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·Original Article·

# Aquaporin-4 deficiency attenuates acute lesions but aggravates delayed lesions and microgliosis after cryoinjury to mouse brain

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**Abstract: Objective** To determine whether aquaporin-4 (AQP4) regulates acute lesions, delayed lesions, and the associated microglial activation after cryoinjury to the brain. **Methods** Brain cryoinjury was applied to AQP4 knockout (KO) and wild-type mice. At 24 h and on days 7 and 14 after cryoinjury, lesion volume, neuronal loss, and densities of microglia and astrocytes were determined, and their changes were compared between AQP4 KO and wild-type mice. **Results** Lesion volume and neuronal loss in AQP4 KO mice were milder at 24 h following cryoinjury, but worsened on days 7 and 14, compared to those in wild-type mice. Besides, microglial density increased more, and astrocyte proliferation and glial scar formation were attenuated on days 7 and 14 in AQP4 KO mice. **Conclusion** AQP4 deficiency ameliorates acute lesions, but worsens delayed lesions, perhaps due to the microgliosis in the late phase.

**Keywords:** aquaporin-4; gene deficiency; cryoinjury; microglia; astrocyte

## 1 Introduction

Aquaporin-4 (AQP4), a water channel protein primarily localized in astrocytes, plays a critical role in brain water homeostasis[1,2]. Investigations in AQP4-deficient mice revealed that AQP4 promotes cytotoxic edema but attenuates vasogenic edema after brain injury[3]. Moreover, AQP4 is also involved in processes unrelated to edema, such as astrocyte migration and glial scar formation[4,5], astrocytic $Ca^{2+}$ signaling[6] and striatal neurotransmitter release[7].

In addition to ischemic brain injury, astrocyte AQP4 expression is up-regulated after traumatic brain injury both *in vivo*[8,9] and *in vitro*[10], and this is related to the insults and brain edema. In a freeze-induced traumatic brain injury model (cryoinjury), AQP4-deficient mice exhibit remarkably worse vasogenic edema, suggesting that AQP4 facilitates reabsorption of excessive fluid after the injury[11]. Cryoinjury is one of the well-established models of traumatic brain injury, and is considered to be a model of cortical contusion trauma[12]. Cryoinjury can mimic some characteristics of traumatic brain injuries and the related repair responses, such as microglial activation and inflammatory responses[13-17] as well as astrocyte proliferation and glial scarring[18-20]. However, the roles of AQP4 in acute lesions and late lesions/inflammation (mainly microglial activation) after brain injury have not been reported. To address this question, we investigated the development of lesions and inflammation 1–14 days after brain cryoinjury in AQP4 knockout (KO) mice.

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## 2 Materials and methods

**2.1 Animals** AQP4 KO mice and matched CD1 littermates were kindly provided by Professor Gang HU, Nanjing Medical University, China. AQP4 KO mice were generated as previously described[21]. The mice were housed at 22 ± 1°C under a 12:12 h light/dark cycle, with free access to food and water. All experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang University School of Medicine.

**2.2 Brain cryoinjury** Mice (25–35 g, 8 weeks old) were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg), and placed on a stereotactic frame (SR-5, Narishige, Tokyo, Japan). Rectal (core) temperature was maintained at 37 ± 0.5°C by a heating pad and a heating lamp during the surgery. Brain cryoinjury was induced according to the reported method[12,20]. Briefly, the scalp was incised along the midline to expose the skull. A metal probe (weight, 100 g; tip diameter, 3 mm) cooled in liquid nitrogen was applied onto the surface of the intact skull above the left parietal lobe (1.5 mm lateral to the midline, −3.0 mm from bregma) for 30 s. Sham-operated mice underwent the same operation except that the metal probe was at room temperature. The incision was sutured after cryoinjury and mice recovered from anesthesia in a warm box.

