Original Article

Inhibition of connexin 43 hemichannels improves postoperative cognitive function in aged mice

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Abstract: Aims: Postoperative cognitive dysfunction (POCD) is a neurological disorder associated with neuroinflammation. Connexin 43 (Cx43), an essential component of gap junction, plays a crucial role in neuroinflammation. The present study was designed to investigate the role of Cx43 in the process of POCD. Methods: POCD model was established in aged mice with internal fixation of tibial fractures. Cognitive function was examined using the Morris water maze test. Hippocampus was collected for reverse transcription polymerase chain reaction (RT-PCR), western blotting, and immunofluorescence assays. Results: In the water maze test, mice undergoing surgery took longer time to reach target platform than the controls. IL-1β and TNF-α mRNA expressions in the hippocampus were significantly increased in surgery mice. Cx43 protein presence in the hippocampus was increased in the surgery group. Treatment of Gap26, a specific blocker of Cx43 hemichannel, reduced the Cx43 protein presence, decreased mRNA expressions of IL-1β and TNF-α, and improved cognitive score in the maze test. Conclusion: Internal fixation of tibial fractures in aged mice induces Cx43 hemichannels opening and enhances neuroinflammation in the hippocampus, leading to cognitive impairment. Administration of Gap26 reduces neuroinflammation in the hippocampus and improves postoperative cognitive function.

Keywords: Cognitive dysfunction, connexin 43, hemichannel, neuroinflammation, hippocampus

Introduction

Postoperative cognitive dysfunction (POCD) presents a decline in memory and learning ability after anesthesia and surgery, which often occurs in senile people. Chronic inflammation in the central nervous system is an important mechanism underlying cognitive impairment, including Alzheimer’s disease, stroke, and epilepsy [1, 2]. Accumulating evidence indicates that microglia and astrocytes play a key role in the process of neuroinflammation.

Gap junctions are important for intercellular connections. Connexins (Cxs) are transmembrane channel proteins, which are assembled to juxtaposed gap junctions (GJs) or non-juxtaposed hemichannels (HCs) [3]. Under physiological condition, GJs play a dominant role in connecting two neighbor cells for transferring molecules, ions and electrical impulses. Under disease conditions, HCs act as a conduit between cytoplasm and extracellular space, leading to cell cytotoxicity. Connexin43 is widely expressed in astrocytes and microglia [4, 5]. Local inflammation increases Cx43 expression and modifies gap junctions’ structure and functions in the central nervous system [6-9]. Inhibition of Cx43 HCs improves survival rates of neuron [8, 10, 11] and restores cognitive function in rats [12].

In the present study, POCD model was development by performing surgical fixation of tibial fractures in aged mice [13]. It was hypothesized that the surgery opens Cx43 HCs in glia in the hippocampus, leading to cognitive impairment. If so, inhibition Cx43 HCs improves cognitive function.
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Materials and methods

Animals

Aged C57BL/6 male mice (16-18 months old, 33-35 g) were purchased from Shanghai Jiesi-jie Company (Shanghai, China) and housed separately in the Laboratory Animal Unit of Zhongshan Hospital (Shanghai, China). Mice were given free access to food and water. The experimental design was approved by the Animal Ethics Committee of Zhongshan Hospital, Fudan University.

POCD model

POCD model was set up by conducting internal fixation of tibial fractures in mice [13]. Aged mice were anaesthetised by 1% sodium pentobarbital (10 ml/kg, intraperitoneal injection). A 0.3-0.6 cm vertical incision was made near the tibial tubercle. The needle (#26) was inserted into the tibial tubercle. Gap26 (50 µg/kg) was administrated intraperitoneally one hour before the surgery [12]. After the surgery, mice were intraperitoneally given butorphanol (2 mg/kg) to relieve pain.

Morris water maze test

Morris water maze test was used to examine learning ability and memory. Spatial acquisition training was conducted for five continuous days. Escape latency was measured as time spent before mice climbed to the target platform. The exploratory experiment was performed on Day 6. Mice were gently placed into the pool from the contralateral quadrant of the target platform. Both swimming tracings and time spent on each quadrant were recorded.

Real-time PCR

Primers used in this study were designed and synthesized by Sangon. GAPDH (forward, 5’-GTT CAA CGG CAC AGT CAA G-3’; reverse, 5’-GCC AGT AGA CTC CAC GAC AT-3’); Cx43 [13] (forward, 5’-GAG TTT GCC TAA GGC GCT C-3’; reverse, 5’-AGG AGT TCA ATC ACT TGG CG-3’), IL-1β (forward, 5’-GAA ATG CCA CCT TTT GAC AGT G-3’; reverse, 5’-TGG ATG CTC TCA TCA GGA CAG-3’); TNF-α (forward, 5’-CAG GCG GTG CCT ATG TCT C-3’; reverse, 5’-CGA TCA CCC CGA AGT TCA GTA G-3’). Total RNA was extracted with Trizol (TAKARA, Japan), and reverse transcribed into cDNA with the TAKARA reverse transcription kit. A two-step PCR reaction program was set as follows: degeneration at 95°C for 5 s, 60°C annealing for 30 s, and 70°C extension for 30 s, (40 cycles).

