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Inhibition of NADPH oxidase 2 improves cognitive abilities by modulating aquaporin-4 after traumatic brain injury in mice

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ABSTRACT

Traumatic brain injury (TBI) is caused by acquired damage that includes cerebral edema after a mechanical injury and may cause cognitive impairment. We explored the role of nicotinamide adenine dinucleotide phosphate oxidase 2 (NADPH oxidase 2; NOX2) and aquaporin-4 (AQP4) in the process of edema and cognitive abilities after TBI in *NOX2*$^{-/-}$ and *AQP4*$^{-/-}$ mice by using the Morris water maze test (MWM), step-down test (STD), novel object recognition test (NOR) and western blotting. Knockout of *NOX2* in mice decreased the AQP4 and reduce edema in the hippocampus and cortex after TBI in mice. Moreover, inhibiting AQP4 by 2-(nicotinamide)-1,3,4-thiadiazole (TGN-020) or genetic deletion of AQP4 could attenuate neurological deficits without changing reactive oxygen species (ROS) levels after TBI in mice. Taken together, we suspected that inhibiting NOX2 could improve cognitive abilities by modulating ROS levels, then affecting AQP4 levels and brain edema after in TBI mice. Our study demonstrated that NOX2 play a key role in decreasing edema in brain and improving cognitive abilities by modulating AQP4 after TBI.

1. Introduction

Traumatic brain injury (TBI) often causes emotional, cognitive deficit and affects the quality of patients' daily life [1]. About 2 million patients undergo TBI annually in the United States, and more than 125,000 patients experience depression, anxiety, and cognitive deficit after TBI, which places an economic burden on society [2]. Although most of the TBI patients exerted a good recovery, a larger number of patients need a long time to recover [3]. In fact, treatments for edema and cognitive deficit in TBI patients are seriously inadequate [4].

Subunits of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase; NOX) exerts a key role in producing ROS and other superoxide anions by catalyzing nicotinamide adenine dinucleotide phosphate [5]. NOX complex is composed by four cytosolic subunits with two transmembrane structures. Recently, studies have shown that NOX is involved in central nervous system (CNS) impairment and prevents secondary complications by inducing brain edema and ROS levels [6]. Mao et al. showed that superoxide dismutase (SOD) levels and the expression of aquaporin-4 (AQP4) were similar among all NOX2$^{-/-}$ mice. In the pathogenesis of

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edema, inflammation, neurological deficits and arthritis, nicotinamide adenine dinucleotide phosphate oxidase 2 (NADPH oxidase 2; NOX2) produces uncontrolled ROS and affects the development of these processes [7,8]. Chen et al., reported that NOX2 mediates progressive neurodegeneration and reactive microgliosis-generated chronic neuroinflammation [9], suggesting that NOX2 may become a potential therapeutic target for treating neurodegenerative disease. Researchers have found that NOX2 is upregulated and then induces neural oxidative damage in ischemic, traumatic, and degenerative CNS diseases [10,11]. Hervera et al., showed that NOX2 is involved in axonal regeneration by modulating ROS levels [12]. Using a peptide that specifically inhibits NOX2 protein or genetic deletion of NOX2, NOX2 knockout mice (*NOX2*$^{-/-}$) exert neuroprotective effects in ischemia/reperfusion injury and TBI [13,14]. Moreover, Lu et al., showed that brain edema in TBI rats could be regulated by AQP4 [15]. Although amount of studies have been performed to explore the mechanisms of edema, emotional and neurological deficits after TBI, the efforts of treating TBI should be strengthen [4]. Pecorelli et al., showed that oxidative damage induced by NOX2 promotes neuronal hyperexcitability, affecting water and ion balance by aquaporin-4 (AQP4) modulation and thus generating a vicious cycle [16]. However, role of NOX2 and AQP4 in edema after TBI remains unknown, and the relationship between NOX2 and AQP4 in edema process after TBI in mice should be studied in future.

In this study, we try to explore whether NOX2 and AQP4 were involved in the edema process after TBI in mice, and then explore its potential mechanisms of edema between NOX2 and AQP4. Morris Water Maze, step-down test and novel object recognition test were applied to evaluate learning and memory ability. Brain water content was applied to evaluate brain edema after TBI in mice. Inhibition of AQP4 with 2-(nicotinamide)-1,3,4-thiadiazole (TGN-020) according to previous studies [17–19] and then explore the role of AQP4 in edema after TBI in mice. Moreover, *NOX2*$^{-/-}$ and *AQP4*$^{-/-}$ mice were used in this study to explore the mechanisms of edema in TBI mice.

2. Materials and methods

2.1. Animals

All C57BL/6J mice were purchased from Guangdong Provincial Medical Experimental Animal Center. Here, all experiments were performed using either male C57BL/6 mice, male NADPH oxidase 2 (*NOX2*) knockout mice (*NOX2*$^{-/-}$; B6.129S-Cybbtm1Din/J, Jackson Laboratories) or male AQP4$^{-/-}$ mice.

AQP4$^{-/-}$ mice were obtained by using GenOway according to a previous study [20]. Briefly, the genetic strategy aimed to delete exons 1–3. A flippase (FLPo) recognition target (FRT)-neomycin-FRT-LoxP-validated cassette was inserted into exon 3, and a LoxP site was added into exon 1. ES cells were injected into blastocytes and then induced chimeras after homologous recombination. The heterozygous floxed mice were bred from chimeras with females of the C57BL/6J strain. A heterozygous floxed mice were crossed with Cre-expressing C57BL/6J mice to obtain heterozygous for AQP4$^{+/-}$ mice or AQP4$^{+/+}$ mice (wild-type mice).

