Protective Effects of Aquaporin-4 Deficiency on Longer-Term Neurological Outcomes in a Mouse Model of Traumatic Brain Injury

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Research

Keywords: Traumatic brain injury (TBI), Aquaporin-4 (AQP4), Glymphatic system, Inflammation

Posted Date: November 13th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-104428/v1

Abstract

Background: Traumatic brain injury (TBI) has been a crucial health problem, with more than 50 million patients worldwide each year. Glymphatic system is a fluid exchange system that relies on the polarized water channel aquaporin-4 (AQP4) at the astrocytes, accounting for the clearance of abnormal proteins and metabolites from brain tissues. However, the dysfunction of glymphatic system and alteration of AQP4 polarization during the progression of TBI remain unclear.

Methods: $AQP4^{-/-}$ and Wild Type (WT) mice were used to establish the TBI mouse model respectively. Morris water maze (MWM) was used to establish the cognitive functions of $AQP4^{-/-}$ and WT mice post TBI. Western-blot and qRT-PCR assays were performed to demonstrate protective effects of AQP4 deficiency to blood-brain barrier (BBB) integrity and amyloid-β clearance. The inflammation of cerebral tissues post TBI was estimated by ELISA assay.

Results: AQP4 deficiency alleviated the brain edema and neurological deficit in TBI mice. AQP4-knockout led to improved cognitive outcomes in mice post TBI. The BBB integrity and cerebral amyloid-β clearance were protected by AQP4 deficiency in TBI mice. AQP4 deficiency ameliorated the TBI-induced inflammation.

Conclusion: AQP4 deficiency improved longer-term neurological outcomes in a mouse model of TBI.

Introduction

Traumatic brain injury (TBI) is defined as traumatic structural damage and/or brain dysfunction caused by external forces, which may induce the loss or reduction of consciousness and memory, mental disorders, neurological dysfunction, and intracranial damage [1, 2]. Although with the rapid development of emergency medicine and critical care technology, the success rate of TBI emergency treatment has increased significantly, and the overall mortality rate of TBI has dropped from 50–30% [3]. The fatality rate of TBI is still the highest among all types of trauma [4]. The mortality of severe TBI (sTBI) patients is still as high as 30%, and 10% of mild TBI patients end up with permanent neurological dysfunctions [5]. Therefore, it is urgent for us to identify the physiological mechanisms of TBI and alleviate its symptoms.

Glymphatic system is defined as a rapid switching system between the interstitial fluid and cerebrospinal fluid mediated by aquaporin-4 (AQP4) located on the astrocyte endfeet [6]. Glymphatic system mainly functions to remove metabolites (e.g. lactic acid) and abnormal proteins (e.g. β-amyloid) from brain tissue [7]. Accumulating evidence has demonstrated that the activity of glymphatic system is markedly enhanced during sleep and general anesthesia, and the function of the system can be impaired by aging, TBI, type 2 diabetes and Alzheimer's disease [8, 7]. Previous research reveals that the influx of mouse cerebrospinal fluid tracer (OA-45) can be significantly inhibited by TBI, which reduces the function of the glymphatic system by about 60% [9]. Impaired cerebrospinal fluid influx and β-amyloid clearance can be observed in the ipsilateral hippocampus less than 1 day after TBI in mice, and this damage can last up to a month [10].

As a core protein in the glymphatic circulatory system, AQP4 is also involved in the pathological process of TBI [11]. It is reported that TBI caused the loss of polarity distribution of AQP4 on the end feet of astrocytes in the mouse brain parenchyma, which is similar to the disorder of AQP4 distribution in the brain caused by aging [11]. Previous studies reported that AQP4-knockout inhibits the incidence of brain edema or neuroinflammation, and alleviates the activation of glial cells and neuronal apoptosis post cerebral ischemia injury in mouse model [12].

In this study, we systematically investigated the relationship between AQP4 and TBI-induced neurological disorders, cognitive impairment, blood-brain barrier integrity damage, changes in Aβ clearance, and inflammatory response in an AQP4-knockout mouse model. And we reported that AQP4 deficiency led to improved longer-term neurological outcomes in mice post TBI.

Methods

Animals

Adult male AQP4$^{-/-}$ and Wild Type (WT) mice (aged approximately 8–12 weeks) weighing between 20 to 25 g were purchased from Nanjing Biomedical Research Institute (Nanjing University, China). All mice used in this research were cultured in the virus/antigen-free system with permanent humidity and constant temperature and free to get the pathogen-free food and water. The protocol was approved by Ethical Committee in Hebei Medical University. Cortical impact injury (CCI) animal model was established according to our previously published paper [13].

Brain water content assay

The brain water weight of mice was measured 24 hours post TBI. TBI and Sham mice were sacrificed and their brains were removed from skull immediately. The collected cerebral tissues were then cut into sections with a side length of 4 mm. And isometric cerebellum tissues were also collected as the internal control. An electronic analytical balance was used to weigh the tissues and their weight was recorded as wet weight (WW). Then all tissue sections were placed in a dryer at 100 °C for 24 to dry the moisture thoroughly. The tissues were then weighed by the same analytical balance and their weight was recorded as dry weight (DW). Thus, the brain water content was calculated as [(WW − DW) / WW] × 100%.

