout exacerbated depressive-like behaviors in mice. Using immunohistological staining, western blot, and enzyme-linked immunosorbent assay (ELISA), we found a significant loss of astrocytes, aggravated downregulation of excitatory amino acid transporter 2 (EAAT2), synapsin-1, and glial cell line-derived neurotrophic factor (GDNF) in the hippocampus of AQP4^{-/-} mice. Moreover, even less hippocampal neurogenesis was identified in corticosterone-treated AQP4^{-/-} mice *in vivo* and hippocampus-derived adult neural stem cells (ANSCs) *in vitro*. **Conclusions:** The present findings suggest AQP4 involves the pathogenesis of depression by modulating astrocytic function and adult neurogenesis, highlighting a novel profile of AQP4 as a potential target for the treatment for depression.

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Introduction

Depression is one of the leading causes of disability worldwide. Till now, several dysfunctions such as the monoamine variation, hypothalamic-pituitary-adrenal (HPA) axis dysfunction, neurodegeneration and inflammation, and neurogenesis inhibition have been presumed to be involved in the pathogenesis of depression [1]. Despite the extensive research, the neurobiology of depression remains poorly understood due to low rates of heritability and heterogeneity of precipitating factors [2]. Therefore, understanding both the pathophysiology of depression and the neuro-mechanisms of antidepressants will open new perspectives for the management of depression.

As a major component of the neural network, astrocytes play an important role in the neuropathology of major depression. Postmortem histological analysis of the frontal cortex and hippocampus demonstrated a decreased number of astrocytes in patients suffering from major depression [3]. In animal models, selective injury to astrocytes in prefrontal cortex is sufficient to

induce depressive-like behaviors [4]. Moreover, inhibited astrocytic function in hippocampus is sufficient to block the antidepressant effects of imipramine in an animal model of depression [5]. To date, astrocytes have been implicated to contribute to pathogenesis of depression by modulating neurotransmission [6,7], supporting energy metabolism [8], releasing neurotrophin and ATP [7,9], and regulating hippocampal neurogenesis [10]. Thus, astrocytic dysfunction could be a general mechanism contributing to the pathogenesis of depression.

Aquaporin-4 (AQP4), the predominant isoform of aquaporins in adult brain, is primarily expressed in astrocytes throughout the central nervous system, suggesting its involvement in the modulation of astrocytic function. Accumulating data have shown that AQP4 knockout or downregulation caused astrocytic dysfunction such as attenuated brain fluid and ion homeostasis [11–14], impaired cell migration and glial scar formation [11,15], altered neural transmission and synaptic plasticity [16,17], and pro-inflammatory factor secretion [18]. Thus, AQP4 has gained sufficient attention in a wide range of neurological disorders [19], as well as

some psychiatric diseases, including major depressive disorder [3]. However, little is known about the underlying mechanism.

Our previous studies showed that AQP4 knockout abolishes chronic fluoxetine-treatment-induced hippocampal neurogenesis and antidepressant effects [20]. However, the role of AQP4 in the pathogenesis of depression remains obscure. In the present study, using AQP4 knockout mice, we investigated the role of AQP4 in a mouse model of depression induced by repeated corticosterone injections [21].

Materials and methods

All experiments were approved by Institutional Animal Care and Use Committee of Nanjing Medical University.

Drug Administration and Behavioral Tests

Aquaporin-4 gene knockout (AQP4^{-/-}) CD1 mice were generated as described previously [22]. Mice were housed in humidity-(40%) and temperature-controlled (21 ± 2°C) rooms with free access to water on a 12 h light/dark cycle. Age-matched male mice (2–3 month old) were used in the experiments. Mice were given 5-bromo-2-deoxyuridine (BrdU, 150 mg/kg twice a day for three consecutive days; Sigma, St Louis, MO, USA) intraperitoneally 3 days prior to corticosterone treatment. Five wild-type (AQP4^{+/+}) and AQP4^{-/-} mice were killed and perfused with 4% paraformal-

dehyde after 3-day BrdU injection to determine the basal level of cell proliferation in the subgranular zone (SGZ) of hippocampus. Other BrdU-injected mice were randomly assigned to vehicle or corticosterone-treated groups (n = 14–16/group). Corticosterone was injected subcutaneously (20 mg/kg, suspended in physiological saline containing 0.1% dimethyl sulfoxide [DMSO] and 0.1% Tween-80; Sigma) once daily in a volume of 5 ml/kg at random times during the light phase, while the control groups were administrated only with vehicle (Figure 1A). On the 21st day of the paradigm, depression-like behaviors was observed in forced swimming test (FST) at 8:00 am and tail suspension test (TST) at 11:00 am, and last corticosterone was injected at 14:00 pm. Two hours after last corticosterone injection, nine or 10 mice of each group were sacrificed and blood was collected for corticosterone assay and hippocampus for neurochemical measurement. All other mice were killed to isolate the brains for immunohistological study. FST and TST were carried out as described previously [20].