All the mice (3-months-old) were randomly selected and housed in a 12 h light/dark cycle with controlled ambient temperature (24 ± 1 °C) and humidity (50 ± 10 %). All the mice were allowed to acclimatize for 3 days before experiments. The mice were given a standard diet and sterile drinking water *ad libitum* during experiments. In this study, the animal experiments were performed with the approval of the Laboratory Animal Ethics Committee (Approval #201655) of Nanjing Medical University. In order to investigate the effects of TBI, mice were divided into two groups including control (C57BL/6 mice without Traumatic brain injury (TBI)) group and TBI group. In order to investigate the role of 2-(nicotinamide)-1,3,4-thiadiazole (TGN-020) in the TBI, mice were divided into two groups including TBI group and TBI + TGN-020 group. In order to explore the role of *AQP4* in the TBI, mice were divided into two groups including wild-type (C57BL/6 mice) + TBI group and *AQP4*−/− + TBI group. n = 6 mice in each group in this study.

2.2. Preparation of TBI model mice

Male mice (3 months old, body weight range from 20 to 25 g) were applied in this study. Chloral hydrate (10 %) was intraperitoneally injected, and mouse brain was fixed on the stereo positioner with prone position of mice. A scalp separated the periosteum and exposed the skull of mice. Keeping the dura mater intact, a dental drill was used to drill the skull in the middle of the right front sill and the herringbone sulcus. A focal trauma was then induced to the right brain hemisphere 2 mm lateral to the midline in the midcoronal plane according to previous study [21]. The weight drop was fixed 3.0 cm above the skull of mice. The anus temperature was controlled at 37 ± 0.5 °C during the operation by using a thermostatic operating table. In this study, the mortality rate of TBI mice was 19.2 %. In the control group, all the mice performed the same protocols without the weight drop injury. TGN-020 (200 mg/kg) was administered intraperitoneally 1 h after TBI for 22 consecutive days. The mice were sacrificed after the behavioral experiments on day 22 after TBI.

2.3. Quantitative PCR (Q-PCR)

RNA isolation and quantitative PCR of the right brain without an olfactory bulb were harvested and placed in a 5 ml tube at −80 °C for storage until protein extraction were performed on day 24 after TBI (Fig. S1). Briefly, the mice were decapitated, and the hippocampus was removed. After homogenization, the samples were subjected to RNA isolation using TRIzol and chloroform. The experiments were carried out by using a Peltier Thermal Cycler (MyGene™ Series). The PCR primers were *NOX2*-F (TCCTGCTGCCAGTGTGTCGAAA) and *NOX2*-R (TGCAATTGTGTGGATGGCGGTG); *AQP4*-F (CGGTTCATGGAAACCTCACC), *AQP4*-R (CATGCTGGCTCCAGTATAAT); GAPDH-F (AGGTCGGTGTGAACGGATTTG) and GAPDH-R (GGGGTCGTTGATGGCAACA).

TransScript Top Green Real-Time PCR Supermix (TransGen, Beijing, China) and the Bio-Rad CFX Manager system (Bio-Rad, USA) were applied in this study to perform Q-PCR.

2.4. Western blotting

Mice were decapitated, then right brains without olfactory bulbs were harvested on day 24 after TBI (Fig. S1). The tissues were homogenized at 4 °C by using glass mortar in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 5 mM EDTA, 2 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. Primary antibodies against NOX2 (ab129068, Abcam, Cambridge, UK), AQP4 (ab259318, Abcam, Cambridge, UK), and β-actin (ab8226, Abcam, Cambridge, UK) were diluted using blocking solution to the suggested concentrations and incubated with the membrane at 4 °C overnight. After incubation with a secondary antibody for 1 h at room temperature, the membrane was washed. Then the blots were incubated with anti-mouse IgG conjugated to horseradish peroxidase (1 : 5000) for 1 h at 37 °C. Membrane exposure and data acquisition were achieved by the ImageQuant™ RT ECL System (GE Healthcare).

2.5. ELISA

Mice were decapitated, then right brains without olfactory bulbs were harvested on day 24 after TBI (Fig. S1). The harvested tissues were ground into a homogenate in a glass mortar. SOD and Malondialdehyde (MDA) contents were detected using mouse superoxide dismutase (SOD) and MDA ELISA kits according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.6. ROS measurement

Mice were decapitated, then right brains without olfactory bulbs were harvested on day 24 after TBI (Fig. S1). Dichloro-dihydro-fluorescein diacetate (DCFH-DA)-based ROS measurement: A DCFH-DA solution was prepared using DMSO with a concentration of 10 mM. The harvested tissues were ground into a homogenate in a glass mortar, and the lysate was diluted to 1/10 the original concentration using PBS. The stocking solution was diluted in lysate to 10 μM. The mixtures were incubated for about 15 min, and samples were subjected to fluorescence-activated cell sorting (FACS) Canto II (Becton, Dickinson and Company, Sparks, MD), and experimental data were analyzed by using FlowJo 7.6.

2.7. Brain water content determination

The method of measuring brain water content was performed according to a previous study on day 24 after TBI (Fig. S1) [22]. Wet and dry weights of right cortex or right hippocampus were measured respectively. 100 % × [(wet weight−dry weight)/wet weight] was used to calculate the water percentage in cortex or hippocampus.