Evans blue staining assay

The brain water weight of mice was performed 24 hours post TBI. Mice were anesthetized with 10% chloral hydrate (0.3 ml / 100 g). Evans blue (EB) (2%, 2 ml/kg) was injected from the tail vein 0.5 hours before the perfusion. The EB leakage through the blood-brain barrier (BBB) was used to assess the permeability of the BBB. Mice were perfused through the heart with 0.9% NaCl until the lavage fluid from the right atrium was clear. The brain tissue of each mouse was removed. Each sample was weighed and then homogenized in 0.75 ml of PBS and 0.25 ml of 100% TCA solution. The sample was cooled at 4 °C overnight, and then centrifuged at 1,000 xg for 30 minutes at 4 °C. Subsequently, the EB in the 100 µl supernatant of each sample was measured at 620 nm using a 96-well plate reader.

Modified Neurological Severity Scores (mNSS)

The mNSS test was performed to score the neurobehavior of mice pre, 1, 7 and 14 days post-TBI. The motor, sensory, reflex and balance abilities of each mouse were estimated and a higher score represented a better neurofunction. NSS test included the motor tests (6 points), sensory tests (2 points), beam balance tests (6 points), and reflexes absent and abnormal movement tests (4 points). Each point was awarded for the inability of the rat to perform a task in the NSS test. A score between 13 and 18 represented severe nerve damage. A score between 7 to 12 represented moderate injury. And a score between 1 to 6 represented mild injury. All scores used in this research were collected and analyzed by doctors who were blind to the grouping of the mice.

Morris water maze (MWM) assay

MWM assay was used at 18, 19, 20, 21-day post TBI. The mice were divided into 4 groups randomly (N = 12). The MWM assay in this study took 4 days and mice in each group were trained 3 times a day at the same time. Trained mice were located at the four corners of a big pool. The mice were trained to look for a platform in the pool and stay on it, and the whole time of the process was recorded. The mice were allowed to stand on the platform if they could find it. Mice that were unable to find it in one minute were gently pulled onto the platform for 10 s by the researchers. The swimming path, swimming speed, time in the target quadrant in 60 s and the number of platform site crossings of each mouse were recorded after they were placed in four quadrants of the pool during the training.

Quantitative real-time PCR

qRT-PCR assay was performed 3 days post-TBI. The cerebral tissue of each mouse was thoroughly ground and mixed with 1 mL Trizol (Life Technologies, Carlsbad, CA, USA) in a centrifuge tube. Chloroform and isopropanol (Life Technologies, Carlsbad, CA, USA) were used to extract RNA from tissues. FastKing cDNA reverse transcription kit (TIANGEN Biotech, Beijing, China) was used to remove genomic DNA from RNA and reverse transcribe RNA into cDNA. SuperReal PreMix Plus (TIANGEN Biotech, Beijing, China) kit was used to perform qRT-PCR, in which SYBR green (TIANGEN Biotech, Beijing, China) was used as the fluorescent signal. The mixture configured according to its instructions was placed in a Roche fluorescent quantitative PCR machine, and the LightCycler&reg480 software is used to analyze the data with a built-in algorithm. GAPDH was a negative control. The primers were as follows:

AQP4: primer F, 5'-CTGGAGCCAGCATGAATCCAG-3'

primer R, 5'-TTCTTCTCTTCTCCACGGTCA-3'

IL-6: primer F, 5'-ACAACCACGGCCTTCCCTACTT-3'

primer R, 5'-CACGATTTCCCAGAGAACATGTG-3’

MCP-1 primer F, 5'- TAAAAACCTGGATCGGAACCAAA-3'

primer R, 5'- GCATTAGCTTCAGATTTACGGGT-3'

MIP-2: primer F, 5'- CCAACCACCAGGCTACAGG-3'

primer R, 5'- GCGTCACACTCAAGCTCTG-3'

GAPDH primer F 5’-ACCCCAGCAAGGACACTGAGCAAG-3’

primer R 5’-GGCCCCTCCTGTTATTATGGGGGT-3’

Western-blotting assay

Western-blotting assay was performed 3 days post-TBI. The mouse brain tissue was thoroughly ground and mixed with 200 μl RIPA buffer. 200 μl of 2 × loading buffer was added to the mixture. The mixture was placed in a metal bath at 100 degrees Celsius to denature the protein. A 10% SDS-PAGE was utilized to separate the proteins in an electrophoresis apparatus with a constant voltage of 140V for 120 min. The protein on SDS-PAGE was then transferred to a polyvinylidene difluoride membrane (Real-Times Biotechnology Co.,Ltd, Beijing, China) in a transfer tank with a constant current of 300A.The membrane was co-incubated with Anti-Amyloid Precursor Protein antibody (ab32136), Anti-Occludin (ab167161), Anti-ZO1 tight junction (ab96587), Anti-Aquaporin 4 (ab46182) (1:1000, Abcam, Beijing, China) at 37℃ for 1 hour and washed by phosphate buffered saline for 3 times. Then, the goat anti-rabbit secondary antibody (1:10000, Cell Signaling Technology, Beijing, China) was incubated with the membrane at 37 °C for 1 hour. An AEC Peroxidase Substrate Kit (Solarbiotech, Shanghai, China) was used for blot imaging.

ELISA assay

ELISA assay was performed 3 days post-TBI. The enzyme-linked immunosorbent assay (ELISA) assays was performed to demonstrate the cytokine levels in serum of rats according to the manufacturer’s protocol. The serum inflammatory factors including MCP-1, IL-6 and MIP-2 were measured by commercially available kits (Life Technologies, Carlsbad, CA, USA).

