The α2δ-1/NMDA receptor complex is involved in brain injury after intracerebral hemorrhage in mice

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Abstract

Background: Intracerebral hemorrhage (ICH), a common cerebrovascular disease, seriously threatens human health and has severe secondary injuries, while existing treatment methods have many limitations. α2δ-1 is a subunit of voltage-gated Ca²⁺ channels (VGCCs) and can act on glutamate receptor N-methyl-D-aspartate receptors (NMDARs) to relieve neuropathic pain. Methods: We first performed ICH modeling on WT mice and Cacna2d1 knockout (KO) mice. The expression levels of GluN1 and α2δ-1 were measured by Western blot and q-PCR, and the interaction between the two proteins was evaluated by co-precipitation. The neuronal apoptosis was detected by the TUNEL assay, and the expression levels of inflammatory factors were assessed by ELISA. The nerve functions of mice were evaluated using behavioral experiments including corner turn test and forelimb use asymmetry. Cerebral hematoma was indicated by brain water content and lesion volume. Results: ICH up-regulated the expression levels of α2δ-1 and GluN1. KO of Cacna2d1 significantly reduced the ICH-induced apoptosis. The treatment of gabapentin on α2δ-1 also significantly reduced the occurrence of apoptosis. KO of Cacna2d1 also reduced the ICH-induced levels of inflammatory factors. Furthermore, neural functions were also significantly improved. Conclusion: Cacna2d1 KO alleviates cerebral hematoma in ICH mice, exhibits a significant regulating effect on its secondary injuries such as neuronal apoptosis and inflammation, and restores the nerve functions of ICH mice. Loss of Cacna2d1 can provide useful therapeutic clues for ICH treatment.
half an hour of ischemia, caspase-1 and caspase-8 in the nerve cells of the infarct center are significantly activated, and the cell morphology also exhibits the characteristics of early apoptosis. Cell apoptosis is triggered immediately in the ischemic center area, which in turn damages nerve cells.14

ICH-induced neuronal damage is closely related to neurotoxicity caused by the massive release of glutamate from ischemic neurons.15 Glutamate is the main excitatory nerve mediator of the central nervous system, which participates in excitatory synaptic transmission thereby playing an indispensable role in maintaining the normal signal transmission of neurons.12,15 However, under pathological conditions, excitatory amino acids have a toxic effect on nerves. When the brain contracts, it releases a large amount of glutamate transmitters, inhibits its supplementation, and causes glutamate to accumulate between cells and interact with surrounding normal neurons. The role of glutamate is mediated by different types of glutamate receptors. Among the known glutamate receptors, the most critical one involved in glutamate-mediated nervous system damage is N-methyl-D-aspartate receptor (NMDAR).16,17 NMDAR is a gated ion channel receptor consisted of two structural subunits GluN1 and two regulatory subunits GluN2, which is closely related to synaptic plasticity, learning and memory, and participates in the process of various central nervous system diseases. Glutamate accumulation leads to over-activation of NMDAR, which can mediate the influx of calcium ions, causing delayed damage to neurons.18

Voltage-dependent calcium channel protein a2δ subunit 1 (a2δ-1) is encoded by the Cacna2d1 gene, which plays an important role in muscle development and synapse generation.19 Down-regulation of a2δ-1 can cause migration, adhesion, and spread of myoblasts.20 As a subunit of voltage-gated Ca2+ channels (VGCCs) and the action site of gabapentin, a2δ-1 can be used to treat neuropathic pain. It has been reported that a2δ-1 can act on NMDARs, thereby affecting the progression of neuropathic pain. However, the role of a2δ-1/NMDAR complex in nerve damage after ICH is unknown.21–23

We hypothesized that a2δ-1/NMDAR was a key complex that mediated nerve damage, and found that the loss of a2δ-1 improved the nerve functions and reduced the apoptosis in ICH mice, and also suppressed the activation of GluN1.

Methods and Materials

Human tissues

Human brain tissues were collected at the Second Hospital of Hebei Medical University. The protocol was approved by the institutional review boards in the Second Hospital of Hebei Medical University. Table S1 lists the information (including age, gender, cause of death, post-mortem interval and ICH score) of the six patients and five normal cadavers. The ICH score was first designed as a clinical grading scale that would allow for mortality prediction in patients with ICH. A score of 0 correlates with 0% 30-day mortality; a score of 1, 13% mortality; a score of 2, 26% mortality; a score of 3, 72% mortality; a score of 4, 94% mortality; a score of 5, 100% mortality; and a score of 6, 100% mortality.24 Brain samples from six patients who died within 3 days of ICH were included in the ICH group. Autopsy and histological validation of the presence of ICH were conducted in all cases by an experienced neuropathologist. Samples from five individuals who died of non-hemorrhagic diseases and had no history of neurologic or neuropsychiatric diseases were used as controls. The specimens were collected with informed consent from the patients’ family.