2.8. Morris Water Maze (MWM)

Morris water maze test (MWM) was applied to evaluate the cognitive ability according to a previous study [23] on day 15 after TBI (Fig. S1). Mice received 4 training sessions per day for three consecutive days, separated by intervals of 20 s. Time was recorded for mice to find the platform from four different inlets in each quadrant, referred to as escape latency. If the mouse cannot achieve the platform within 2 min, its latency was calculated as 120 s. The average of the 4 training latencies were applied as the final statistic for judging the mouse's acquisition of memory. After removing the platform, the mouse was placed in the water from any water inlet point to record the swimming trajectory of the mouse within 120 s both 1 h and 1-day post-training: the latency required for the mouse to find the position of the platform and times it shuttles through the target quadrant.

2.9. Step-down test (SDT)

The step-down test was applied to evaluate the cognitive ability after the Morris water maze test on day 19 after TBI (Fig. S1). The animals were trained for 5 min in this way, and the number of received electric shocks per mouse was recorded as learning results. The test was repeated 1-day post-training, referred to as the memory retention test. The incubation period of the animal was recorded after the first shock, and the total number of errors was recorded within 3 min.

2.10. Novel object recognition test (NOR)

The novel object recognition test (NOR) was applied to asses the cognitive ability after TBI (Fig. S1) according to previous study [23]. Briefly, the habituation phase, familiarization phase, and discrimination phase were included in this test. In the habituation phase, all the mice were allowed to move and explore freely for 5 min. In the familiarization phase, all the mice were subjected to the two identical objects. In the discrimination phase, the familiar objects (TF) and the time of novelty object (TN) were recorded. The discrimination index (DI) was obtained by using the following formula: DI = (TN - TF)/(TN + TF).

2.11. Statistics

GraphPad Prism version 7.0 (GraphPad, San Diego, CA, USA) was applied to statistical analysis. Our sample size was calculated by setting the type I error as 0.05, with a power of 90 %, and the superiority margin was set to 0. We performed a series of behavior study on three mice to determine the number of animals required; to detect a 1.00 difference of absolute deviation (AD) of Osteoarthritis Research Society International (OARSI) score between control group and TBI group, 6 mice would be required in each group. In this study, the data distribution was assessed by using *Shapiro-Wilk* test for normality. All data were expressed as the mean ± standard error (SEM). The 2ANOVA followed by *Tukey* test was applied in the analysis of escape latency of the MWM and DI in the NOR test. Moreover, two-tailed Student's unpaired t-tests was used to compared the statistical significance between two groups. $P < 0.05$ was considered to a indicate statistical significance.