Statistical analysis

The categorized variables were shown as frequency or percentage. Data were presented as mean ± SD. Two-way ANOVA test with a Tukey’s or Sidak's post hoc test was acquired for the statistical analysis. GraphPad Prism 10.0 was used for the plotting and analysis of the data in this research.

Results

Protective Effects of AQP4 deficiency on the TBI-induced brain edema and neurological deficit

To demonstrate the protective effects of AQP4 deficiency on TBI mice, Brain water content measurement was performed 24 hours post-TBI. Cerebral edema is defined as increased brain volume caused by

increased water content in the brain, which is a typical reflection of TBI-induced cerebral damage. Compared to the Sham group, TBI could not induce the significant increase of brain water content in $AQP4^{-/-}$ mice (Fig. 1a). To demonstrate the neuroprotective effects of quercetin on TBI rats, mNSS test was performed pre, 1, 7 and 14 days post-TBI. As shown in Fig. 1b, AQP4 deficiency induced the decrease of mNSS, indicating that AQP4 deficiency ameliorated the nerve damage caused by TBI and protected the motor function of model mice.

Protective Effects of AQP4 deficiency on the TBI-induced cognitive impairments

To further identify the function of AQP4 in the progression of TBI, MWM test was performed to investigate the alteration of the learning ability and memory of the $AQP4^{-/-}$ mice. As shown in Fig. 2a, the mice in WT-TBI groups took the longest time to escape from the maze, indicating that the knockout of AQP4 promoted the recovery of learning ability of TBI mice. On the other hand, there was no significant difference between the swimming speed of mice in all groups (Fig. 2b), indicating that AQP4 had no protective effect on the motor ability of TBI mice. In addition, AQP4 deficiency induced the increase of time in the target quadrant in 60 s (Fig. 2d) and the number of platform site crossings (Fig. 2c) compared to the WT group. These figures totally demonstrated that AQP4 deficiency significantly ameliorated the TBI-induced cognitive impairments.

Protective Effects of AQP4 deficiency on BBB integrity

To demonstrate the protective effects of AQP4 deficiency on the integrity of BBB, Evans blue staining assay was performed 24 h post-injury. As presented in Fig. 3a, AQP4 deficiency induced a marked decrease of Evans blue content post TBI compared to the WT mice, suggesting that AQP4 deficiency protected the BBB integrity of TBI mice. The expression of BBB junction proteins including ZO-1 and occludin were evaluated by Western blot assay. TBI treatment induced the significant downregulation of ZO-1 and occludin, while AQP4 deficiency induced the upregulation of both ZO-1 and occludin (Fig. 3b, c and d).

AQP4 deficiency promoted amyloid-β clearance in the ipsilateral cortex of TBI mice

Glymphatic system mainly functions to remove abnormal proteins such as β-amyloid from brain tissue. qRT-PCR and Western blotting assays were used to analyzed the mRNA and proteins levels of AQP4 in the cortex between wild type and $AQP4^{-/-}$ mice (Fig. 4a, b and c). Amyloid-β 1–40 and amyloid-β 1–42 in the ipsilateral cortex of experimental mice at 3 days post-injury was evaluated by ELISA assay. As shown in Fig. 4d and e, AQP4 deficiency induced the downregulation of both Amyloid-β 1–40 and amyloid-β 1–42 in the cortex of TBI mice. Similarly, the amyloid precursor protein (APP) also decreased significantly in

$AQP4^{-/-}$ mice post TBI (Fig. 4f and g), suggesting the protective effects of AQP4 deficiency on amyloid-β clearance in the ipsilateral cortex of TBI mice.

Protective Effects of AQP4 deficiency on the TBI-induced inflammation in the ipsilateral cortex

To investigate the protective effect of AQP-4 deficiency on the inflammatory responses induced by TBI, ELISA and qRT-PCR assays were performed. As shown in Fig. 5a, b and c, TBI induced the upregulation of cytokines including IL-6, MCP-1 and MIP-2 in in the ipsilateral cortex. On the contrary, AQP-4 deficiency induced the significant downregulation of cerebral cytokine levels, suggesting that AQP-4 deficiency attenuated the systemic inflammation induced by TBI in mouse model. The inflammatory response in the brain tissues of TBI mice was evaluated by qPCR assays. The relative RNA levels of IL-6, MCP-1 and MIP-2 in the brain tissues of TBI mice were all decreased with the deficiency of AQP-4 (Fig. 5d, e and f).

Discussion

TBI is a global issue that severely affects public health and social economy [14, 15]. It is estimated that about 52,000 people die from TBI in the United States each year, and about 530,000 people are disabled by it [16]. The brain damage caused by TBI is time-dependent, and its pathophysiological process can be divided into three major stages, which sometimes overlap with each other [17, 18]. The early stage of TBI usually occurs within 24 hours after injury, and mainly includes a series of energy metabolism disorders such as ischemic cascade caused by decreased cerebral blood flow, calcium overload, and mitochondrial dysfunction [19]. The intermediate stage of TBI occurs within a few days after brain trauma [20]. The occurrence and development of neuroinflammation further lead to vascular damage and destruction of the blood-brain barrier, causing the formation of cerebral edema. The final stage of TBI occurs within a few weeks or even months after the trauma, and this stage is related to the adverse neurological outcome of TBI patients [21]. The deterioration of nerve function leads to convulsions and seizures. Neuroinflammation is an important part of TBI secondary injury, including the release of endogenous harmful substances and the activation of the innate immune system, which plays a key role in the recovery of TBI [22]. However, the disorders of immune response regulation can also lead to secondary damage to the central nervous system. Many cytokines are released from neutrophils, microglia, immune cells and glial cells in the injured area, including anti-inflammatory factors such as IL-10, TGF-β, neurotrophic factors, IL-4, IL-13, prostaglandins and pro-inflammatory substances such as IL-10, CXCL1, IL-1, IL-6, TNF-α, etc [23].

