EPHRIN-B2 EXPRESSION IN THE ASTROCYTE MEMBRANE AROUND MOUSE BRAIN INJURIES AND THE NEUROPROTECTIVE EFFECT CONFERRED AFTER EPHRIN-B2 KNOCKOUT

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ABSTRACT

Objective: *To analyze Ephrin-B2 ligand expression in the astrocyte membrane around brain injuries in mice and the neuroprotective effect conferred after Ephrin-B2 knockout.*

Methods: *A mouse brain injury model was established, and Ephrin-B2 gene knockout mice were cultured. The Ephrin-B2 gene knockout mice expressed glial fibrillary acidic protein (GFAP)-positive astrocytes. Ephrin-B2 and GFAP protein expression was detected by western blotting, and GFAP gene expression was detected by polymerase chain reaction (PCR).*

Results: *Western blotting results showed good initial astrocyte growth with no significant difference in Ephrin-B2 knockout mice and control mice. After the brain trauma model was established, GFAP expression in the cerebral cortex of Ephrin-B2 knockout mice was significantly higher than that in the control group ($P<0.05$). Western blotting results showed that phosphorylated Ephrin-B2 protein expression increased significantly after brain injury in the control group, reached the highest level on the seventh day after injury, and subsequently declined until the 21st day; however, it remained higher than that in mice without brain injury ($P<0.05$). On the third day after brain injury, the total Ephrin-B2 protein in the control group decreased to a level slightly lower than that in mice without brain injury, after which Ephrin-B2 protein expression increased gradually. In Ephrin-B2 knockout mice, the expression of GFAP increased significantly after brain injury. On the seventh day after brain injury, the astrocyte volume was significantly increased in the knockout mice, and the processes were thickened. On the 21st day after brain injury, astrocytes were evenly distributed to fill the injured area, resulting in very small injury holes. Real-time quantitative polymerase chain reaction results showed that within 21 days after brain injury, GFAP expression in the hippocampus and ipsilateral hippocampus of Ephrin-B2 knockout mice and control mice was significantly different ($P<0.05$). On the seventh day after brain injury, GFAP mRNA expression was the highest in the hippocampus and around the injured area in the control group, after which the levels decreased gradually. In Ephrin-B2 knockout mice, GFAP expression was the highest in the ipsilateral hippocampus on the seventh day post-injury and remained significantly increased around the injured area on the 21st day, when the expression level was three times higher than that in control mice.*

Conclusion: *After brain injury, Ephrin-B2 ligand expression in the astrocyte membrane and GFAP mRNA expression around the injured area were significantly increased. The resulting reactive astrocytes provided an increased neuroprotective effect.*

Keywords: *Brain injury, Ephrin-B2 ligand, astrocyte, neuroprotection.*

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Introduction

Brain injuries are a common neurological problem and are some of the most frequently addressed critical issues in neurosurgery. Falls and collisions are the most common causes of brain injuries, which can include soft tissue, skull, and intracranial tissue injuries and are often accompanied by mental disorders that constitute the main causes of brain injury-associated disability and death(1). Continuous improvement and innovation in neurosurgical treatment methods can significantly improve the mortality rate and prognoses of patients. Some studies show a high incidence of neurological dysfunction after brain injury. Most patients experience weakened learning and memory abilities and limb dysfunction, which seriously impact the families and lives of the patients(2). Therefore,

reducing the sequelae of brain injury after treatment and improving the cognitive and motor abilities of patients have become the focus of clinical scholars. The recently discovered Ephrin-B2 ligand is a member of the Eph receptor family and an important angiogenic factor. It participates in embryonic nervous system development and angiogenesis and plays very important roles in various nerve cells[3, 4].

In this study, Ephrin-B2 ligand expression in the astrocyte membrane around a brain injury and the neuroprotective effect conferred after Ephrin-B2 knockout were analyzed by establishing a brain injury mouse model.

Materials and methods

Laboratory animals

Two mouse lines (glial fibrillary acidic protein CRE mice and Ephrin-B2 ligand-loxp mice) were hybridized to obtain Ephrin-B2 knockout mice, and wild-type mice were selected as the control group. All mice were housed in the laboratory at 20-24°C, with 50%-60% humidity, a 12-hour day/night cycle, and unrestricted food and water intake. Mice at 10-12 weeks of age weighing 25-40 g were used in the experiment.