Western blotting

Mouse hippocampi were minced and lysed by RIPA buffer. Protein concentration was measured by bicinchoninic acid assay (Beyotime, China). A total of 75 µg protein was loaded for electrophoresis and transferred to polyvinylidene difluoride membrane. After blocking with skimmed milk, the membrane was incubated with primary antibodies (1:1000) overnight at 4°C. On the second day, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:10000) for one hour at room temperature. Target protein was normalized with the housekeeping gene β-Tubulin and analyzed using software ImageJ.

Immunofluorescence assay

Frozen mouse hippocampus was cut into 4-µm thickness and rewarmed at room temperature for about 30 min. After blocking for 20 min at room temperature, samples were incubated with primary antibody against Cx43 (1:100) and glial fibrillary acidic protein (GFAP, 1:100) overnight at 4°C. On the second day, samples were incubated with secondary antibodies (1:300, Fluor 488-labeled anti-rabbit and Fluor 594-labeled anti-rat, respectively) for one hour. Hoechst was stained for nuclei. Images were taken by laser-scanning microscopy (Leica Microsystems, German).

Reagents

Gap26 was purchased from Shanghai Botai Company (Shanghai, PRC). Primers were designed and synthesized by Sangon (Shanghai, PRC). Trizol and reverse transcription kit were bought from TAKARA (TAKARA, Japan). Primary antibodies against Cx43 phosphorylated Cx43 and β-Tubulin, as well as secondary antibodies, were purchased from Cell Signaling Technology (CST, Danvers, MA). GFAP antibody was purchased from Servicebio Biotechnology (Hubei, PRC). Chemiluminescence reagent ECL was bought from Xinsaiamei Biotechnology (Shanghai, PRC).

Statistical analysis

Data are presented as means ± SEM. The statistical analysis was done by one-way ANOVA
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followed by post hoc comparison using the Bonferroni test (GraphPad Prism 5 software San Diego, CA, USA). \( P < 0.05 \) was considered statistically significant.

**Results**

*Mice undergoing surgery exhibit impaired learning ability and memory loss*

Under basal condition, control and surgery mice had similar performances in swimming pool. Since Day 3, control mice spent less time to reach the target quadrant than surgery mice (Figure 1A). In the exploratory experiment (Day 6), control mice spent most of their exploring time in the target quadrant, while the surgery mice spent equal time in each quadrant (Figure 1B and 1C). There were no statistical differences in swimming speed between the two groups (Figure 1D).

**Surgical fixation of tibial fractures increases expressions of inflammatory factors in the hippocampus**

Neuroinflammation plays a crucial role in the development of POCD. After the surgery, mRNA expressions of IL-1β and TNF-α were significantly increased in the hippocampus on Day 1 and 3, compared with those in control mice (Figure 2).

**Surgical fixation of tibial fractures increases Cx43 expressions on astrocytes in the hippocampus**

Cx43 protein was mainly expressed on astrocytes in the hippocampus since most Cx43 signals were co-localized with GFAP, a lineage mark of astrocytes. Immunofluorescent signal of Cx43 was significantly increased on Day 1 when compared with control mice (Figure 3).

Cx43 mRNA expression in the surgery group was increased on Day 1 and Day 3, compared with the control group (Figure 4A). Consistently, western blotting showed that the presence of total Cx43 protein was significantly increased in surgery mice (Figure 4B and 4C). On Day 1, phosphorylated level of Cx43 on s368 residue was significantly increased in the surgery group.

Figure 1. Surgery of tibial fractures fixation impairs learning ability and induces memory loss in aged mice. A. The latency time during acquired training. B. Time spent in each quadrant in the exploratory experiment. C. Mouse swimming trajectories in control (left) and surgery (right) groups. D. Mouse swimming speed in control and surgery groups. L: left, R: right, O: opposite, and T: target quadrant of the swimming pool. N=8 *\( P < 0.05 \) compared with the controls, \#\( P < 0.05 \) compared with the control group on the target quadrant.

Figure 2. Surgery of tibial fractures fixation increases mRNA expressions of TNF-α (A) and IL-1β (B) in the hippocampus. N=6. c: control; s: surgery. *\( P < 0.05 \) versus the controls.

Figure 3. Surgical fixation of tibial fractures significantly increases Cx43 expressions on astrocytes in the hippocampus.
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when normalized with the total Cx43 protein (Figure 4B and 4D).