Immunohistological Staining

Immunohistochemistry staining was carried out according to a method previously described [20]. Serial sections were cut (30 μm) through each entire hippocampus. Every sixth section was kept for BrdU (1:500; Millipore, Temecula, CA, USA), neuronal nuclei (NeuN, 1:1000; Millipore), glial fibrillary acidic protein

Figure 1 Aquaporin-4 (AQP4) knockout exacerbated corticosterone-induced depressive-like behaviors. **(A)** Schematic representation of the experimental procedure. **(B)** Corticosterone concentration in the plasma 2 h after last corticosterone injection of 21-day paradigm (n = 9–11 for each group). **(C)** Body weight loss in AQP4^{+/+} and AQP4^{-/-} mice after chronic corticosterone injection (n = 14–16 for each group). **(D, E)** Corticosterone treatment significantly increased immobility times of tail suspension test (TST) **(D)** and Forced swimming test (FST) **(E)** in both genotypic mice, while the immobility times of TST and FST in AQP4^{-/-} mice were significantly longer than AQP4^{+/+} mice (n = 13–15 for each group). *P < 0.05; #P < 0.01.

(GFAP, 1:800; Millipore) immunohistochemistry. The other set of tissue was stained with doublecortin (DCX, 1:500; Cell Signaling Technology, Beverly, MA, USA), Ki67 (1:2500; Abcam, Cambridge, MA, USA) immunofluorescence.

For labeling of BrdU and cell-specific markers, sections were incubated with mouse anti-BrdU and rabbit anti-microtubule-associated protein 2 (MAP2, 1:200; Cell Signaling Technology) or rabbit anti-GFAP (1:1000; Millipore) antibodies followed by incubation with corresponding Alexa Fluor[®] secondary antibodies. Sections were observed under a confocal microscope (Zeiss Axiovert LSM510; Carl Zeiss Co., Jena, Germany) for visualization and photography. At least 50 BrdU-labeled cells per animal were analyzed for double labeling.

For cultured cell immunofluorescence, cells were incubated with rabbit anti-AQP4 antibody (1:800; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-nestin antibody (1:1000; Millipore), mouse anti-GFAP antibody (1:500; Abcam), or rabbit anti-GFAP antibody (1:1000; Millipore), and mouse anti- β -tubulin antibody (TUJ1; 1:500; Santa Cruz). Then, incubated with Alexa Fluor[®] secondary antibodies, cells were observed under a confocal microscope.

Cell Counting

BrdU-positive (BrdU⁺) and Ki67-positive (Ki67⁺) cells were counted using the Optical Fractionator method with Microbrightfield StereoInvestigator software (Stereo Investigator software; Microbrightfield, Williston, VT, USA) as our previously reported [20]. Density of GFAP-positive (GFAP⁺) cells in the hippocampus, NeuN-positive (NeuN⁺) cells in CA1, CA3, and dentate gyrus, and DCX-positive (DCX⁺) cells in the subgranular zone were measured on six to seven sections per animal using StereoInvestigator software. Results are expressed as mean $\pm$ SEM number of cells per mm² [4] (Microbrightfield).

Enzyme-Linked Immunosorbent Assay (ELISA)

The serum corticosterone concentrations after 21-day corticosterone treatment were measured using a commercially available ELISA kit (Abcam). Left lobes of hippocampus were isolated for hippocampal brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) assay by ELISA kits following the instructions provided by the manufacturer (Promega, Madison, WI, USA).

Western Blot Analysis

Right lobe of hippocampus was excised and homogenized in homogenization buffer supplemented with a protease inhibitor cocktail (Life technologies, Gaithersburg, MD, USA). Western blot was performed by standard protocols using anti-AQP4 (1:800; Santa Cruz), anti-excitatory amino acid transporter 1 (EAAT1, 1:1000; Cell Signaling Technology), anti-EAAT2 (1:1000; Cell Signaling Technology), anti-connexin 43 (Cx43, 1:1000; Cell Signaling Technology), anti-postsynaptic density protein 95 (PSD95, 1:750; Abcam), and anti-synapsin 1 (1:500; Abcam). The blots were incubated with HRP-conjugated secondary antibodies, and signals were detected by enhanced chemiluminescent reagents

and analyzed with Bio-Rad Gel Doc/Chemi Doc Imaging System and Quantity One software (Hercules, CA, USA).