In this study, we mainly studied the role of AQP4 in the progression of TBI. We showed that AQP4 knockout mice ended up with better neurological consequences after suffering from TBI. We found that the absence of AQP4 could improve the symptoms of TBI mice, protect the integrity of the BBB, promote the clearance of brain amyloid beta, and inhibit the inflammatory response in the mouse brain tissue.

Brain tissue produces a large number of potentially neurotoxic proteins, cell fragments and other metabolites in the process of metabolism. In order to maintain a steady state, these metabolic wastes need to be cleared from the brain in time. It is estimated that the adult brain needs to remove about 7 g of junk protein every day. In 2012, Iliff and Nedergaard et al. reported that a rapid cerebrospinal fluid-brain tissue fluid exchange flow system is widely distributed in the brain, which can promote the clearance of soluble proteins such as β-amyloid protein in the brain [24]. The flow system has the function of flushing and cleaning brain tissue, and is one of the ways for the brain to remove metabolites and foreign bodies. This system is called the glymphatic system, because it is similar in function to the peripheral lymphatic system and relies on astrocytes [25]. Recent studies have shown that in addition to removing brain metabolites (such as lactic acid), soluble proteins (such as Aβ and Tau protein) and foreign bodies, the glymphatic system also has the ability to transport glucose, lipids and apolipoprotein E(ApoE) and other nutrients and neuroactive substances to the brain tissue [26]. Therefore, the glial lymphatic system is an important fluid flow system that maintains the homeostasis of the cerebral environment. Iliff et al. reported that TBI can significantly inhibit the influx of the mouse cerebrospinal fluid tracer (OA-45), reducing the function of the glial lymphatic system by about 60% [27]. They also found that cerebrospinal fluid influx and Aβ clearance can be impaired markedly in the ipsilateral hippocampus 1 day after TBI, and the glial lymphatic system function inhibition caused by TBI is still significant after 28 days [27].

In this research, we also reported the impact of the absence of the glymphatic system on the TBI process. The glymphatic system in the brain of AQP4 knockout mice is inactivated. However, the dysfunction of the glymphatic system protected the brain tissue of TBI mice. The absence of AQP4 and the obstruction of glymphatic circulation alleviated the symptoms of cerebral edema in TBI mice, improved their long-term neurological outcomes, and reduced the inflammatory response of the cerebral cortex induced by TBI.

3-Amyloid precursor protein (3-APP) is widely distributed in the central nervous system as a transmembrane protein. It has neurotrophic and neuroprotective effects, and can promote neurite growth and synapse formation. β-amyloid is the digestion product of its precursor APP under pathological conditions, and the main component of senile plaques, which is the main pathological change of Alzheimer's disease [28]. Primary injury of TBI can induce changes in biochemical and cell biology functions, leading to continuous damage and death of neurons [29]. This continuous damage acts as a secondary injury and causes the activation of multiple apoptosis and inflammatory pathways. After TBI, β-amyloid and APP quickly accumulate in the cerebral cortex, and the expression of APP-related secretases such as BAC-1, PS-1 and other proteins also increase accordingly [30]. A large number of previous studies have shown that TBI is an independent risk factor for AD [31]. AD plaques are formed within a few hours after TB1, and it has nothing to do with age. This may be related to AD-like pathological changes and AD-like cognitive impairment. In this study, we also explored the effect of the absence of AQP4 on the clearance of β-amyloid in the mouse brain. We reported that AQP4 knockout improved the clearance of β-amyloid in the cerebral cortex after TBI. AQP4-deficient mice lack the glymphatic system, which allows them to clear β-amyloid in other ways that are not easily affected by TBI.

Conclusion

In conclusion, we demonstrated the protective effects of AQP4 deficiency on TBI mouse model in this research. We reported that AQP4 deficiency alleviated the brain edema induced by TBI and thus improved the longer-term neurological outcomes of TBI mice. Mechanistically, AQP4 deficiency protected the BBB integrity, enhanced the clearance of β-amyloid, and ameliorated the inflammatory responses induced by TBI. We hope that our findings can assist researchers to understand the mechanism of TBI progression and the role AQP4 plays in this process, and provide new possibilities for the treatment and clinical care of TBI.

Abbreviations

Traumatic brain injury (TBI); aquaporin-4 (AQP4); Cortical impact injury (CCI)

Declarations

Ethical Approval

The protocol was approved by Ethical Committee in Hebei Medical University.

Consent for publication

All of the authors have consented to publication of this research.

Availability of supporting data

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

All authors declare that they have no conflict of interest.

Funding

The study was supported by the Natural Science Foundation of Hebei Province, China (H2018206325 and H2020206543).

Authors' contributions

Study Design; Manuscript Preparation: Xiaosong Liu, Yingxin Xie, Xiangdong Wan, Jianliang Wu, Zhenzeng Fan and Lijun Yang. Literature Search; Data Collection; Statistical Analysis; Data Interpretation: Xiaosong Liu, Yingxin Xie, Xiangdong Wan, Jianliang Wu, Zhenzeng Fan and Lijun Yang.