Animal model

A total of 65 male C57BL/6 mice and 44 Cacna2d1 knockout (KO) mice (8–10 weeks old) were purchased from Shanghai Laboratory Animal Center. ICH was induced according to previously described methods. After anesthetization via injecting 10 mg/kg promethazine and 60 mg/kg ketamine intraperitoneally, mice were fixed on the mouse stereotaxic apparatus. Then a 0.5-mm-diameter burr hole was drilled at the following coordinates relative to bregma (0.5 mm anterior, 2.5 mm right lateral, 4 mm deep) at a speed of 2 μL/min. Thereafter, 0.075 U of collagenase VII-S (Sigma-Aldrich, St Louis, MO) dissolved in 0.5 μL saline was administered by a 1-μL microsyringe. After anesthetization by ketamine/xylazine overdose, mice were killed and tissues were obtained on day 3 post-ICH for ELISA, IP, and Western blot experiments. All the procedures were performed with the approval of the Administrative Committee of Experimental Animal Care and Use of the Second Hospital of Hebei Medical University. All surgeries were performed under anesthesia and efforts were made to minimize the number of animals used in the present study. Two WT mice and one Cacna2d1 KO mouse died during anesthesia.

Neurological outcomes

Two rating systems were applied in order to assess the neurological outcomes from day 0 to day 10 after ICH as described previously. In the corner turn test, the mice were first allowed to proceed into a 30°-angled corner. After that, the animals had to turn to either the left or right limb to get out of the corner, and the choice was...
recorded. Another behavioral test involved was forelimb placing test to evaluate the sensory and motor impairments. The percentage of trials in which the animal placed the appropriate forelimb on the edge of the countertop when stimulated by the vibrissae was calculated. Behavioral assessments were done by researchers blind to group assignment. All of the experiments were repeated for at least 10 times. The percentage of right turns was calculated and only turns that involved full rearing along each wall were included.

Brain water content

The dissected mouse brain was divided into three parts: ipsilateral hemisphere, contralateral hemisphere, and cerebellum, which were then weighed into the wet weight, and then dry at 110°C for 24 h to measure the dry weight. The following formula was used to calculate the brain water content: (wet weight − dry weight)/wet weight × 100%.

Magnetic resonance imaging

Lesion volume in the brains was determined at day 3 after ICH using a 7-T small animal MRI. T2-weighted image sequence was scanned to detect lesion volume.

Drug treatment

Two doses of gabapentin (100 mg/kg, IP) were injected: one at 30 min after ICH and another at 6 h after ICH. The same volume of saline as the gabapentin was administered intraperitoneally in the WT + ICH group as control.

Co-immunoprecipitation

We collected the hypothalamic tissues of two patients with ICH and mice from each group (three mice per sample). The tissue was dissected and homogenized, and ice-cold hypotonic buffer (1 mmol/L CaCl₂, 20 mmol/L Tris, pH 7.4, 1 mmol/L MgCl₂, and protease inhibitor mixture) was prepared for membrane preparation. The supernatant was immunoprecipitated using samples were washed three times, and then immunoblotted with anti-z2δ-1 antibody (1:500).

ELISA

IL-1β and TNF-α ELISA kits (Abcam Inc. Cambridge, MA, USA) were applied to determine the expression levels of inflammatory cytokines in ICH mice. Tissues were treated in accordance with the manufacturer’s instructions, and the supernatants were added into 96-well plates pre-coated with corresponding primary antibodies. After washing four times, appropriate biotinylated antibodies were added and incubated for 30 min at 37°C, followed by detecting via horseradish peroxidase (HRP)-conjugated streptavidin and chromogen reagent. Finally, absorbance at 570 nm was immediately detected on an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Transferase-mediated nick end labeling (TUNEL) assay