Cell Cultures and Treatments

Primary hippocampal astrocytes from newborn mice (within 3 days old) and hippocampal adult neural stem cells (ANSCs) from adult mice (8–10 weeks age) were cultured as previously reported [11,23]. Astrocytes were cultured at the density of 5×10^4 cells/ml and treated with corticosterone (0.01, 0.1, 1, 10, 100 μ M) for 48 h. Cell viability was accessed by cell counting kit-8 (CCK-8; Beyotimes, Shanghai, China), and the values of 50% concentration of inhibition (IC₅₀) were calculated. Dissociated ANSCs from neurospheres were plated at 1×10^4 cells/ml and administrated with growth medium containing vehicle (DMSO) or 5 μ M corticosterone for 48 h. BrdU incorporation assays were carried out as described previously for proliferation assessment [11]. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) kits (Beyotimes) were used to evaluate cells apoptosis. For ANSCs differentiation, 1×10^4 cells/ml ANSCs were seeded on coated 24-well culture plates in DMEM/F12 with vehicle or 5 μ M corticosterone, containing 1% fetal bovine serum. After 7 days of differentiation, cells were probed with antibodies against immature neuron marker β -tubulin type III (TUJ1; Abcam) and astrocyte marker GFAP. The percentages of TUJ1⁺ and GFAP⁺ cells were quantified (approximately 300 cells counted per well, $n = 4$ dissections).

Statistics

All values are expressed as the mean $\pm$ SEM. Differences among means were analyzed using two-way analysis of variance (ANOVA) with genotype and treatments as independent factors. Tukey's *post hoc* tests were performed when significant effects were found. $P < 0.05$ was defined as significant.

Results

AQP4 Knockout Exacerbates Corticosterone-Induced Depressive-Like Behaviors

A similar corticosterone injection paradigm was reported to cause a persistent elevation of plasma corticosterone levels with peak levels occurring within 4 h after the injection [24]. As shown in Figure 1B, peak levels of serum corticosterone significantly increased in both AQP4^{+/+} and AQP4^{-/-} mice after 21-day corticosterone injection ($n = 9$ for each group; $P < 0.001$ vs. respective vehicle-treated control). Body weight loss was observed in both corticosterone-treated genotypes, while greater loss of weight was observed in AQP4^{-/-} mice than that in AQP4^{+/+} mice (Figure 1C, genotype $F_{1,54} = 10.839$, $P < 0.001$; treatment $F_{1,54} = 216.421$, $P < 0.001$; interaction $F_{1,54} = 16.467$, $P < 0.001$). The immobility times of FST and TST were longer in corticosterone-treated AQP4^{-/-} mice than that in AQP4^{+/+} mice ($P < 0.05$) (Figure 1C, D; FST: genotype $F_{1,48} = 6.269$, $P = 0.016$; treatment $F_{1,48} = 49.895$, $P < 0.01$, interaction $F_{1,48} = 7.516$, $P = 0.009$; TST: genotype $F_{1,51} = 2.204$, $P = 0.144$; treatment $F_{1,51} = 36.136$, $P < 0.01$; interaction $F_{1,51} = 4.833$, $P = 0.032$).

AQP4 Knockout Exacerbates Corticosterone-Induced Astrocytic Dysfunction in Hippocampus

Corticosterone treatment caused a significant decrease in hippocampal GFAP⁺ cell density in AQP4^{-/-} mice ($P < 0.001$ vs. AQP4^{-/-} vehicle) but not in AQP4^{+/+} mice ($P = 0.503$ vs. AQP4^{+/+} vehicle; Figure 2A,B; genotype $F_{1,16} = 8.334$; $P = 0.011$; treatment $F_{1,16} = 128.445$; $P = 0.004$, and interaction $F_{1,16} = 4.780$; $P = 0.044$). A significant upregulation of AQP4 was also detected in the hippocampus of AQP4^{+/+} mice ($n = 5$, t -test, $P = 0.038$, Figure 2C) while no band was visualized in that of AQP4^{-/-} mice.

Cx43 is a functional marker of intercellular communication in astrocytic networks [25]. As shown in Figure 2E, corticosterone significantly downregulated Cx43 expression in the hippocampus of both genotypes (genotype $F_{1,16} = 0.284$, $P = 0.602$; treatment, $F_{1,16} = 10.701$, $P = 0.005$, and interaction $F_{1,16} = 0.011$, $P = 0.916$). EAAT1/EAAT2 are two major glutamate transporters expressed in astrocytes, mediating glutamate clearance and metabolism [6]. Corticosterone also downregulated EAAT1 expression in the hippocampus of both genotypes (Figure 2D; genotype $F_{1,16} = 2.059$, $P = 0.171$; treatment $F_{1,16} = 40.701$, $P < 0.001$; interaction $F_{1,16} = 0.101$, $P = 0.755$) while only inhibited EAAT2 expression in AQP4^{-/-} mice ($n = 5$, $P = 0.011$) but not AQP4^{+/+} mice ($n = 5$, $P = 0.461$). As previously reported [26], AQP4 knockout significantly decreased EAAT2 expression in the hippocampus (Figure 2C; genotype $F_{1,16} = 67.720$, $P < 0.001$, treatment $F_{1,16} = 6.727$, $P < 0.02$; interaction $F_{1,16} = 2.986$, $P = 0.103$).