Acknowledgments

None.

Authors' information

Not applicable.

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Figures

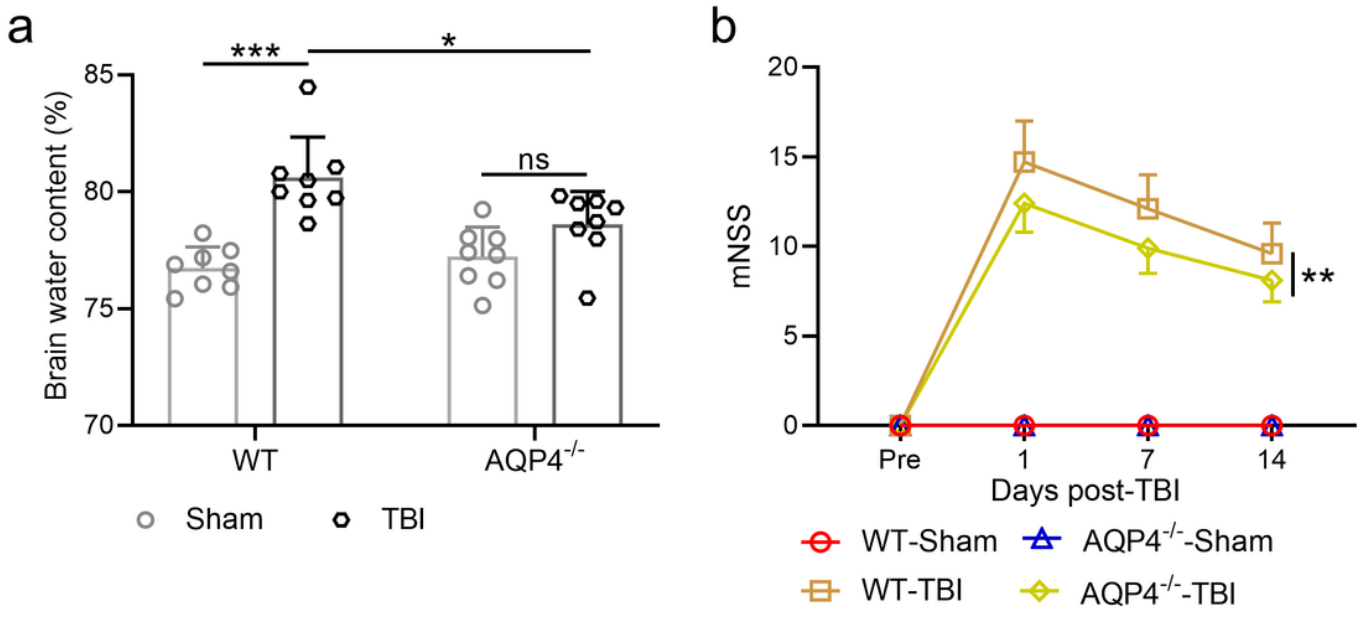

Figure 1

Effects of AQP4 deficiency on the TBI-induced brain edema (a) and neurological deficit (c) in mice. Brain water content was compared 24 h post-TBI and neurological deficit scores were measured pre, 1, 7 and 14 days post-TBI. Data are presented as mean ± SD with all data points showing in a. N = 8 for each

group. *p < 0.05, ***p < 0.001, ns means no significance. Two-way ANOVA followed Sidak's multiple comparisons test. Data are presented as mean ± SD in b. N = 12 for each group. **p < 0.01. Two-way ANOVA followed Tukey's multiple comparisons test.