**Gap26 treatment reduces Cx43 expression in the hippocampus**

A specific blocker of Cx43 hemichannel Gap26 [10, 18, 19], was administrated one hour before the surgery.

Gap26 treatment did not alter Cx43 presences, either phosphorylated level or total protein, in control mice (Data not shown).

**Discussion**

The present study reports that surgery of tibial fractures fixation [13] increases the phosphorylated level of Cx43 s368 residue and elevates inflammatory responses in the hippocampus, resulting in cognitive dysfunction in 15-month old mice. Blockade of Cx43 HJs significantly improves cognitive function in mice.

Chronic neuroinflammation is a hallmark of a neurological disorder, including stroke, Par-
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Figure 5. Gap26 treatment downregulates Cx43 presences and decreases mRNA levels of TNF-α and IL-1β in the hippocampus of surgery mice. A. Representative western blots and densitometric quantification of Cx43 protein. B. Quantitative real-time PCR of Cx43 mRNA expression. C. Protein presence Cx43 in the hippocampus on day 1 treated with (lower panel) or without (upper panel) Gap26. In immunofluorescence images, the nuclei was stained with DAPI-stained (blue), the astrocyte was stained with GFAP (red), and Cx43 was stained in red, respectively. D. Protein presence phosphorylated Cx43 s368 in the hippocampus on day 1 treated with (lower panel) or without (upper panel) Gap26. In immunofluorescence images, the nuclei was stained with DAPI-stained (blue), the astrocyte was stained with GFAP (red), and Cx43 was stained in red, respectively. E. TNF-α mRNA expression in the hippocampus of surgery mice. F. IL-1β mRNA expression in the hippocampus of surgery mice. c: control; s: surgery. N=6. *P < 0.05 versus the controls. #P < 0.05 versus S1.

Figure 6. Gap26 treatment improves cognitive function in surgery mice. A. The latency time during the water maze test. B. Time spent in the exploratory experiment. C. Mouse swimming trajectories in control (left) and Gap26 (right) groups. D. Mouse swimming speed in control and treated groups. L; left, R: right, O: opposite, and T: target quadrants of the pool. N=6. *P < 0.05 versus the controls.

Kinsson’s disease, Huntington’s disease and Alzheimer’s disease. It is characterised by the activation of astrocytes and microglia in the central nervous system [18, 20, 21]. In the present study, enhanced mRNA expressions of IL-1β and TNF-α indicated that inflammatory responses took place in the central nervous system [13]. Taken together with the cognitive impairment in these mice, it suggests that neuroinflammation plays a vital role in the occurrence of POCD [22].

Connexins are transmembrane channel proteins, which are assembled to form gap junctions or hemichannels. In the central nervous system, Cx43 protein is mainly expressed on astrocytes and plays a crucial role in channel gating, ion conduction [23], synaptic transmission and plasticity [19], as well as blood-brain barrier integrity [24]. In the present study, increased presences of Cx43 protein, both phosphorylated form and total protein, indicates that Cx43 plays a key role in the process
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Inflammation is an important mechanism of HCs opening [7, 25]. Proinflammatory cytokines, such as IL-1β and TNF-α, are essential to increase Cx43 HCs activity [6, 8, 21]. In a cerebral ischemic model, Cx43 HCs are open when sheep are subjected to cerebral ischemia [10]. In the present study, the surgical fixation of tibial fractures increased productions of proinflammatory cytokines and enhanced protein presences of HCs, confirming that proinflammatory cytokines and Cx43 HCs are team players in the process of POCD.

Gap26 is an HC peptide mimetic, which specifically blocks Cx43 HCs, but not GJs [9, 12, 26]. It is reported that Gap26 treatment reduces the area of cerebral infarction and improves cognitive function in the rat, shown as better performances in water maze tests [12]. In an ischemic sheep model, blockade of Cx43 HCs promotes electroencephalographic power recovery and improves the prognosis of stroke [10]. In the present study, Gap26 treatment reduced mRNA expressions of IL-1β and TNF-α in the hippocampus, suggesting that the activation of astrocyte is essential for the upregulation of proinflammatory cytokines. Gap26 treatment improved postoperative cognitive function, confirming the critical role of Cx43 HCs in the occurrence of POCD.

In summary, aged mice undergoing tibial fractures fixation exhibit a cognitive defect. Blockade of Cx43 HCs reduces inflammatory responses in the hippocampus and improves cognitive function in aged mice. Thus, the opening of Cx43 HCs plays a prominent role in the neuroinflammation as well as in the occurrence of POCD. Blockade of Cx43 hemichannels shed a light of a promising future strategy for the treatment of POCD.

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Disclosure of conflict of interest

None.

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