AQP4 Knockout Aggravates Corticosterone-Induced DownRegulation of Synapsin-1 in Hippocampus

To investigate the effects of corticosterone on hippocampal neuroplasticity, the neuronal density, expressions of postsynaptic marker PSD-95 and presynaptic marker synapsin-1 were determined. As shown in Figure 3A, corticosterone did not alter the neuronal density in the hippocampus subregions CA1, CA3, and dentate gyrus (DG) ($n = 5$ for each group). AQP4 knockout as well as corticosterone did not alter PSD-95 expression in the hippocampus (Figure 3B; genotype $F_{1,16} = 0.167$, $P = 0.689$; treatment $F_{1,16} = 0.129$, $P = 0.724$; interaction $F_{1,16} = 1.036$, $P = 0.324$). However, corticosterone treatment significantly downregulated synapsin-1 expression in the hippocampus of both genotypes (genotype $F_{1,16} = 14.727$, $P = 0.001$; treatment $F_{1,16} = 39.572$, $P < 0.001$; interaction $F_{1,16} = 7.851$, $P = 0.013$), while even less synapsin-1 expression was found in corticosterone-treated AQP4^{-/-} mice (Figure 3C, $P < 0.001$ vs. AQP4^{+/+} mice).

AQP4 Knockout DownRegulates GDNF Levels in Corticosterone-Induced Depression

ELISA data demonstrated that 21-day corticosterone treatment downregulated BDNF levels in the hippocampus of both AQP4^{+/+} and AQP4^{-/-} mice (Figure 4A; treatment $F_{1,36} = 16.019$, $P < 0.001$; genotype $F_{1,36} = 0.001$, $P = 0.976$; interaction $F_{1,36} = 0.181$, $P = 0.673$). Compared with wild mice, corticosterone sig-

nificantly decreased the GDNF level in the hippocampus of AQP4^{-/-} mice but not in that of AQP4^{+/+} mice (Figure 4B; treatment $F_{1,36} = 6.111$, $P = 0.018$; genotype $F_{1,36} = 1.940$, $P = 0.172$; interaction $F_{1,36} = 4.133$, $P = 0.049$).

AQP4 Knockout Aggravates Corticosterone-Induced Neurogenesis Impairment in Hippocampus

As shown in Figure 1A, AQP4 knockout did not alter the basal cell proliferation (BrdU⁺) in SGZ of the hippocampus in normal mice. Administered with corticosterone, both genotypes exhibited significantly decreases in the number of survival BrdU⁺ cell in SGZ (Figure 5B; genotype (1,16) = 0.638, $P = 0.436$; treatment (1,16) = 40.685, $P < 0.01$; interaction (1,16) = 5.381, $P = 0.034$) while far less BrdU⁺ cells were observed in the SGZ of AQP4^{-/-} mice ($P = 0.015$).

Moreover, the phenotype of survival BrdU⁺ cells was identified by double labeling of BrdU and MAP2 or GFAP. AQP4 knockout and corticosterone did not alter the neuronal (MAP2⁺/BrdU⁺) and astrocytic (GFAP⁺/BrdU⁺) differentiation ratio among various treatment group (Figure 5C) (MAP2⁺/BrdU⁺: genotype (1,16) = 0.826, $P = 0.377$; treatment (1,16) = 0.936, $P = 0.348$, interaction (1,16) = 0.003, $P = 0.995$; GFAP⁺/BrdU⁺: genotype (1,16) = 0.916, $P = 0.353$; treatment (1,16) = 0.183, $P = 0.674$; interaction (1,16) = 0.117, $P = 0.737$). However, coincident with the decline of BrdU⁺ cells, these data indicate a net reduction in new mature neurons (MAP2⁺) in the DG of corticosterone-treated AQP4^{-/-} mice compared with AQP4^{+/+} mice.

Cell proliferation in the SGZ at the end of 21-day corticosterone treatment was also assessed by endogenous mitotic marker Ki-67 [27]. Corticosterone treatment significantly decreased the number of Ki67⁺ cells in the hippocampus of both AQP4^{+/+} and AQP4^{-/-} mice (genotype $F_{1,16} = 5.314$, $P = 0.035$; treatment $F_{1,16} = 128.445$, $P < 0.001$; interaction $F_{1,16} = 4.632$, $P = 0.047$), while even less Ki67⁺ cells were found in AQP4^{-/-} mice (Figure 2D; $P = 0.005$ vs. corticosterone-treated AQP4^{+/+} mice).