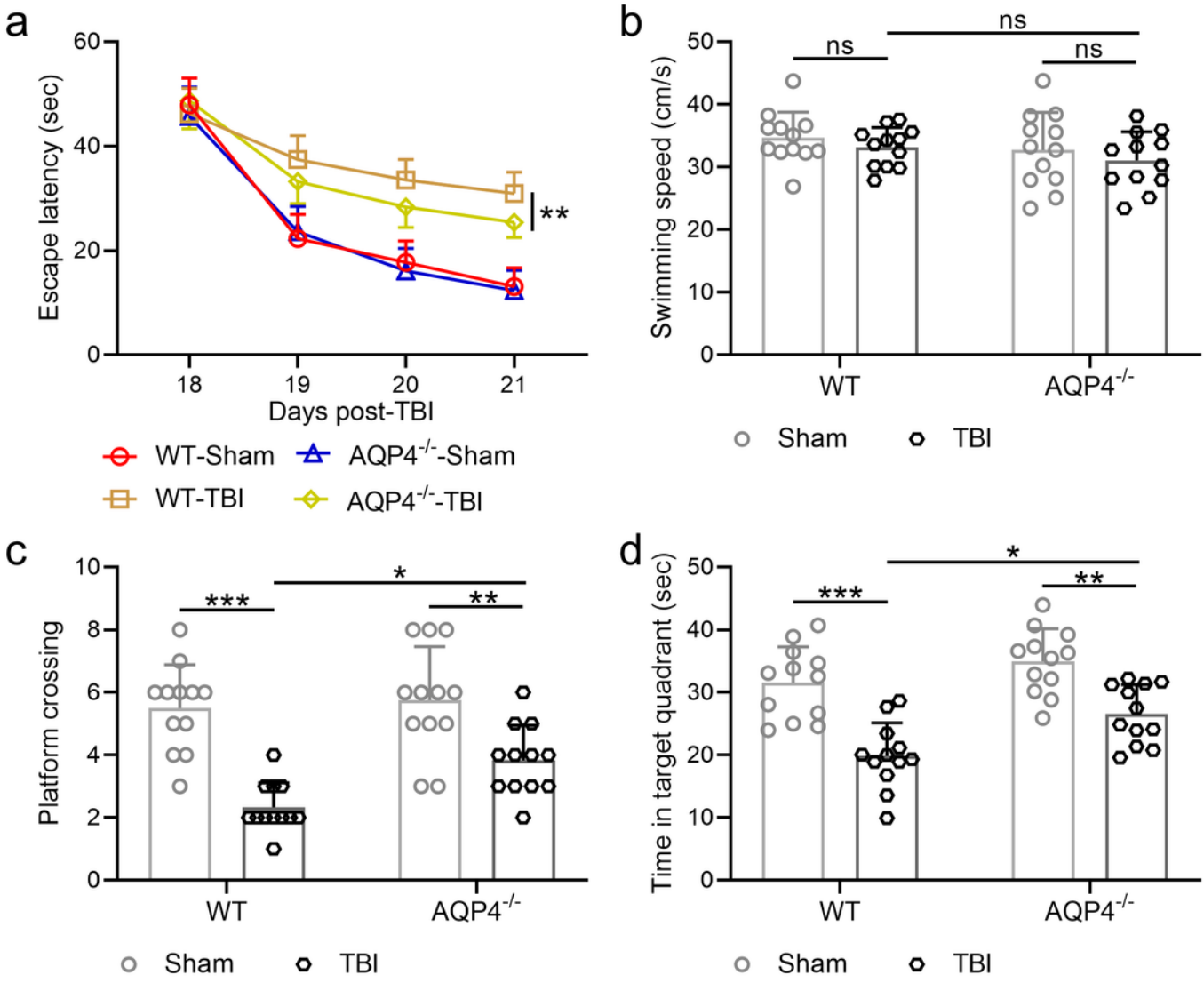

Figure 2

Effects of AQP4 deficiency on the traumatic brain injury induced cognitive dysfunction. In 4 days of training sessions, the mice's escape latencies (a) and the average swim speed (b) were measured. In the probe trial, the time in the target quadrant in 60 s (d) and the number of platform site crossings (c) were recorded. N = 12 for each group. Data are presented as mean ± SD in a. **p < 0.01. Two-way ANOVA followed Tukey's multiple comparisons test. Data are presented as mean ± SD with all data points showing in b-d. *p < 0.05, **p < 0.01, ***p < 0.001, ns means no significance. Two-way ANOVA followed Sidak's multiple comparisons test.