**2.3 Histopathological examination** Mice were re-anesthetized 1, 7 or 14 days after cryoinjury, and perfused transcardially with 4% paraformaldehyde after a pre-wash with saline. The brain was removed, fixed in 4% paraformaldehyde overnight, and then transferred to 30% sucrose and incubated for 3–5 days. The whole brain was photographed with a digital camera (FinePix S602 Zoom, Fuji, Japan) to record the changes on the surface. The damaged brain area was then dissected into 1-mm-thick coronal slices. Sequential 10-μm coronal sections were cut from the slices (from bregma -2.3 to -2.45 mm) by cryomicrotomy (CM1900, Leica, Wetzlar, Germany), and stained with 1% toluidine blue. The lesion area was calculated by ImageTool 2.0 software (University of Texas Health Science Center, San Antonio, TX, USA); lesion volume was calculated as the product of the sum of lesion area and thickness (sum of lesion area × thickness). The surviving neurons (cells with a pyramidal appearance and larger size but without a shrunken appearance) in temporoparietal cortical layers III and IV were counted in five 200-μm$^2$ squares of the region between 100 and 500 μm from the lesion boundary. The investigators were blinded to the genotypes of mice when they measured lesion volume and counted cell numbers.

**2.4 Immunohistochemical examination** For immunostaining, the sections were treated with 0.3% $H_2O_2$ in methanol for 30 min, hydrated gradually to distilled water, and incubated for 2 h with 5% goat serum to block non-specific immune reactions. Sections were then incubated at 4°C overnight with rabbit polyclonal antibody against ionized calcium-binding adaptor molecule 1 (Iba-1, a marker of microglia; 1:1 000; Wako, Osaka, Japan) or against glial fibrillary acidic protein (GFAP, a marker of astrocytes; 1:600; Chemicon, Billerica, MA, USA). After washes, the sections were incubated with biotinylated goat anti-rabbit IgG (1:200; ZhongShan Goldenbridge Biotechnology, China) for 2 h followed by incubation with avidin-biotin-peroxidase complex (1:200; ZhongShan Goldenbridge Biotechnology) for 2 h. Finally, the sections were exposed to 0.01% 3, 3'-diaminobenzidine for 0.5–2 min, followed by examination under a light microscope (Olympus BX51, Tokyo, Japan). Iba-1-positive microglia in the lesion core and boundary were counted as described above. The optical density of GFAP immunoreactivity was evaluated in an area of 1 × 1 mm$^2$ in the boundary zone, and compared to that in the same location on the contralateral side. The glial scar thickness in the lesion boundary was measured using ImageTool 2.0 software (University of Texas Health Science Center). Normal goat serum was used instead of the primary antibody as a control, and no positive staining was detected.

**2.5 Statistical analysis** Data are expressed as mean ± SEM. Statistical analyses were performed using one-way analysis of variance followed by Newman-Keuls *post-hoc* Multiple Comparison using SPSS 11.5 for Windows. $P < 0.05$ was considered to be statistically significant.

## 3 Results

**3.1 Confirmation of AQP4 gene deficiency** Genotyping of AQP4 KO mice has been reported elsewhere[21]. In another study, we found that AQP4 mRNA expression and AQP4-positive cells are detectable in wild-type mice but not in AQP4 KO mice; in a test of water intoxication, the survival of AQP4 KO mice is higher than that of wild-type mice (100% *vs* 58.3% at 60 min; 91.7% *vs* 50% at 90 min) (unpublished data), which is consistent with a previous report[22]. These results confirmed a deficiency of the AQP4 gene in AQP4 KO mice.

**3.2 Brain lesions after cryoinjury** Lesion areas were examined at 24 h and on days 7 and 14 after cryoinjury. At 24 h, apparent bleeding was found on the brain surface of wild-type mice but not on that of AQP4 KO mice (Fig. 1A). Toluidine blue staining revealed that the lesion was marked at 24 h, and gradually decreased on days 7 and 14 after cryoinjury in both types of mice (Fig. 1B). The lesion volume was smaller at 24 h but larger on days 7 and 14 after cryoinjury in AQP4 KO than in wild-type mice (Fig. 1C).

**3.3 Neuronal loss after cryoinjury** In the core of the lesion in the cortex, there were almost no surviving neurons at 24 h and on days 7 and 14 after cryoinjury. In the boundary zone adjacent to the lesion core, the numbers of neurons were reduced. AQP4 KO mice lost fewer neurons at 24 h but more on days 7 and 14 compared with wild-type mice (Fig. 2). These findings indicated that the acute lesion was attenuated but the delayed lesion was aggravated in AQP4 KO mice.