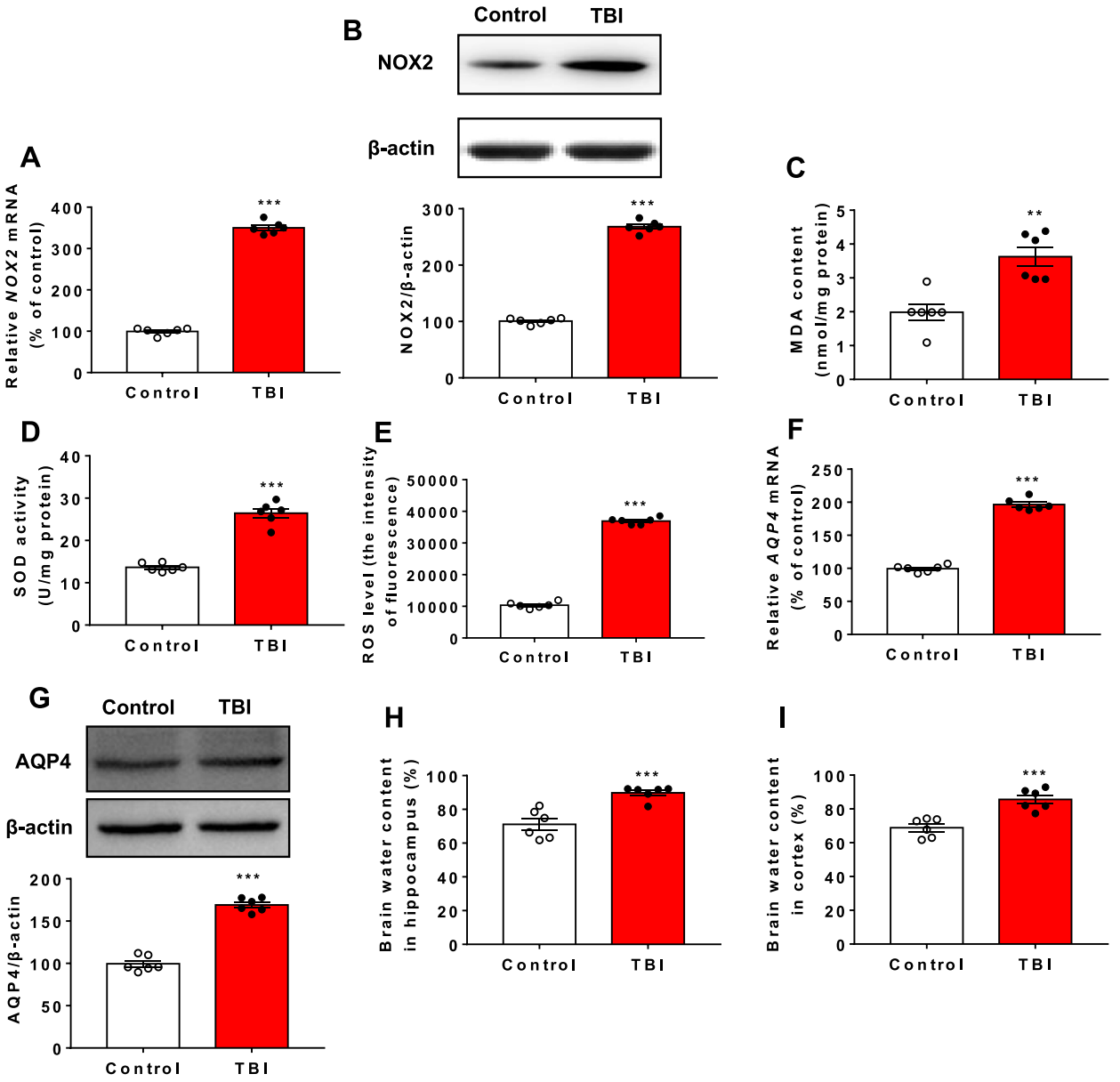

Fig. 1. Effects of TBI on the expression of NOX2 and AQP4, ROS production and brain edema in mice.
NOX2 mRNA levels (**A**), NOX2 protein levels (**B**), MDA content (**C**), SOD activity (**D**), ROS levels (**E**), *AQP4* mRNA levels (**F**), AQP4 protein levels (**G**), brain water content in hippocampus (**H**) and brain water content in cortex (**I**) in the mouse models of TBI. n = 6 mice in each group. Data represent the mean ± SEM. Error bars indicate SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control group.

3. Results

3.1. TBI induced the expression of NOX2, AQP4, and ROS production and increased brain edema in mice

Our results revealed that TBI significantly induced the upregulation of both *NOX2* mRNA levels (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 1A) and NOX2 protein levels (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 1B). Thus, we measured the malondialdehyde (MDA) content, SOD activity and ROS level after TBI. TBI significantly increased MDA content, SOD activity and ROS levels compared to control treatment (Unpaired *t*-test, df = 10, $P = 0.0012$ for Fig. 1C, Unpaired *t*-test, df = 10, $P < 0.0001$ for Fig. 1D and E, Fig. 1C–E).

Edema is associated with unfavorable prognosis in traumatic brain injury (TBI). Thus, we studied AQP4 expression levels and brain edema in the hippocampus and cortex after TBI. Our results showed that TBI significantly induced the upregulation of *AQP4* mRNA levels (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 1F) and AQP4 protein levels (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 1G). Moreover, we found that the water content in the hippocampus (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 1H) and cortex (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 1I) of TBI mice was significantly increased compared to control mice.