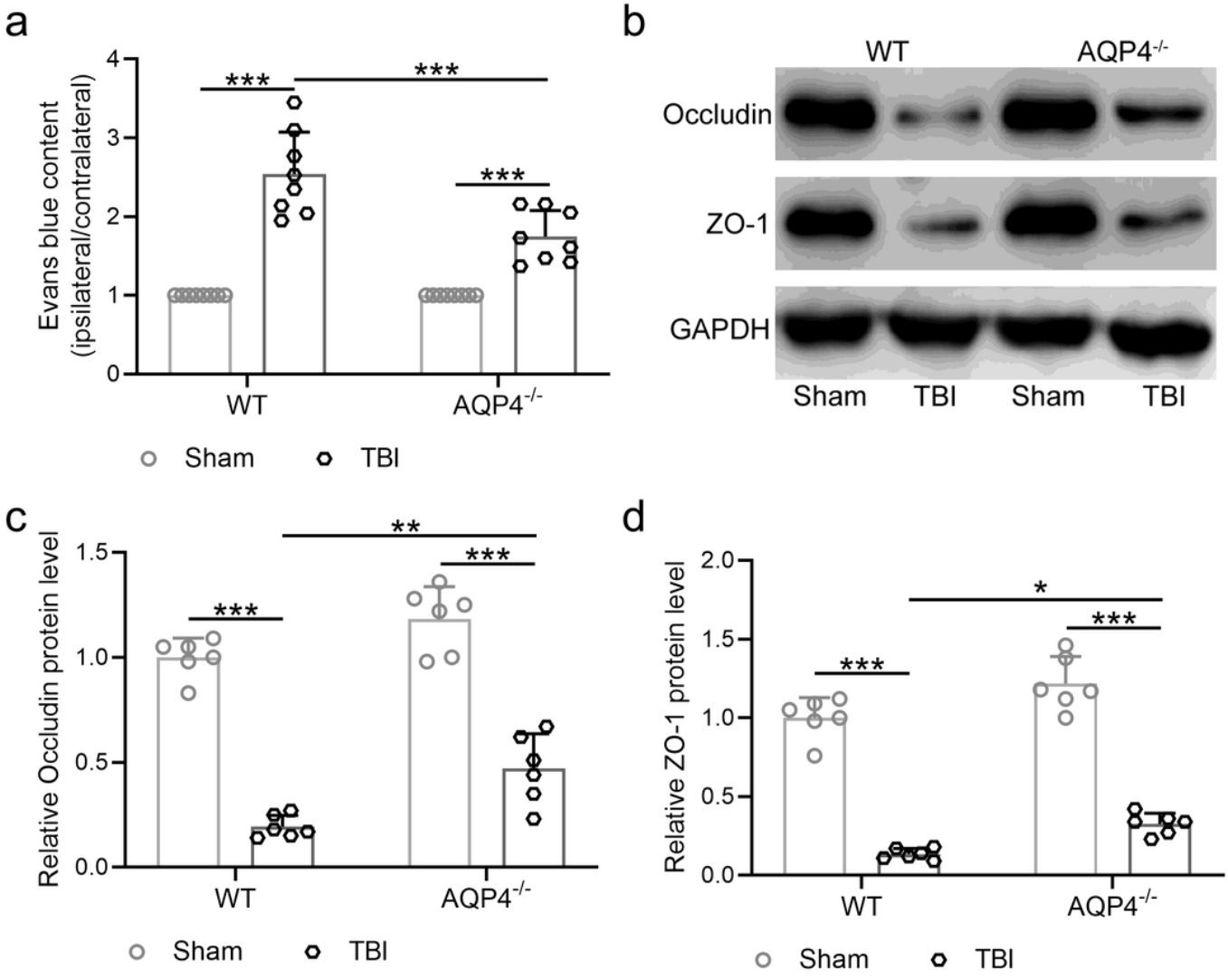

Figure 3

Effects of AQP4 deficiency on blood-brain barrier (BBB) integrity in TBI mouse model. a, Evans blue extravasation was performed in TBI mice 24 h post-injury. N = 8 for each group. b, the protein levels of ZO-1 and occludin in the ipsilateral cortex 3 days post-injury. Data of WT-sham group was used to normalized the quantitative analysis (c and d). N = 6 for each group. Data are presented as mean ± SD with all data points showing. *p < 0.05, **p < 0.01, ***p < 0.001. Two-way ANOVA followed Sidak's multiple comparisons test.

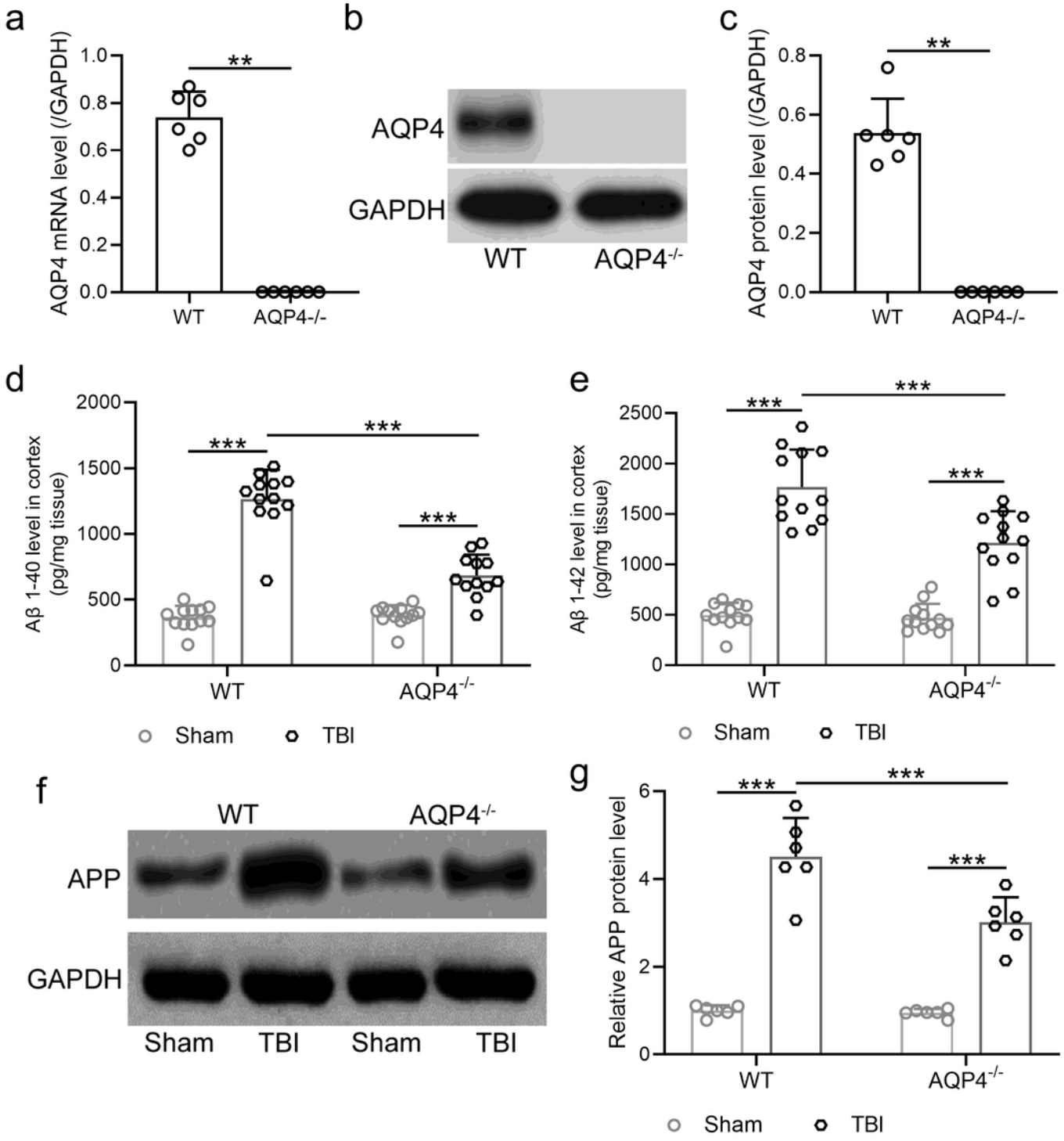

Figure 4

AQP4 deficiency promotes amyloid-β clearance in the ipsilateral cortex of traumatic brain injury mice. qRT-PCR and western blotting were used to analyzed the mRNA and proteins levels of AQP4 in the cortex between wild type and AQP4-/- mice (a-c). N = 6 for each group. Data are presented as mean ± SD with all data points showing. **p < 0.01. Student t tests followed Mann Whitney test. d and e, ELISA analysis of amyloid-β 1-40 and amyloid-β 1-42 in the ipsilateral cortex of mice 3 days post-TBI. N = 12 for each

