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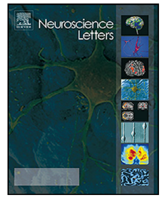

Research article

Deletion of aquaporin-4 is neuroprotective during the acute stage of micro traumatic brain injury in mice

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HIGHLIGHTS

- Two-photon microscopy was use to examine the role of AQP4 in micro TBI.
- AQP4 deletion reduced cells death, astrocytes swelling and lesion volume.
- Treatment inhibiting AQP4 can be used to protect micro brain injury.

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ABSTRACT

Micro traumatic brain injury (TBI) is the most common type of brain injury, but the mechanisms underlying it are poorly understood. Aquaporin-4 (AQP4) is a water channel expressed in astrocyte end-feet, which plays an important role in brain edema. However, little is known about the role of AQP4 in micro TBI. Here, we examined the role of AQP4 in the pathogenesis of micro TBI in a closed-skull brain injury model, using two-photon microscopy. Our results indicate that AQP4 deletion reduced cell death, water content, astrocyte swelling and lesion volume during the acute stage of micro TBI. Our data revealed that astrocyte swelling is a decisive pathophysiological factor in the acute phase of this form of micro brain injury. Thus, treatments that inhibit AQP4 could be used as a neuroprotective strategy for micro TBI.

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1. Introduction

Traumatic brain injury (TBI) is a significant health burden worldwide [1]. Although the clinical manifestation of TBI varies extensively—from death to neurological deficits or short-term brain damage, depending on the severity of the injury—concussion or micro TBI is the most common type [2]. Micro TBI, which often occurs in contact sports and explosive blasts, can contribute to the development of cognitive impairment later in life [3]. Unfortunately, the mechanisms underlying micro TBI are poorly understood, because it is often neglected because of its lack of critical impact within a short space of time. As a result, there are no effective drugs to attenuate the formation and progression of micro TBI.

Cerebral edema is a major contributor to morbidity associated with severe TBI [4], and a reduction in its severity can decrease morbidity. However, the role of cerebral edema is unclear in the pathophysiology of micro TBI. Aquaporin 4 (AQP4) is a bidirectional transport channel for water. In astrocytes, it is mainly present in the end-feet at the blood–brain and brain–cerebrospinal fluid barriers [5]. Phenotypic analysis of transgenic mice lacking AQP4 has provided compelling evidence for its involvement in cerebral water balance, astrocyte migration, and neural signal transduction [6]. AQP4 can be beneficial or detrimental depending on the nature of the disease. For example, AQP4 deletion can reduce brain swelling and improve neurological outcome in models of cytotoxic cerebral edema, such as water intoxication, focal cerebral ischemia [7] and bacterial meningitis [8]. In contrast, brain swelling and clinical outcome are worse in AQP4-null mouse models of cortical freeze-injury, brain tumor, and hydrocephalus [6,9]. Research on TBI has

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provided substantial evidence that the effect of AQP4 modulation is largely dependent upon both the type of brain injury and the time following injury onset [3]. However, little is known about the role of AQP4 in micro TBI because of the lack of an appropriate model system. Recently, a closed-skull brain injury model has been developed that has revealed novel insights into micro TBI pathogenesis using two-photon microscopy [10]. In the present study, we examined the role of AQP4 in the pathogenesis of micro TBI using the same closed-skull brain injury model with two-photon microscopy.

2. Materials and methods

2.1. Mice

$AQP4^{-/-}$ and $AQP4^{+/+}$ mice were purchased from Nanjing Biomedical Research Institute, Nanjing University, China. The present experiments were performed on 2–3-month-old, male $AQP4^{+/+}$ and $AQP4^{-/-}$ mice, weighing 20–25 g. All animals were treated according to protocols approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University, China. Investigators were blinded to genotype information for all experiments.

2.2. Surgery preparation

Mice were deeply anesthetized with 5% isoflurane, which was maintained with 2% isoflurane in a mixture of 20% oxygen and 80% air. Rectal temperature was maintained at $37 \pm 0.5\,^{\circ}$C, using a regulated heating pad with a rectal probe (Reward, China).