**3.4 AQP4 deficiency aggravates microgliosis** Iba-1-positive microglia were activated from 24 h following brain injury, showing a hypertrophic or an amoeboid morphology. In both the lesion core and boundary zone, Iba-1-positive microglia were mildly increased at 24 h, and greatly increased on days 7 and 14 after cryoinjury (Fig. 3A). The

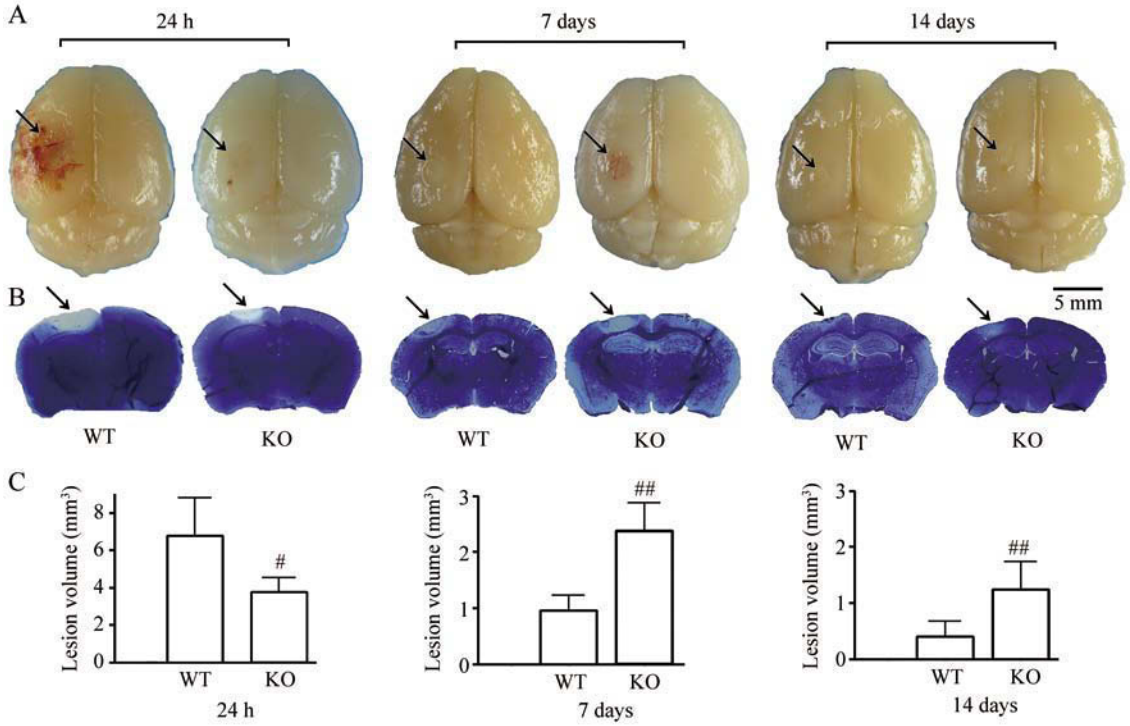


**Fig. 1. Brain lesions after cryoinjury in aquaporin-4 (AQP4) knockout (KO) and wild-type (WT) mice. A: Photographs of whole brains show the lesions (arrows) on the brain surface at 24 h and on days 7 and 14 after cryoinjury. B: Coronal sections after toluidine blue staining showing the lesions were used to calculate lesion area. C: Summary data are presented as mean ± SEM ($n$ = 6). $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 *vs* WT mice.**