group. f, Immunoblot analysis of amyloid precursor protein (APP) expressions in the ipsilateral cortex of experimental mice at 3 days post-injury. Data of WT-sham group was used to normalized the quantitative analysis (g). N = 6 for each group. Data are presented as mean ± SD with all data points showing. ***p < 0.001. Two-way ANOVA followed Sidak's multiple comparisons test.

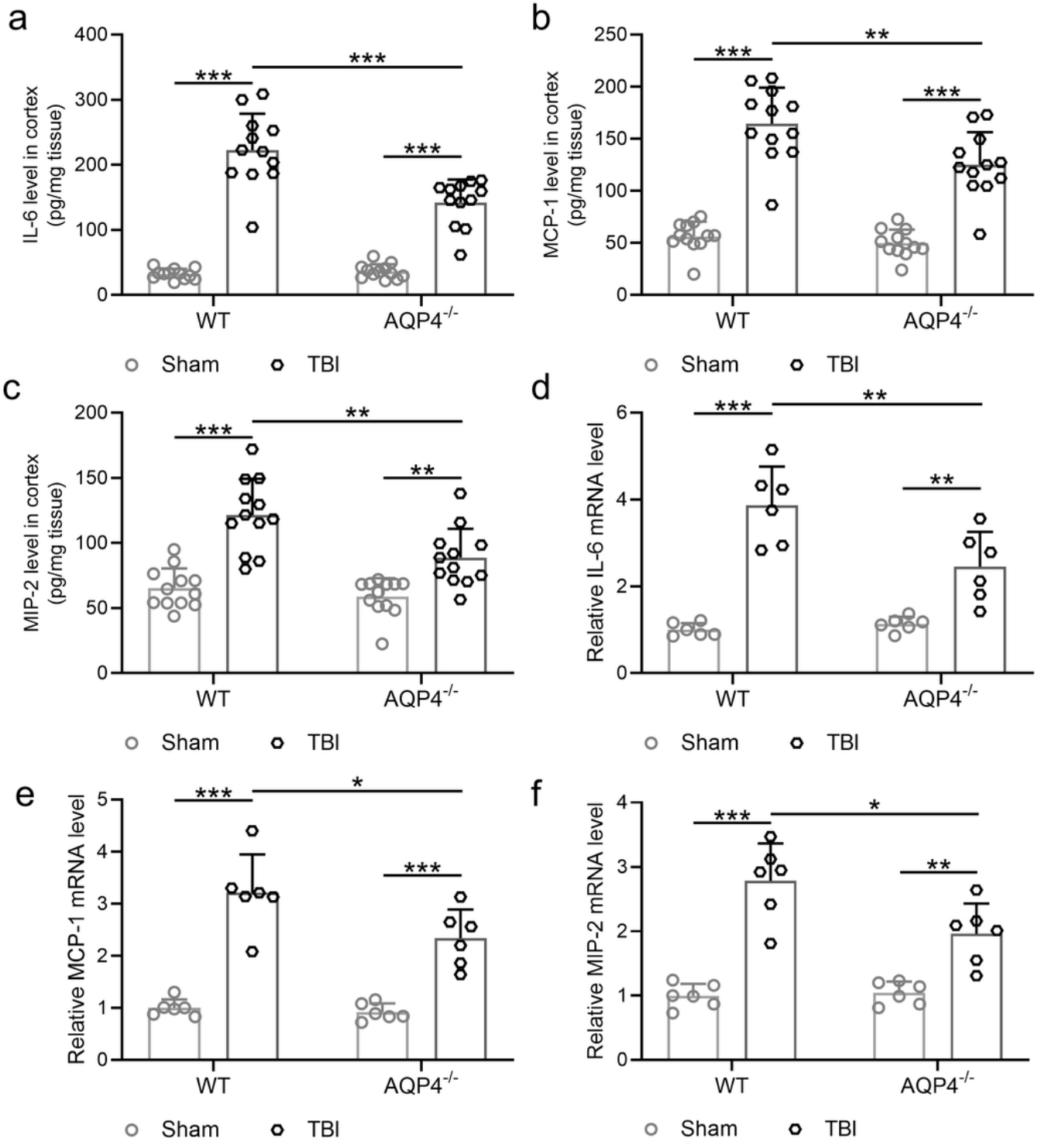

Figure 5

Effects of AQP4 deficiency on TBI-induced inflammation in the ipsilateral cortex of model mice 3 days post-TBI. ELISA assay of IL-6 (a), MCP-1 (b) and MIP-2 (c). mRNA levels of IL-6 (d), MCP-1 (e) and MIP-2 (f). N = 12 for each group in a-c. N = 6 for each group in d-f. *p < 0.05, **p < 0.01, ***p < 0.001. Two-way ANOVA followed Sidak's multiple comparisons test.