Laboratory reagents and instruments

Upright and inverted fluorescence microscopes were purchased from Olympus Corporation, Japan. The spectral enzyme marker was purchased from Semerfly Technology Co., Ltd., USA. The miniature centrifuge, PCR, and microsurgery instruments were purchased from Fisher Scientific Worldwide (Shanghai) Co., Ltd. Micropipettes were purchased from Haimen Aibende Biotechnology Co., Ltd., Germany. The frozen section machine was purchased from Leica Biotechnology Co., Ltd., Germany. The ultrasonic cell homogenizer was purchased from Bole Life Medical Products Co., Ltd.

Goat anti-mouse Ephrin-B2 antibodies were purchased from R&D Systems. Ephrin-B2 polyclonal antibodies were purchased from Emergency Technology Co., Ltd. Mouse anti-GFAP antibodies were purchased from Semerfly Technology Co., Ltd. Goat anti-mouse beta-actin antibodies, fetal bovine serum, and phosphate buffer solution were purchased from Yingjie Life Technology Co., Ltd.

Experimental method

• The brain injury model was established in mice under anesthesia. Each mouse was fixed on a stereotactic frame, and the dorsal head skin was incised. Holes were drilled into the skull using a tip diameter of 3 mm and an impact depth of 0.5 mm, causing damage to the cerebral cortex. After the holes were drilled, the skin was sutured, and the mouse was placed in a rewarming box to recover from anesthesia.

• Anesthetized mice were injected in the left ventricle with 50 ml heparin 1X-PBS and 20 ml 4% polyformaldehyde on days 3, 7, 14, and 21 after brain injury. After injection, the brain tissue of each anesthetized mouse was carefully dissected, stored in a refrigerator at 4°C, and subsequently dried in 15% and 30% sucrose solutions. Some of the brain tissues were stored in a refrigerator at -80°C. The remaining tissue samples were embedded, immediately sliced into 20 μm coronal slices using a freezing slice machine, and then stored in a refrigerator at -80°C 30 minutes after slicing.

• Ephrin-B2 protein and glial fibrillary acidic protein (GFAP) expression in mice after brain injury was detected by western blotting. Fresh damaged tissue (50 mg) was added to 1 ml precooled RIPA buffer and then centrifuged at 3000 r/min and 4°C for 15 minutes. The supernatant was collected and stored at -80°C. The protein samples were subjected to vertical electrophoresis in 30% polypropylene-G gels at 80 V and then transferred to 10% sodium dodecyl sulfate (SDS) gels at 120 V (90 min). The proteins were subsequently transferred from the 10% SDS gel to a nitrocellulose membrane (120 V, 90 min). Odyssey blocking buffer was added to the membrane, sealed, and allowed to stand for 1 hour. Anti-goat anti-mouse renin B2 antibodies (1:100) and rabbit anti-mouse beta-actin antibodies (1:2000) were placed in Tris-buffered saline (TBS) overnight at 4°C. After the blocking buffer was washed from the membrane, fluorescence-labeled antibodies were added to the goat antibody (1:5000) and rabbit antibody (1:5000) TBS solutions and incubated with the membrane at room temperature for 2 hours. After rinsing the membrane three times, the Odyssey infrared imaging system was used to perform fluorescence scanning. The Ephrin-B2 antibodies (green) and beta-actin antibodies (red) were detected at 680 nm and 800 nm, respectively. Semi-quantitative analysis of the obtained images was performed using Odyssey 3.0 software.

• GFAP gene expression was determined by polymerase chain reaction (PCR). The total RNA was extracted from 50 mg fresh damaged tissues using the RNEAsy Mini Kit (Ca, Valencia, Qiagen,

CA) according to the manufacturer's instructions. The total RNA was then analyzed by an ND-1000 spectrophotometer system (Thermo Fisher Scientific Inc., ND-1000 3.7.1). A high-capacity DNA reverse transcription kit, the GFAP RN01460868_M1 fluorescent probe, and beta-actin were used.

As an endogenous reference, each sample was repeated three times. Quantitative analysis was carried out using SDS7000 System software for biological systems.

Statistical method

Statistical Product and Service Solutions (SPSS) 19.0 software was used for statistical analysis. Measurement data were expressed as the mean±standard deviation ($\bar{x}\pm s$), and the independent samples t-test was used for comparisons between the two groups. Count data were expressed as percentages, and comparisons between the two groups were performed using the χ^2 test. Differences were defined as statistically significant at $P<0.05$.