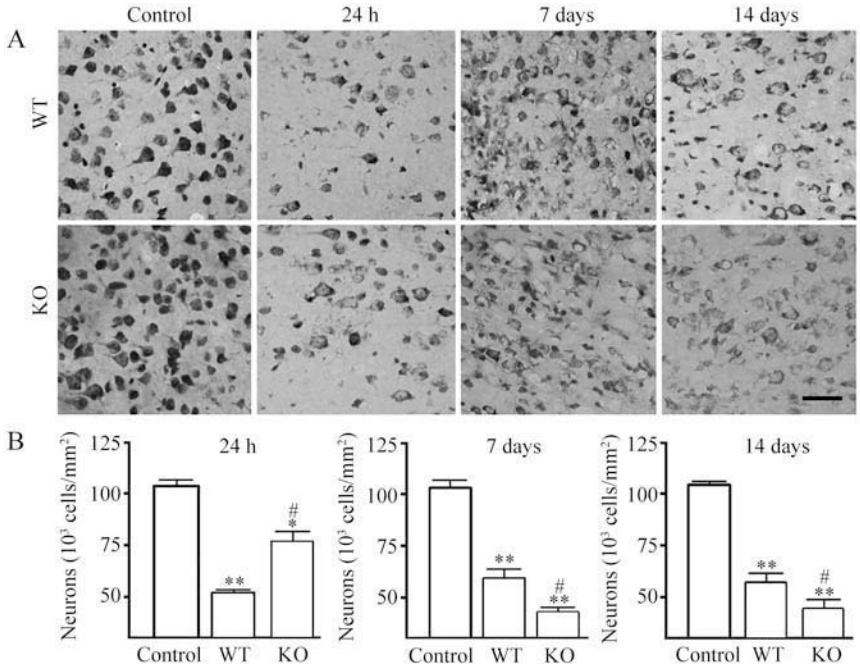


**Fig. 2. Neuronal loss after cryoinjury in aquaporin-4 (AQP4) knockout (KO) and wild-type (WT) mice. A: Neurons are reduced in the boundary zone adjacent to the lesion core at 24 h and on days 7 and 14 after injury. AQP4 KO mice lost fewer neurons at 24 h but more on days 7 and 14 than wild-type mice. Scale bar, 20 μm. B: Quantitative analysis of toluidine blue-stained cells. Summary data are presented as mean ± SEM ($n$ = 6). $^{*}P$ <0.05, $^{**}P$ <0.01 *vs* Control. $^{\#}P$ <0.05 *vs* WT mice.**

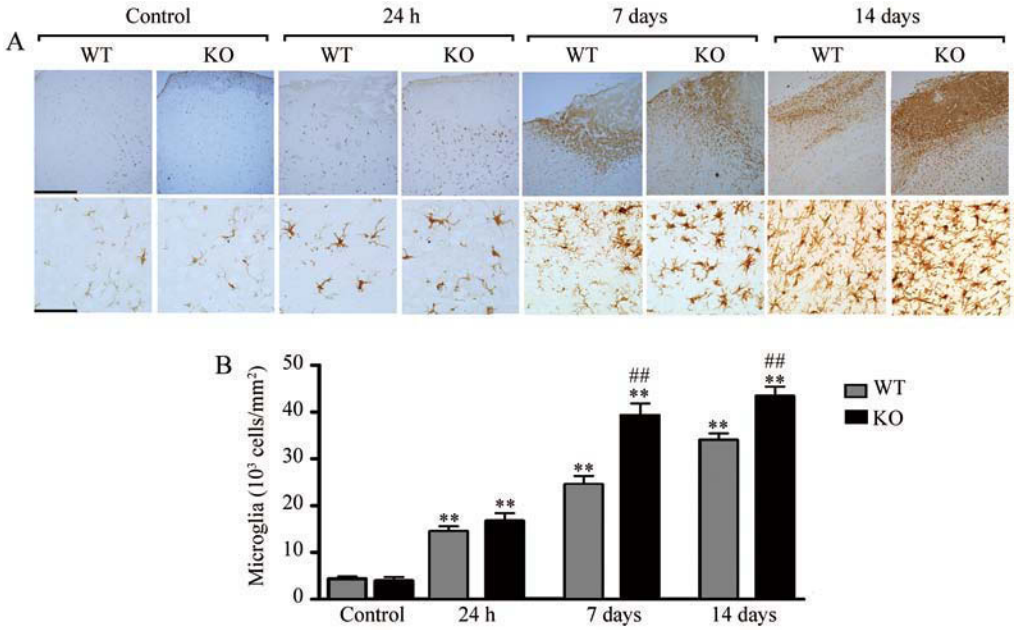


**Fig. 3. Microglial density after cryoinjury in aquaporin-4 (AQP4) knockout (KO) and wild-type (WT) mice. A: Photographs show that the density of Iba-1-positive microglia was markedly higher at 24 h, and remained so on days 7 and 14. The number of microglia was increased more in AQP4 KO mice than in WT mice at 7 and 14 days. Scale bar for upper, 50 μm; for lower, 25 μm. B: Quantitative analysis of Iba-1-positive microglia ($n$ = 6). Data in control WT and control KO groups are pooled together. Data are presented as mean ± SEM. $^{**}P$ <0.01 *vs* Control, $^{\#\#}P$ <0.01 *vs* WT mice.**

number of microglia in AQP4 KO mice was significantly higher than that in wild-type mice on days 7 and 14 (Fig. 3B). Furthermore, the microglial density on day 14 was markedly higher in the lesion core in AQP4 KO mice than in WT mice (Fig. 3A).