DCX is another marker of neurogenesis for representing a snapshot of cells undergoing a neuronal maturation [27]. Although corticosterone significantly reduced DCX⁺ cells density in the SGZ of both genotypes (treatment $F_{1,16} = 35.420$, $P < 0.001$; genotype $F_{1,16} = 2.467$, $P = 0.136$; interaction $F_{1,16} = 5.214$, $P = 0.036$), the density in corticosterone-treated AQP4^{-/-} mice was significantly lower than that in AQP4^{+/+} mice (Figure 5E; $n = 5$, $P = 0.008$).

AQP4 Knockout Exacerbates Corticosterone-Induced Astrocyte Injury and Neurogenesis Inhibition *in vitro*

To determine the direct effects of AQP4 knockout on corticosterone-treated astrocytes and ANSCs, astrocytes viability and ANSCs neurogenesis *in vitro* were investigated (Figure 6A,C). CCK8 analysis indicated that corticosterone inhibited the cell viability of both AQP4^{+/+} and AQP4^{-/-} astrocytes in concentration-dependent way (Figure 6B), and revealed a higher IC₅₀ of corticosterone on the cell viability in AQP4^{+/+} astrocytes (IC₅₀: 476.4 μ M) than that in AQP4^{-/-} astrocytes (IC₅₀: 6.7 μ M).

Figure 2 Aquaporin-4 (AQP4) knockout exacerbated corticosterone-induced hippocampal astrocytic dysfunction. **(A, B)** Corticosterone treatment significantly decreased density of astrocyte in the hippocampus of AQP4^{-/-} mice but not in that of AQP4^{+/+} mice ($n = 5$; Scale bar 200 μ m). **(C)** The expression of AQP4 was significantly increased in the hippocampus of AQP4^{+/+} mice followed by 21-day corticosterone injection ($n = 5$). **(D)** AQP4 knockout downregulated expression of EAAT2 in the hippocampus. Corticosterone treatment significantly inhibited expression of EAAT2 in the hippocampus of AQP4^{-/-} mice but not in that of AQP4^{+/+} mice ($n = 5$). **(E, F)** Corticosterone treatment significantly reduced the expression of EAAT1 and Cx43 in the hippocampus of both AQP4^{+/+} and AQP4^{-/-} mice ($n = 5$). * $P < 0.05$; # $P < 0.01$.

Figure 3 Aquaporin-4 (AQP4) knockout aggravated corticosterone-induced hippocampal neuroplasticity inhibition by downregulating synapsin-1 expression. **(A)** Corticosterone-treated for 21 days did not alter the neuronal density in the dentate gyrus (DG), CA1, and CA3 subregions of hippocampus in both AQP4^{+/+} and AQP4^{-/-} mice ($n = 5$; Scale bar 100 μm). **(B)** AQP4 knockout and 21-day corticosterone treatment did not alter the expression of postsynaptic marker PSD95 in the hippocampus ($n = 5$). **(C)** AQP4 knockout enhanced corticosterone-induced downregulation of presynaptic marker synapsin-1 in the hippocampus ($n = 5$). * $P < 0.05$; # $P < 0.01$.

Figure 4 (A) Corticosterone treatment significantly reduced protein levels of brain-derived neurotrophic factor in the hippocampus of both aquaporin-4 (AQP4)^{+/+} and AQP4^{-/-} mice ($n = 9-10$). (B) The protein level of glial cell line-derived neurotrophic factor (GDNF) in the hippocampus was significantly decreased in AQP4^{-/-} mice but not in AQP4^{+/+} mice after 21-day corticosterone treatment ($n = 0$). * $P < 0.05$; # $P < 0.01$.

Considering that serum concentration of corticosterone was in the range of 2.5–6 μM after 21-day injection in both genotypes (Figure 1B), 5 μM corticosterone was used for ANSCs proliferation, apoptosis, and differentiation studies. As indicated in Figure 6D and G, both AQP4 knockout and corticosterone treatment significantly inhibited the proliferation of ANSCs ($n = 4$; genotype, $F_{1,12} = 22.978$, $P < 0.001$; treatment, $F_{1,12} = 17.29$, $P = 0.001$; interaction $F_{1,12} = 0.573$, $P = 0.464$). When normalized to the level of respective vehicle control, much worse proliferation in AQP4^{-/-} ANSCs ($63.6 \pm 6.19\%$) was detected compared with AQP4^{+/+} ANSCs ($29.7 \pm 10.3\%$, $n = 4$, $P = 0.03$). TUNEL assay revealed that both AQP4 knockout and corticosterone significantly induced the apoptosis of cultured ANSCs (genotype, $F_{1,12} = 31.781$, $P < 0.001$; treatment, $F_{1,12} = 35.335$, $P < 0.001$; interaction $F_{1,12} = 5.183$, $P = 0.042$). However, no significant difference in corticosterone-induced apoptosis was found (fold change of respective vehicle control) between AQP4^{+/+} ANSCs (2.9 ± 0.72 -fold) and AQP4^{-/-} ANSCs (2.55 ± 0.24 -fold, $n = 4$, $P = 0.66$). Furthermore, ANSCs differentiation assay also showed that AQP4 knockout and corticosterone significantly