Results

GFAP expression in ephrin-B2 knockout mice and control mice

Western blotting results showed good astrocyte growth with no significant difference in Ephrin-B2 knockout and control mice. After the brain trauma model was established, GFAP expression in the cerebral cortex of Ephrin-B2 knockout mice was significantly higher than that in the control group ($P<0.05$) (see Figure 1).

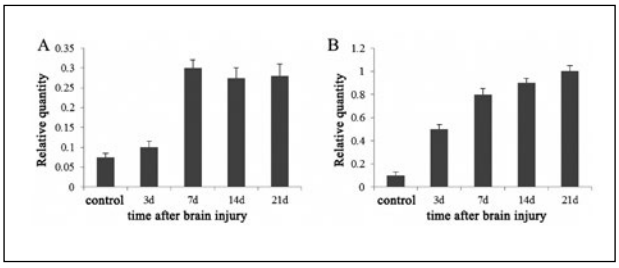

Figure 1: GFAP expression in Ephrin-B2 knockout mice and control mice.
A: GFAP expression level in control mice; B: Ephrin-B2 gene knockout mice.

Ephrin-B2 protein expression in the astrocyte membrane around brain injuries in mice

Western blotting results showed that phosphorylated Ephrin-B2 protein expression in the control group increased significantly after brain injury, reached the highest level on the seventh day after injury, and subsequently declined until the 21st day; however, it remained higher than that in mice without brain injury ($P<0.05$). On the third day after brain injury, the total Ephrin-B2 protein decreased to a level slightly lower than that in mice without brain injury, and subsequently, Ephrin-B2 protein expression increased gradually (see Figure 2).

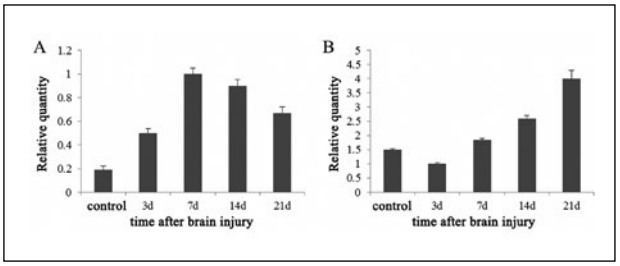

Figure 2: Ephrin-B2 protein expression in the astrocyte membrane around brain injuries in mice.
A: Phosphorylated Ephrin-B2 protein expression; B: Total Ephrin-B2 protein expression.

Changes in astrocytes after brain injury in ephrin-B2 knockout mice

In Ephrin-B2 gene knockout mice, GFAP expression increased significantly after brain injury. On the seventh day after brain injury, the astrocyte volume was significantly increased, and the processes were thickened. On the 21st day after brain injury, astrocytes were evenly distributed to fill the injured area, resulting in very small injury holes (see Figure 3).

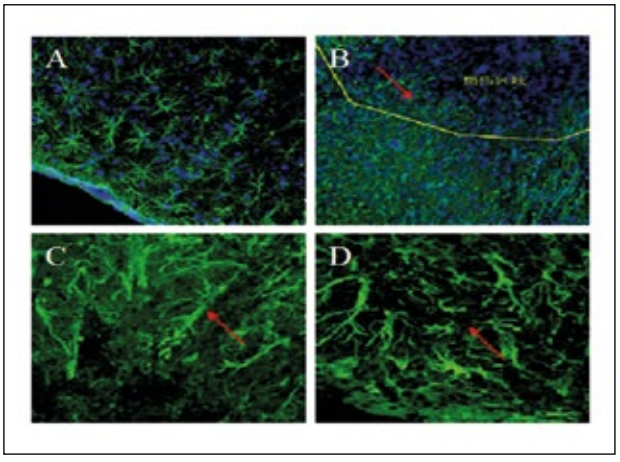

Figure 3: Changes in astrocytes after brain injury in Ephrin-B2 knockout mice.
A-D are 3, 7, 14, and 21 days after brain injury, respectively.

GFAP mRNA expression changes in ephrin-B2 gene knockout mice after brain injury

Real-time quantitative PCR results showed that GFAP expression in the hippocampus and ipsilateral hippocampus was significantly different between Ephrin-B2 knockout mice and control mice within 21 days after brain injury ($P<0.05$). On the seventh

day after brain injury, GFAP mRNA expression in the hippocampus and around the injured area in the control group was the highest, after which it decreased gradually. In Ephrin-B2 knockout mice, GFAP expression in the ipsilateral hippocampus was the highest on the seventh day and remained significantly increased around the injured area on the 21st day. On the 21st day, GFAP expression in Ephrin-B2 knockout mice was three times higher than that in control mice (see Figure 4).