**3.5 AQP4 deficiency attenuates astrogliosis** GFAP-positive astrocytes were increased mildly at 24 h and on day 7, and a glial scar wall was found on day 14 in the boundary zone after cryoinjury (Fig. 4). The increases in the optical density of GFAP staining were 41.6 ± 7.5% ($n$ = 6) and 35.0 ± 4.8% ($n$ = 6, $P$ = 0.13, $t$-test) on day 7, and 71.3 ± 4.4% ($n$ = 6) and 47.6 ± 9.4% ($n$ = 6, $P$ <0.001, $t$-test) on day 14 in wild-type and AQP4 KO mice, respectively. The thickness of the glial scar was 28.6 ± 4.6 μm ($n$ = 6) and 22.6 ± 5.6 μm ($n$ = 6, $P$ = 0.003, $t$-test) on day 14 in wild-type and AQP4 KO mice, respectively. These findings

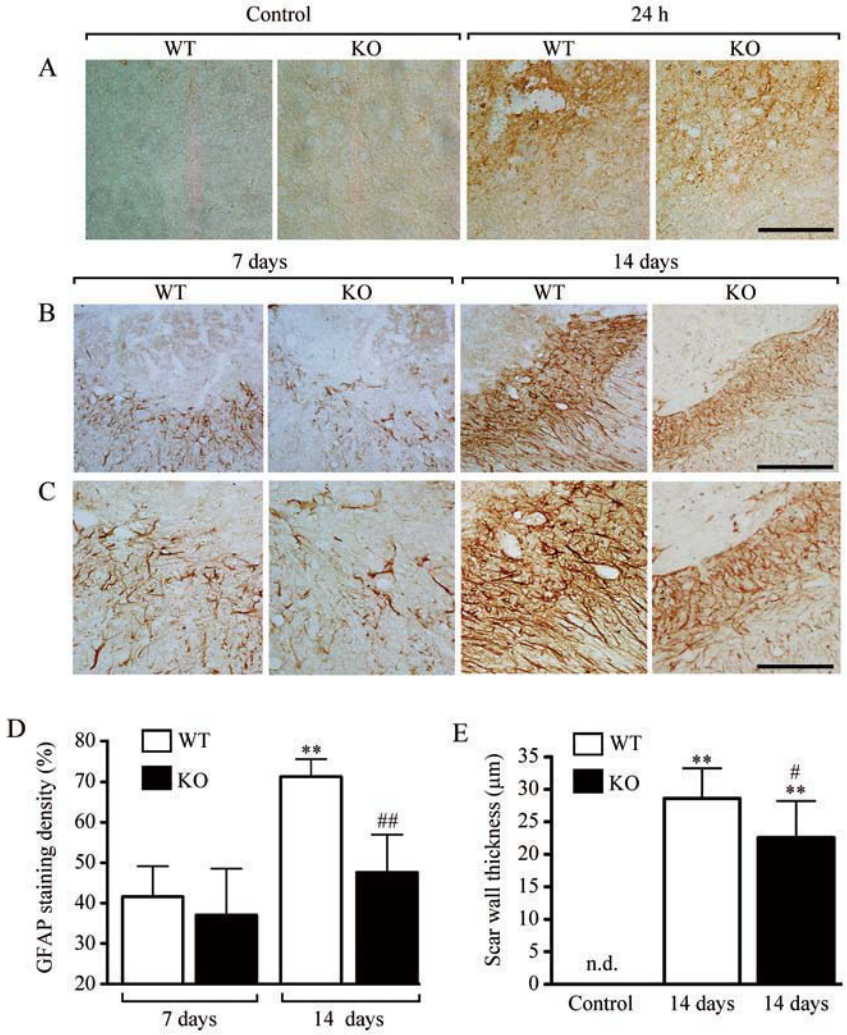


**Fig. 4. Astrocyte proliferation after cryoinjury in aquaporin-4 (AQP4) knockout (KO) and wild-type (WT) mice. A and B: Photographs showing glial fibrillary acidic protein (GFAP)-positive astrocytes in the hemispheres from control mice as well as from cryoinjured mice at 24 h, and on days 7 and 14 after injury. Few GFAP-positive cells were found in sham-operated control mice, while intense proliferation of astrocytes was found in the periphery of the lesion. Scale bars, 50 μm. C: Magnifications of B, showing hypertrophied astrocytes. In AQP4 KO mice, astrocyte proliferation and GFAP-positive cell density were attenuated on days 7 and 14, compared with those in WT mice. Scale bar, 25 μm. D and E: Quantitative analysis of GFAP staining density on days 7 and 14 and of scar wall thickness on day 14 after cryoinjury. The intensity of GFAP staining density was evaluated as the percentage increase of the gray intensity in the ischemic hemisphere: (Gi−Gc)/Gc × l00%, where Gi is the gray intensity of the ischemic hemisphere and Gc is that of the contralateral non-ischemic hemisphere. Summary data are presented as mean ± SEM; $n$ = 6. $^{**}P$ <0.01 *vs* Control. $^{\#}P$ <0.01, $^{\#\#}P$ <0.001 *vs* WT mice.**

suggested that AQP4 KO mice developed more severe microgliosis and milder astrocytosis than wild-type mice in the late phase after cryoinjury.

## 4 Discussion

Our results in AQP4 KO mice indicate dual roles of AQP4 in acute and delayed lesions after brain cryoinjury. AQP4 may on one hand be essential for inducing acute lesions, while on the other hand it attenuates delayed lesions and the associated microglial inflammation after cryoinjury. However, it was reported that AQP4 KO mice exhibit more severe vasogenic brain edema in the earlier phase (2–6 h) following cryoinjury[11], but less severe lesions and cytotoxic brain edema at 24 h after focal cerebral ischemia[22]. Because brain cryoinjury induces focal ischemia with a 50% reduction in regional blood flow[23], the acute lesions at 24 h might be induced by ischemia and related cytotoxic edema[23,24] in addition to vasogenic edema[11]. Thus, our finding may primarily reflect the regulatory role of AQP4 in ischemic insults at 24 h after cryoinjury.