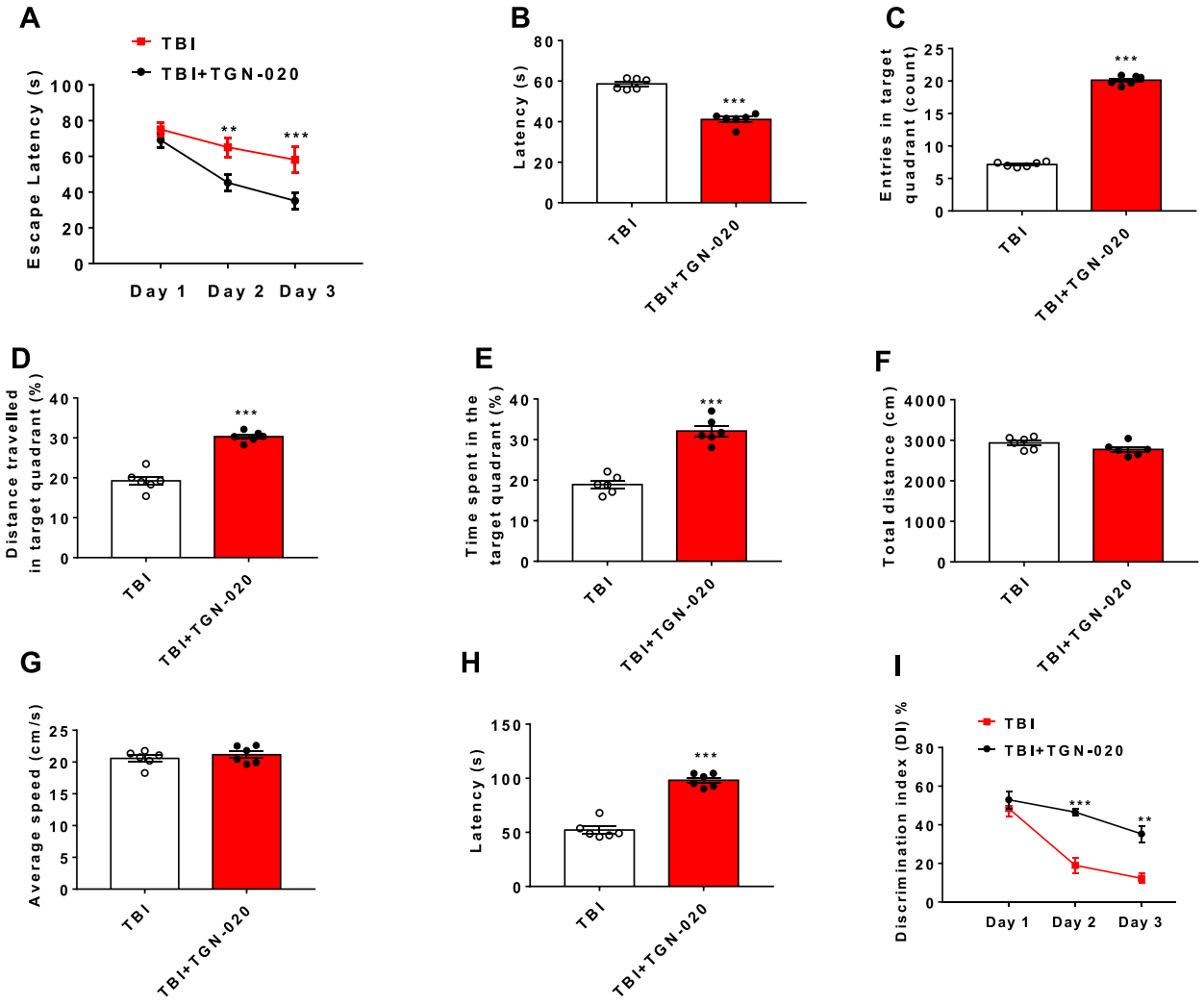

Fig. 2. Effects of TGN-020 on cognitive ability after TBI in mice.
(A) TGN-020 treatment required less time to find the platform in the MWM after TBI. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the TBI group. The latency (**B**), entries into the target quadrant (**C**), distance traveled in the target quadrant (**D**), time spent in the target quadrant (**E**), total distance traveled (**F**) and average speed (**G**) in the MWM were measured after TGN-020 treatment in TBI mice. (**H**) In SDT, latency was recorded after TGN-020 treatment in TBI mice. **(I)** In the NOR test, the discrimination index was measured after TGN-020 treatment in TBI mice. n = 6 mice in each group. Data represent the mean ± SEM. Error bars indicate SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to TBI group.

3.2. TBI induced learning and memory impairment in mice

In order to evaluate the learning and memory ability after TBI, the MWM was performed. For learning ability, we found that TBI mice significant increase the latency to achieve the platform compared to control mice (2ANOVA, $F(1, 42) = 89.59$, $P < 0.0001$, Fig. S2A). Moreover, latency (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S2B), entries in the target quadrant (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S2C), distance traveled in the target quadrant (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S2D) and time spent in the target quadrant (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S2E) were significant decreased in TBI mice compared to control mice. Meanwhile, no significant statistical difference of total distance (Unpaired *t*-test, df = 10, $P = 0.1061$, Fig. S2F) or average speed (Unpaired *t*-test, df = 10, $P = 0.7473$, Fig. S2G) was found in the two groups. Furthermore, latency (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S2H) in the SDT and discrimination index (2ANOVA, $F(1, 30) = 25.63$, $P < 0.0001$, Fig. S2I) in the NOR after TBI were decreased compared to control mice.

3.3. Genetic deletion of NOX2 decreased ROS production, AQP4 expression and brain edema in TBI mice

We had revealed that TBI significantly up-regulated the gene and protein levels of NOX2 and AQP4. To explore whether NOX2 is involved in modulating AQP4 and brain edema, *NOX2* knockout mice ($NOX2^{-/-}$) were used in this study. Our results revealed that MDA content (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S3A), SOD activity (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S3B) and ROS levels (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S3C) were significantly decreased in $NOX2^{-/-}$ mice compared to wild-type mice after TBI. After TBI, genetic deletion of NOX2 significantly decreased *AQP4* mRNA levels (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S3D) and AQP4 protein levels (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S3E) compared to those in wild-type mice. Moreover, the water content in the hippocampus (Unpaired *t*-test, df = 10, $P = 0.0019$, Fig. S3F) and cortex (Unpaired *t*-test, df = 10, $P = 0.0263$, Fig. S3G) was significantly decreased in $NOX2^{-/-}$ mice compared to wild-type mice in the animal models of TBI.

3.4. Neurological deficits was reversed in $NOX2^{-/-}$ mice after TBI

For learning ability, we found that $NOX2^{-/-}$ mice significant decrease the latency to achieve the platform compared to wild-type +