inhibited neuronal differentiation (genotype: $F_{1,12} = 30.001$, $P < 0.001$; treatment: $F_{1,12} = 19.44$, $P = 0.001$; interaction $F_{1,12} = 0.117$, $P = 0.739$). The inhibition rate (to the respective vehicle control) of neuronal differentiation in AQP4^{-/-} ANSCs ($70.57 \pm 7.5\%$) was significantly higher than that in AQP4^{+/+} ANSCs ($34.28 \pm 9.18\%$, $n = 4$, $P = 0.022$; Figure 6H,I).

Discussion

In the present study, we used AQP4 knockout mice to demonstrate that lack of AQP4 exacerbated depressive-like behaviors in a chronic corticosterone subcutaneous injection model of depression. In the hippocampus, the primary target of glucocorticoids and stress in the brain [28], AQP4 knockout aggravated astrocyte vulnerability and neurogenesis impairment induced by corticosterone. These findings suggest that AQP4 plays a critical role in the pathogenesis of depression.

Stress or stress hormone manipulation-based animal models are leading approaches for analyzing cellular and molecular mechanisms underlying the pathophysiology of depression [29]. In this study, a reliable mouse model of depression was induced by 21-day repeated corticosterone injection paradigm [21,30,31]. We found AQP4 knockout did not alter the metabolism of corticosterone but exacerbated corticosterone-induced depressive symptoms. This result is different from our previous report [20], in which no significant differences in depressive symptoms were found between AQP4^{+/+} and AQP4^{-/-} mice followed 7-week chronic mild stress. Considering a diverse array of variables involved in these animal models of depression [32], the difference may be partially attributed to the distinct duration (3 week vs. 7 week), the different stressors (corticosterone vs. CMS), and limitations of mice depression model paradigm [29].

Mounting evidence in clinical and preclinical studies has implicated glial abnormalities in the pathophysiology of depression. Researches on postmortem brain tissues or animal model of depression have demonstrated the reductions in the numbers and/or sizes of astrocytes in prefrontal cortex, cingulate cortex, and hippocampus are associated with depression [3,33,34]. Notably, results obtained from animal models showed that selectively injury or function inhibition of astrocytes in the prefrontal cortex or hippocampus was sufficient to induce depressive-like behaviors [4,5]. In this study, no significant difference of neuronal density was found in the hippocampus between two genotypic depressive mice while AQP4 knockout led to a reduction in astrocytes density in the hippocampus as well as a decreased IC₅₀ for cell viability of corticosterone-treated astrocytes *in vitro*, implying the lack of AQP4 could increase astrocyte vulnerability to the injury of corticosterone, which may at least partially contribute to the enhanced susceptibility of AQP4^{-/-} mice to corticosterone-induced depression.

Accumulating data suggest that functional impairment of astrocytes is present in the brains of experimentally depressed animals or individuals suffering with depression [3]. Several proteins are served as functional markers of astrocytes, such as EAAT1/2, AQP4, Cx43, and GDNF. [3]. In the hippocampus, EAAT1 and EAAT2 are predominantly expressed in astrocytes and responsible for glutamate clearance and metabolism [6]. In fact, reduced expressions of astrocyte-specific EAATs (EAAT1 and EAAT2)

Figure 5 Aquaporin-4 (AQP4) knockout aggravated corticosterone-induced hippocampal neurogenesis impairment. **(A)** AQP4 knockout did not alter the basal cell proliferation in the subgranular zone of hippocampus ($n = 5$; scale bar 200 μm). **(B)** The number of survival BrdU⁺ cell in the subgranular zone of AQP4^{-/-} mice was significantly less than that of AQP4^{+/+} mice after 21-day corticosterone treatment ($n = 5$; scale bar 200 μm). **(C)** Corticosterone treatment did not alter the ratios of neuronal differentiation (MAP2⁺/BrdU⁺) or astrocytic differentiation (glial fibrillary acidic protein [GFAP]⁺/BrdU⁺) of BrdU⁺ cells ($n = 5$; scale bar 100 μm). **(D)** AQP4 knockout aggravated corticosterone-induced inhibition of cell proliferation (Ki67⁺) in the subgranular zone ($n = 5$; scale bar 200 μm). **(E)** The densities of new-generated immature neuron (DCX⁺) in subgranular zone were decreased by 21-day corticosterone treatment ($n = 5$; scale bar 200 μm). The density of DCX⁺ immature neurons was significantly lower in the hippocampus of AQP4^{-/-} mice than that of AQP4^{+/+} mice ($n = 5$; Scale bar 200 μm). * $P < 0.05$; # $P < 0.01$.