2.3. Micro TBI preparation

After the animal was placed in a stereotaxic apparatus, an incision was made through the middle skin of the skull using a sterile scalpel, and the skull bone was exposed by scraping away the periosteum. A cranial window of 2.0×2.0-mm size was thinned on the left somatosensory cortex, between bregma and lambda sutures, using a microdrill. To induce compression injury as previously described [10], with some modifications, the skull was thinned for 1–2 min to a thickness of 20–30 μm. Once thinned, the blunt end (~1.0 mm width) of a surgical instrument was used to gently press the pliable skull bone downward 0.5 mm under stereotactic guidance to promote a constant concavity in the bone. This process resulted in the skull bone collapsing (without breaking) inward towards the surface of the brain.

2.4. *In vivo* two-photon imaging

For *in vivo* two-photon imaging, the mouse was fixed with a custom-fabricated metal frame that held the head with a cyanoacrylate and dental cement, which was then fixed on the stage of a Leica DM6000 CFS (Leica, Germany). Data acquisition and laser scanning were controlled using Leica LAS AF 2.5 software. For imaging vessel network and counting dead cells, a Leica NA 0.95, 25× water-immersion objective was used. All images were acquired using two-channel NDD detection, with emission filters of 525/50 nm and 585/50 nm, on a TCS SP5 MP System (Leica Microsystems, Mannheim, Germany). The blood serum was labeled by intravenously injecting 0.2 ml of 2% (wt/vol) solution of 2-MDa fluorescein–dextran (FD2000S; Sigma, USA) in saline.

2.5. Transcranial propidium iodide staining

Previous studies [10] have shown that low-molecular-weight compounds are able to pass through intact thinned skull into the meninges and parenchyma to reliably label dead cells by transcranial application of propidium iodide (PI). Therefore, we visualized cell death to evaluate the evolution of injury in the compression site by incubating the thinned skull with PI (1.5 mM) in artificial cerebrospinal fluid (aCSF) for 30 min. This was followed by a single wash with aCSF and imaging with two-photon microscopy, using a Leica NA 0.95, 25× magnification water-immersion objective. Stacks of images were acquired using a step size of 2.0 μm to a depth of 200 μm. Cells labeled by PI exhibiting red fluorescence were considered to be dead cells. For lesion volume detection, PI was incubated by transcranial application to the compression site for 30 min before fixation. The total number of PI-positive cells in the focal lesion was calculated in three randomly-selected images. The number of PI-positive cells was calculated using the Image J software, NIH, Bethesda, MD, USA "analyze particles" tool.

2.6. BBB integrality test

Twenty-four hours after TBI, the integrity of the blood–brain barrier (BBB) was examined, as previously described [11], with some modifications. Dextran-conjugated Rhodamine B (70 kDa MW; Invitrogen) was injected through the tail vein for visualization of the vessels and disruption of BBB, indicated by dye leakage. The blood vessels in the brain were illuminated with a mode-locked Ti:sapphire laser unit set at 860 nm, using a 0.95 NA, 25× water-immersion objective lens. A stack of 10 images, spanning 100–300 mm below the cortical surface, was acquired every 5 min over a 30 min period.

2.7. Brain edema measurement

The percentage brain water content was determined using the wet–dry method, as previously described [12]. The percentage water content (% H_2O) was calculated for each hemisphere as follows: % H_2O = [(wet weight–dry weight)/wet weight] × 100%.

2.8. Lesion volume calculation

Lesion volume were calculated by PI staining, follow the method of lesion volume calculation by TTC in [13,14]. After fixation,brain was sliced into 50 μm thick each section. Lesion site with PI staining was imaged with confocal microscopy and measured by Image J software. Total infarction volume was the sum of PI staining volumes.

2.9. Histological analysis

The brain regions in the thin-skull window from each brain were dissected into 10-μm thick consecutive coronal slices. The following cell types were labelled: neurons (anti-NeuN; 1:400; Millipore, USA), astrocytes (anti-GFAP; 1:400; Sigma, USA), microglia (anti-Iba1; 1:400; Wako, Japan) and AQP4 (anti-AQP4; 1:200; Alomone, Israel).

2.10. Confocal microscopy

Two-dimensional images were captured from the stained 10-μm frozen sections using a Leica SP5 confocal microscope equipped with a 25× objective. Images were collected using sequential scanning with the 405-, 488-, and 561-nm lasers to produce combined overlays.

2.11. Statistical analysis

Statistical values were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by *post hoc*

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