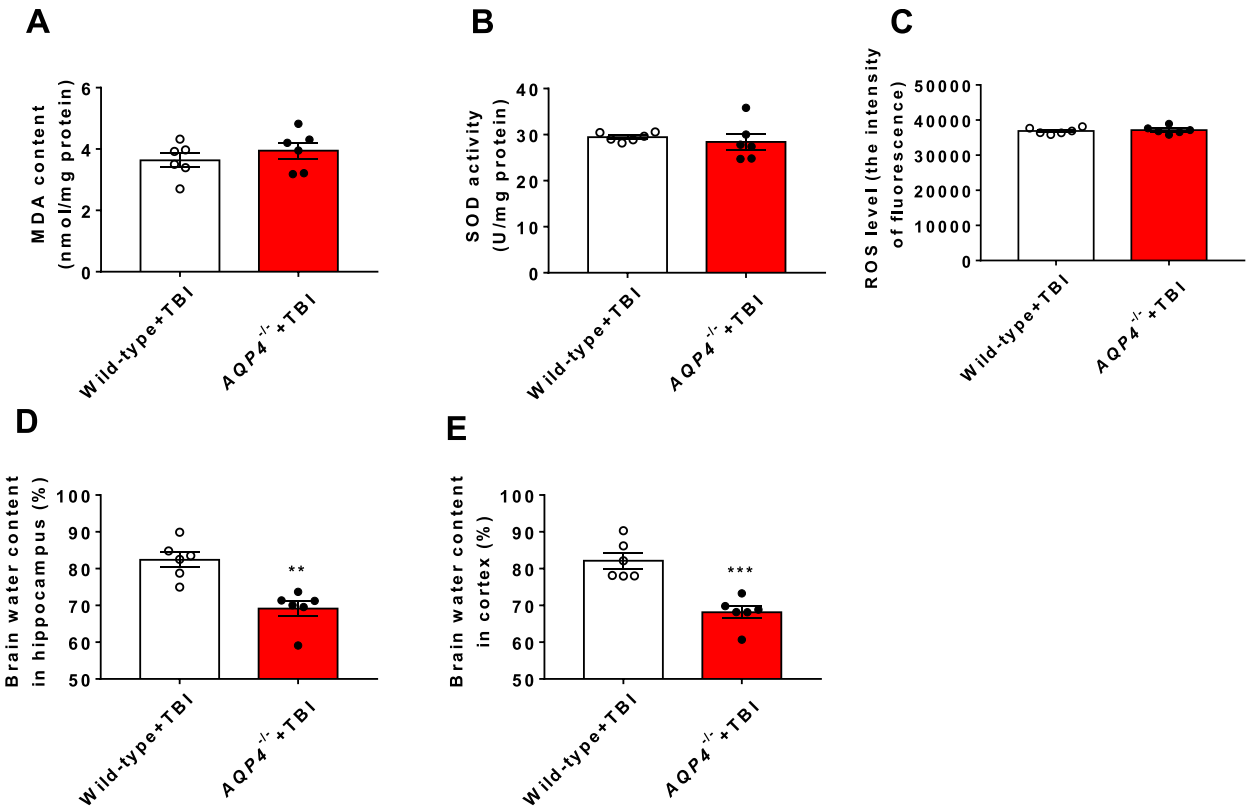

Fig. 3. Effects of TBI on the expression of ROS and brain edema in $AQP4^{-/-}$ mice.
MDA content (**A**), SOD activity (**B**), ROS levels (**C**), brain water content in the hippocampus (**D**) and brain water content in the cortex (**E**) in the animal models of TBI in $AQP4^{-/-}$ mice. n = 6 mice in each group. Data represent the mean ± SEM. Error bars indicate SEM. $^{**}P < 0.01$ compared to wild-type + TBI group.

TBI mice (2ANOVA, F(1,30) = 78.91, $P < 0.0001$, Fig. S4A). Moreover, latency (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S4B), entries in the target quadrant (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S4C), distance traveled in the target quadrant (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S4D) and time spent in the target quadrant (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S4E) were significant increased in *NOX2*$^{-/-}$ mice compared to wild-type mice after TBI. Meanwhile, total distance (Unpaired *t*-test, df = 10, $P = 0.3547$, Fig. S4F) or average speed (Unpaired *t*-test, df = 10, $P = 0.1251$, Fig. S4G) in the two groups have no significant change. Furthermore, latency (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. S4H) in the SDT and discrimination index (2ANOVA, F(1, 42) = 60.94, $P < 0.0001$, Fig. S4I) in the *NOX2*$^{-/-}$ mice were increased compared to wild-type + TBI mice.

3.5. Acute inhibition of AQP4 reduced brain edema without affecting ROS levels

Although Oosuka et al. reported that 2-(nicotinamide)-1,3,4-thiadiazole (TGN-020) inhibits ROS production after high glucose treatments in cultured Müller cells [24], we found that applying TGN-020 to TBI mice did not affect MDA content (Unpaired *t*-test, df = 10, $P = 0.8118$, Fig. S5A), SOD activity (Unpaired *t*-test, df = 10, $P = 0.4711$, Fig. S5B) or ROS levels (Unpaired *t*-test, df = 10, $P =$