Figure 6 Aquaporin-4 (AQP4) knockout exacerbated corticosterone-induced astrocyte damage and neurogenesis inhibition *in vitro*. **(A, C)** Double immunocytochemistry of nestin/AQP4 and glial fibrillary acidic protein (GFAP)/AQP4 indicated AQP4 expressed in ANSCs and astrocytes. **(B)** AQP4 knockout increased the vulnerability of astrocytes to the injury of corticosterone (IC50: AQP4^{-/-} astrocytes 6.7 μ M vs. AQP4^{+/+} astrocytes 476.4 μ M, * P < 0.05 vs. vehicle; # P < 0.05 vs. AQP4^{+/+}). **(D, G)** AQP4 knockout inhibited proliferation of ANSCs. Compared with respective vehicle-treated control, 5 μ M corticosterone-induced 63.6% reduction in proliferation in AQP4^{-/-} ANSCs and 29.7% in AQP4^{+/+} ANSCs (n = 4). **(E, F)** AQP4 knockout enhanced apoptosis of ANSCs. Corticosterone increased 2.9-fold of apoptosis in AQP4^{-/-} ANSCs and 2.55-fold in AQP4^{+/+} ANSCs (n = 4). **(H, I)** AQP4 knockout decreased neuronal differentiation of ANSCs. Corticosterone-induced inhibition rate of neuronal differentiation in AQP4^{-/-} ANSCs (70.57 $\pm$ 7.5%) was significantly higher than that of AQP4^{+/+} ANSCs (34.28 $\pm$ 9.18%, n = 4); Scale Bar 50 μ m; * P < 0.05; # P < 0.01.

were revealed in the brains of depression patients [35] and animal models [36], suggesting that glutamate clearance and metabolism impairment in some regions of brain is a common pathology of depression. Moreover, pharmacological inhibition of EAAT2 in the prefrontal cortex was sufficient to induce depressive symptoms in rat [37]. In the current study, corticosterone treatment caused hippocampal EAAT1 downregulation in both genotypic mice, indicating that EAAT1-mediated impairment of glutamate metabolism in corticosterone-induced depression was independent on the presence of AQP4. Consistent with previous reports, AQP4 knockout resulted in a significant downregulation of EAAT2 in the hippocampus [26,38]. Interestingly, when lacking AQP4, corticosterone treatment downregulated hippocampal expression of EAAT2. It was reported that AQP4 and EAAT2, but not EAAT1, interact as a complex and simultaneously internalize into endosome to downregulate their expressions in the plasma membrane [39]. Additionally, EAAT2 expression is upregulated when AQP4 protein expression is transgenically induced in non-neural cells [39]. These reports postulated that AQP4 is an essential mediator for EAAT2 expression regulation [39]. Together with our results, it could be suggested that (1) chronic corticosterone treatment inhibits EAAT2-mediated glutamate clearance and metabolism and (2) upregulation of AQP4 in the hippocampus of AQP4^{+/+} mice could buffer the inhibitory effects of corticosterone on EAAT2 expression. Cx-43 is another functional marker of astrocyte involving the pathogenesis of depression [25,40]. However, corticosterone treatment decreased expression of Cx43 in the hippocampus, no matter whether AQP4 was present or not. Taken together, the exacerbated corticosterone-induced depressive symptoms in AQP4^{-/-} mice could partially be attributed to the downregulation of EAAT2 rather than EAAT1 or Cx43.

During the past two decades, the importance of neurotrophic factors, especially BDNF and GDNF, in depression have been confirmed. BDNF is mainly produced by neurons, particularly in the hippocampus and cortex, while GDNF is mainly produced by glial cells, in the central nervous system by astrocytes and in the peripheral nervous system by Schwann cells [41]. Studies have repeatedly shown that hippocampal BDNF expression was decreased by long-term corticosterone administration [42]. Similarly, GDNF also participates in the pathogenesis of depression and therapeutic effects of antidepressants [43]. Our data showed corticosterone treatment decreased expression of BDNF independent of AQP4. However, the absence of AQP4 resulted in decrement in GDNF in the hippocampus by corticosterone treatment. Recently, Uchida et al. [44] reported that GDNF played important roles in developing an adaptive stress response, and stress-induced impairment of GDNF synthesis enhanced depressive-like behaviors. Our data suggested that lack of AQP4 leads to neurotrophic function defect of astrocytes under the stress of corticosterone, thereafter contributed to the exacerbated depressive-like behaviors.