With regard to the changes in the late phase (7 and 14 days after cryoinjury), the astrocytosis and glial scar formation were attenuated in AQP4 KO mice, which is consistent with previous findings[4,5]. In addition to regulation of water homeostasis, AQP4 is critical for regulating the normal growth of astrocytes[25] and the initiation of downstream signaling events in astrocytes[6]. Since astrocytes have an inhibitory effect on microglia[26], the attenuated astrocyte functions in AQP4 KO mice might result in aggravated delayed lesions and microgliosis (microglial activation). Either ablation or attenuation of glial scar-forming astrocytes exacerbates the spread of inflammatory cells during locally-initiated inflammatory responses to traumatic brain or spinal cord injury[27-30], or during peripherally-initiated experimental autoimmune encephalomyelitis (EAE)[31,32]. These findings are consistent with our results that microglial density was higher in the lesion core (Fig. 3) with a larger lesion volume in the late phase in AQP4 KO mice, which might result from the attenuated limiting effect of their astrocytes.

Inflammation is the predominant process in the brain following injury, ischemia, autoimmune disorders and viral infection, and astrocyte AQP4 is consistently up-regulated in the brain of humans with inflammatory diseases[33]. Regulatory roles of AQP4 in inflammation have been found in brains with EAE[34,35], neuromyelitis optica (NMO)[36,37], viral infections[38,39], hemorrhage[40], or after intracerebral injection of lipopolysaccharide[41]. In NMO, an inflammatory autoimmune demyelinating disease, AQP4 is a specific tissue target molecule because an IgG1 autoantibody (NMO-IgG) against AQP4 has been identified in the sera of a significant number of NMO patients[36,37]. Here, we provide evidence that astrocyte AQP4 might regulate post-injury microglial inflammation, implying that AQP4 may be a target for the treatment of inflammation after brain injury.

In summary, the present study showed that AQP4 KO mice exhibited milder acute lesions and more severe delayed lesions and associated microglial inflammation after cryoinjury. This may result from astrocyte dysfunction due to AQP4 deficiency.

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