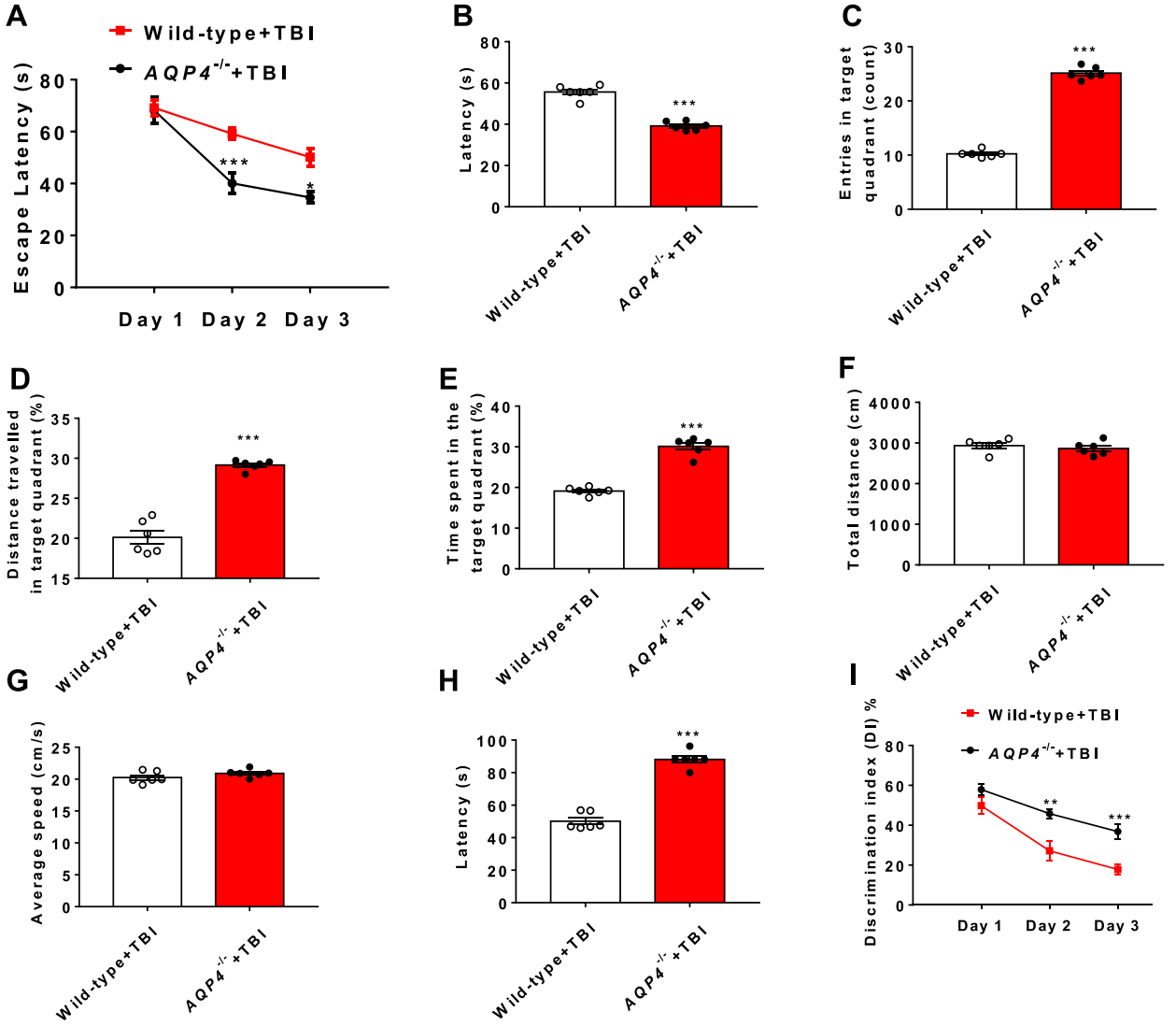

Fig. 4. Effects of TBI on cognitive ability in *AQP4*$^{-/-}$ mice.
(A) The *AQP4*$^{-/-}$ mice required less time to find the platform than wild-type mice on Day 2 and Day 3 in the MWM after TBI. The data are expressed as the mean ± standard error (S.E.M.) of 12 mice per group. The latency (**B**), entries into the target quadrant (**C**), distance traveled in the target quadrant (**D**), time spent in the target quadrant (**E**), total distance traveled (**F**) and average speed (**G**) in the MWM were measured after TBI in mice. **(H)** In the SDT, latency was recorded after TBI in the *AQP4*$^{-/-}$ mice. **(I)** In the NOR test, the discrimination index was measured after TBI in *AQP4*$^{-/-}$ mice. n = 6 mice in each group. Data represent the mean ± SEM. Error bars indicate SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to wild-type + TBI group.

0.8346, Fig. S5C). Moreover, we found that TGN-020 significantly reduced the water content in the hippocampus (Unpaired *t*-test, df = 10, $P = 0.0030$, Fig. S5D) and cortex (Unpaired *t*-test, df = 10, $P = 0.0024$, Fig. S5E) compared to that in TBI mice, those results are similar to previous studies [25].

3.6. Acute inhibition of AQP4 attenuates neurological deficits induced by TBI in mice

For learning ability, we found that TGN-020 treatment significant increase the latency to achieve the platform compared to control mice after TBI (2ANOVA, $F(1,30) = 94.51$, $P < 0.0001$, Fig. 2A). Moreover, latency (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 2B), entries in the target quadrant (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 2C), distance traveled in the target quadrant (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 2D) and time spent in the target quadrant (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 2E) were significant decreased in TGN-020 treatment TBI mice compared to TBI mice. Meanwhile, total distance (Unpaired *t*-test, df = 10, $P = 0.1024$, Fig. 2F) or average speed (Unpaired *t*-test, df = 10, $P = 0.4378$, Fig. 2G) in the two groups have no significant change. Furthermore, latency (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 2H) in the SDT and discrimination index (2ANOVA, $F(1,30) = 97.98$, $P < 0.0001$ Fig. 2I) in the TGN-020 treatment mice were decreased compared to TBI mice.

3.7. Genetic deletion of AQP4 reduced brain edema without affecting ROS levels

$AQP4^{-/-}$ transgenic mice were applied in this study to explore the role of AQP4 in the process of brain edema after TBI. Our results showed that MDA content (Unpaired *t*-test, df = 10, $P = 0.3959$, Fig. 3A), SOD activity (Unpaired *t*-test, df = 10, $P = 0.5632$, Fig. 3B) and ROS levels (Unpaired *t*-test, df = 10, $P = 0.6401$, Fig. 3C) were not significantly changed in $AQP4^{-/-}$ mice compared to wild-type mice in the animal models of TBI. Edema of hippocampus and cortex was significantly decreased in $AQP4^{-/-}$ mice compared to wild-type mice (Unpaired *t*-test, df = 10, $P = 0.0012$ for 3D, Unpaired *t*-test, df = 10, $P < 0.0001$ for 3E, Fig. 3D and E) in the animal models of TBI, which is consistent with previous studies [26].