Until now, hippocampal synaptic plasticity impairment is considered to be a hallmark of depression [2]. Several presynaptic or postsynaptic makers are used to investigate synaptic plasticity, such as synapsins, PSD-95, synaptophysin, and vesicular glutamate transporter [45]. PSD-95 is a member of the synapse-associated protein 90 family and functions as a postsynaptic

scaffolding protein, primarily facilitating glutamatergic signal transduction [46]. Sex- and region-specific alterations of PSD-95 in the brain of stressed animal and individuals suffering with depression have been reported [47–49]. In the present study, 21-day corticosterone injection did not change PSD-95 expression in the hippocampus of two genotypes, indicating the organization and/or function of postsynaptic structure is not affected by corticosterone treatment or AQP4 knockout. Synapsin-1 is the most abundant presynaptic vesicle-associated proteins in mature neurons, playing multiple roles in synaptic transmission, new nerve terminal formation, synaptic contact maturation, and neurite elongation [50]. Our data showed that AQP4 knockout exacerbated corticosterone treatment-induced synapsin-1 downregulation in the hippocampus, which is linked to depressive-like behaviors [51,52]. Therefore, the aggravated decrease in synapsin-1 may mediate presynaptic functional inhibition, which was probably contributed to the exacerbated depressive-like behaviors in AQP4^{-/-} mice. Considering the absence of AQP4 in neurons, this aquaporin could not regulate synapsin-1 expression directly. As discussed above, it is noteworthy that GDNF could promote formation of neuronal synapses and induce expression of synapsin-1 [53]. The lower hippocampal synapsin-1 expression of depressive AQP4^{-/-} mice could be due to the lower GDNF level.

The neurogenesis hypothesis of depression states that a decrease in the production of newborn granule cells in the dentate gyrus is related to the pathophysiology of depression. In our *in vivo* study, AQP4 knockout did not alter the basic cell proliferation in the DG of normal mice as our previous reported [20]. However, in corticosterone-treated mice, AQP4 knockout inhibited proliferation and survival of newborn cells in the DG. Further phenotype analyses of immature and mature neurons demonstrated that AQP4 knockout aggravated corticosterone-induced neurogenesis impairment. Recently studies confirmed that the development of depression-like behavior in corticosterone-treated rats was paralleled by hippocampal neurogenesis [54] and adult hippocampal neurogenesis buffered stress responses and depressive behaviors [55]. Thus, the aggravated neurogenesis inhibition in hippocampus could also contribute to the exacerbated depressive behaviors in AQP4^{-/-} mice. Consistent with our previous results, AQP4 knockout decreased the proliferation and neuronal differentiation, but increased basal apoptosis of hippocampal ANSCs *in vitro* [11]. AQP4 knockout also elicited inhibitory effect of corticosterone on the proliferation and neuronal differentiation of hippocampal ANSCs. These results indicated that lack of AQP4 changed the intrinsic property of ANSCs and enhanced the injurious effects of corticosterone to ANSCs. However, the mechanisms underlying are unclear. The evidence from our laboratory has revealed that AQP4 was essential for the initiation of intracellular calcium event, including calcium spikes and calcium oscillation [11,12]. AQP4 knockout resulted in abnormal expressions of calcium handling proteins in cells [56,57]. Although several investigations suggested corticosterone inhibited proliferation and neuronal differentiation of neural stem cell by acting on glucocorticoid receptor and mineralocorticoid receptor [58], it should also be noted that glucocorticoids were identified as potent modulators of intracellular calcium signaling [59]. Based on these facts, we speculate that

AQP4 modulated the effects of corticosterone on ANSCs by regulating calcium signaling. Further research is required to fully understand the role of AQP4 in corticosterone's effects on neurogenesis *in vitro*.

In conclusion, the present study indicates that AQP4 knockout increases the vulnerability of astrocytes to the injury of corticosterone and exacerbates corticosterone-induced impairment of astrocytic functions, including glutamate uptake and neurotrophin release that subsequently inhibits neuroplasticity in the hippocampus. Moreover, lack of AQP4 enhances inhibitory effects of corticosterone in adult neurogenesis both *in vivo* and *in vitro*. Because astrocyte pathology and adult neurogenesis are two important cellular mechanisms of depression, our findings provide direct evidence that AQP4 participates in the pathogenesis of

depression and modulating AQP4 expression may represent a novel strategy for the treatment of depression.

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Conflict of Interest

The authors declare no conflict of interest.

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