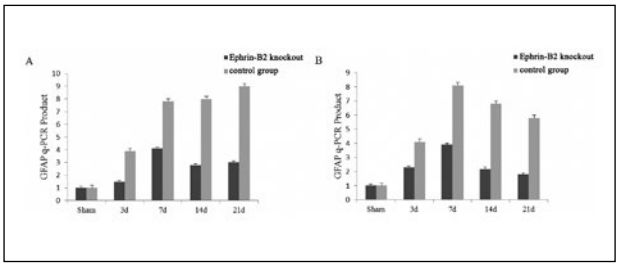

Figure 4: Changes in GFAP mRNA expression after brain injury in Ephrin-B2 knockout mice.
A: Peripheral area of the brain injury in mice; B: Hippocampal area in mice with brain injury.

Discussion

The Ephrin-B2 ligand is an important signal transduction molecule. It is highly expressed in nerve cells, arterial endothelial cells, and other cell types and is a key component of signal pathways by which external stimuli are translated into cellular effects. Accordingly, the Ephrin-B2 ligand exerts a variety of physiological functions[5, 6]. During embryonic development, the Ephrin-B2 ligand is widely expressed in nerve tissue and embryonic artery endothelial cells. It plays an important role in axon orientation, regulating synaptic plasticity to induce the differentiation and migration of neural stem cells. The Ephrin-B2 ligand not only participates in the development of the nervous system but also plays an important role in regulating adult neural stem cells and glial responses after nerve injury.

It is mainly expressed in the membranes of astrocytes and neurons. However, the directional projection of axons to maintain their target positions remains highly important in adulthood[7, 8]. Research has shown that Ephrin-B2 ligand expression occurs in reactive astrocytes after trauma, and its receptor, EPHB2, is expressed in meningeal fibroblasts that invade the injured area. Through two-way signal transduction, the inhibition of contact between the Ephrin-B2 ligand and EPHB2 receptor promotes glial boundary membrane formation after trauma[9]. Immunohistochemical staining showed that the Ephrin-B2 ligand was not expressed in normal brain tissue, but in situ hybridization confirmed Ephrin-B2 expression in whole-brain tissue. Ephrin-B2 is an important component of the central nervous system that is mainly expressed in astrocytes and neurons. Previously, the occurrence of various brain diseases was suggested to be related to abnormalities in the Ephrin-B2 ligand and Eph receptors[10, 11].

Western blotting results showed good initial astrocyte growth without a significant difference in both Ephrin-B2 knockout mice and control mice. After the brain trauma model was established, GFAP expression in the cerebral cortex of Ephrin-B2 knockout mice increased significantly compared with the control group. GFAP is a skeletal protein component specific to astrocytes and can thus be used as an astrocyte marker. Its expression level is related to the proliferation, migration, and hypertrophy of astrocytes. Astrocytes achieve skeletal protein enhancement and cell shape maintenance through interactions between GFAP filaments and the cell membrane[12, 13]. After brain injury, astrocytes showed GFAP regulation and rapid proliferation in response to various factors, and over the course of the injury response, the astrocyte cell volume gradually increased, finally forming glial scarring[14]. In this study, the results showed that GFAP expression increased significantly after brain injury in Ephrin-B2 knockout mice. On the seventh day after brain injury, the astrocyte volume was significantly increased, and the processes were thickened. On the 21st day after brain injury, astrocytes were evenly distributed to fill the injured area, resulting in very small injury holes. Within 21 days after brain injury, real-time quantitative PCR results showed that GFAP expression in the hippocampus and ipsilateral hippocampus of Ephrin-B2 gene knockout mice was significantly different from that in the control group. Our research shows that the Ephrin-B2 ligand plays an important role in regulating reactive astrocyte proliferation. When the Ephrin-B2 ligand was knocked out, reactive astrocytes proliferated in large numbers and invaded the brain injury site, and the processes became thickened. However, the astrocytes were diffusely distributed without excessive aggregation, which was conducive to nerve tissue regeneration and repair in the injured area[15].In conclusion, Ephrin-B2 ligand expression was significantly increased in the astrocyte membrane around brain injury sites in mice, and GFAP mRNA expression was also significantly increased. The resulting reactive astrocytes exhibited an increased neuroprotective effect.

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