3.8. Genetic deletion of AQP4 attenuates neurological deficits induced by TBI in mice

For learning ability, we found that $AQP4^{-/-}$ mice significant decrease the latency to achieve the platform compared to wild-type in the animal models of TBI (2ANOVA, $F(1,30) = 104.3$, $P < 0.0001$, Fig. 4A). Moreover, latency (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 4B), entries in the target quadrant (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 4C), distance traveled in the target quadrant (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 4D) and time spent in the target quadrant (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 4E) were significant increased in $NOX2^{-/-}$ mice compared to wild-type mice in the animal models of TBI. Meanwhile, total distance (Unpaired *t*-test, df = 10, $P = 0.4649$, Fig. 4F) or average speed (Unpaired *t*-test, df = 10, $P = 0.1819$, Fig. 4G) in the two groups have no significant change. Furthermore, latency (Unpaired *t*-test, df = 10, $P < 0.0001$, Fig. 4H) in the SDT and discrimination index (2ANOVA, $F(1,30) = 26.94$, $P < 0.0001$, Fig. 4I) in the $AQP4^{-/-}$ mice were increased compared to wild-type mice in the animal models of TBI.

4. Discussion

Nicotinamide adenine dinucleotide phosphate oxidase 2 (NADPH oxidase 2; NOX2) was up-regulated in the animal models of traumatic brain injury (TBI), and inhibiting NOX2 attenuated neurological deficits after TBI [27]. In this study, we found that NOX2 and reactive oxygen species (ROS) levels were up-regulated after TBI, as previously reported [28]. Although we have demonstrated that NOX2 may be a potential target for treating cognitive impairment by modulating synaptic transmission in the hippocampus in the TBI mice, the role of NOX2 in brain edema after TBI in mice remains unknown. In this study, we explored the potential mechanisms by exploring the effects of NOX2 on edema after TBI by applying $NOX2^{-/-}$ and $AQP4^{-/-}$ mice.

Reports have suggested that ROS can regulate AQP4 protein level in cultured astrocytes [29]. Esposito et al. pointed out that inhibiting AQP4 leads to the production of ROS in cultured astrocytes [30]. However, nicotinamide (TGN-020), an inhibitor of AQP4, could increase intracellular ROS production under high glucose conditions in murine diabetic retinas [31]. Hao et al. pointed out that AQP4 exerts protective effects after 1-methyl-4-phenylpyridinium ion (MPP) and lipopolysaccharide (LPS) treatment by reducing the production of ROS [32]. In this study, we aimed to explore the relationship between ROS, which are primarily produced by NOX2, and AQP4 in the progression of edema after TBI in mice. Although the expression of NOX2 and AQP4 was up-regulated in the animal models of TBI, the relationship between NOX2 and AQP4 remains unknown. Thus, *NOX2* knockout mice ($NOX2^{-/-}$) were applied to probe the potential mechanism between NOX2 and AQP4. Our results showed that knockout of *NOX2* in mice decreases MDA content, SOD activity, ROS levels, and the mRNA and protein levels of AQP4 in the mouse models of TBI. Moreover, brain edema was decreased, and cognitive ability was improved in $NOX2^{-/-}$ mice in the animal models of TBI. Thus, we suspected that AQP4 could be modulated by NOX2 in the mouse models of TBI.

Here we manipulated the activity or expression of AQP4 to explore the role of NOX2 and AQP4 in the process of TBI in mice. TGN-020 was applied to inhibit AQP4 after TBI in mice. Our results showed that MDA content, SOD activity and ROS levels were not significantly changed after TGN-020 treatment after TBI. Moreover, we found that TGN-020 significantly reduced the water content in the brains of TBI mice. Moreover, acute inhibition of AQP4 using TGN-020 improved cognitive ability in the animal models of TBI. These results also illustrate that AQP4 could be modulated by NOX2 and then affect brain edema and cognitive ability in the mouse models of TBI. To verify the relationship between NOX2 and AQP4 in the mouse models of TBI, genetic deletion of AQP4 in mice was applied in this study. The MDA content, SOD activity and ROS level were not significantly altered in $AQP4^{-/-}$ mice compared to wild-

type mice with the animal models of TBI. Moreover, the water content in the brains of *AQP4*$^{-/-}$ mice in the animal models of TBI was significantly decreased compared to that in wild-type mice. Furthermore, cognitive ability in *AQP4*$^{-/-}$ mice was significantly increased compared to that in wild-type mice in the animal models of TBI. Taken together, we suspected that inhibiting NOX2 could decrease the expression of AQP4 and then reduce edema and improve cognitive abilities in TBI mice.

One limitation of this study is that detailed molecular mechanisms of NOX2 and AQP4 in the process of reduce brain edema and improve cognitive abilities after TBI may not be addressed clearly. For example, how does NOX2 affect the expression of AQP4 should be intensively studied in the animal models of TBI.

In summary, our results found that *NOX2*$^{-/-}$ mice could decrease the expression of AQP4, reduce brain edema and improve cognitive abilities after TBI. Moreover, inhibiting AQP4 or deleting *AQP4* in mice attenuated brain edema and neurological deficits without changing ROS levels after TBI. Taken together, we suspected that inhibiting NOX2 could improve cognitive abilities by modulating ROS levels and then affecting AQP4 expression and brain edema in the mouse models of TBI.

PRODUCTION NOTES.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22035.

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