To determine the percentage of neuronal apoptosis, sections were blocked in 10% bovine serum albumin for 1 h at 25°C. Then, these sections were incubated with TUNEL (Cell Death Detection Kit, Roche) and rabbit anti-NeuN (1:200; Abcam) at 4°C overnight. Following incubation, the sections were washed in phosphate-buffered saline (PBS) and incubated with the corresponding secondary antibody at 25°C for 1 h. After that, the sections were incubated in PBS-diluted 4,6-dimidyl-2-phenylindole (DAPI) (10 μg/mL, Sigma) at 25°C for 15 min. All sections were observed under a laser scanning confocal microscope (FV500; Olympus, Tokyo, Japan). The total number of TUNEL and NeuN double-positive cells in five random areas near the damaged area was counted.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The mRNA levels of GluN1 and z2δ-1 in ICH patients or non-hemorrhagic controls were determined by qRT-PCR. Total RNA was extracted using the TRIzol method (Life Technologies), then cDNA was synthesized using the Reverse Transcription Kit (Qiagen). Primers were designed using Primer 5.0 and sequences used were as follows: GluN1(F): AGATGGCTTACAGCGGCA, GluN1(R): GC CATGGCTCCTAGCTCAG; z2δ-1(F): TGATGATATGT GTGCTGCTCATCTT, z2δ-1(R): GTGGCCCTTGTCC TGTG; PS-95(F): ATGAGTTGCTAGTGACGGG, PS-95(R): ATGCTGATGTACCCCGTAGAAGCC.
Western blot
The protein levels of GluN1, z2d-1, and PSD-95 were examined by Western blot. Total protein samples were extracted on ice using RIPA buffer supplemented with 1% protease inhibitor cocktail Complete Mini (Roche). 30 µg protein of each sample was loaded on 10% SDS-PAGE at 100 volts. Then, the protein was transferred to a nitrocellulose membrane at 200 mA current for 2 h. The membrane was probed overnight at 4°C with primary antibodies against GluN1 (#5704, Cell Signaling Technology, Rabbit mAb), z2d-1 (#ACC-015), PSD-95 (p-p65) (#2507, Cell Signaling Technology, Rabbit mAb), respectively. The membrane was incubated with corresponding HRP-conjugated secondary antibodies (1:3000, Bio-world, ST. Louis Park, MN) for 1.5 h at room temperature. Proteins were detected by the Odyssey InfraredImagingSystem. Densitometric analysis was performed using the ScionImagingapplication (Scion Corporation), with PSD-95 as the internal reference.

Statistical analysis
The GraphPad software was employed for the statistical analysis. Student’s t test was used to determine single comparisons between two groups. One-way analysis of variance or two-way ANOVA followed by the Tukey post hoc test were used for multiple comparisons. All data are expressed as mean ± SD, and p ≤ 0.05 was considered statistically significant.

Results
Increased z2d-1/NMDAR complex in the brains of patients with ICH
In order to find out whether the expression of z2d-1/NMDAR complex in ICH patients was altered, we examined the expression levels of GluN1 and z2d-1, an important subunit of NMDAR, in the brain tissues of six patients. The expression levels of both proteins were significantly higher than those of five normal individuals (Fig. 1A–C), indicating that the occurrence of ICH was indeed related with increased expression levels of z2d-1 and NMDAR in the brains of patients.

z2d-1 physically interacts with NMDAR in the brain tissues
Although the expression levels of z2d-1 and GluN1 in the brains of patients with ICH were upregulated, whether these two proteins could directly interact with each other remained unknown. In order to determine the z2d-1/NMDAR interaction in the brain, we performed co-IP analysis using brain extracts from two patients, S1 and S2. Using specific antibodies, z2d-1 co-precipitated with GluN1, while non-specific IgG did not pull down z2d-1 in tissues (Fig. 2A and B). In addition, anti-GluN1 antibody (but not IgG) and z2d-1 in the brain tissues of mouse Sham control and ICH groups could also be co-immunoprecipitated (Fig. 2C and D). These results indicated that z2d-1 in the brain could physically interact with NMDAR both in mice and humans.

z2d-1 KO attenuates neurological deficit and brain injury caused by ICH in mice
Next, a series of neurological tests were conducted to detect the neurological deficit and recovery after ICH. Corner turn test and forelimb use asymmetry test were performed, indicating severe neurological deficits in all ICH mice at 1 day after ICH. In the next 10 days, the scores of Cacna2d1 KO group with ICH and gabapentin-treated WT ICH group changed more significantly than WT mice, indicating that the z2d-1 KO-induced nerve function recovery was faster in these two groups (Fig. 3A and B). However, there was no significant difference in the turn test results between the gabapentin-treated WT ICH and Cacna2d1 KO ICH groups. The lesion volume and brain water content were measured on the 3rd day after ICH. We found that in all ICH groups except KO ICH, the brain water content was increased compared with the WT group. While compared with the WT ICH group, the brain water contents of the KO and KO ICH groups were significantly reduced. Compared with the WT ICH group, gabapentin-treated WT ICH group exhibited significantly decreased brain water content, albeit still significantly higher than WT, indicating that the elimination effect of gabapentin on z2d-1 was not as pronounced as that of the Cacna2d1 KO group (Fig. 3C). Similarly, as shown in Figure 3D, compared with the WT ICH group, the lesion volumes of the Cacna2d1 KO ICH and gabapentin-treated WT ICH groups were significantly reduced. The above results indicated that the elimination of Cacna2d1 relieved the symptoms of nerve defects and greatly reduced the lesions and edema around the hematoma.

ICH increases the synaptic expression level of the z2d-1/NMDAR complex, which could be abolished by z2d-1 KO
In the results of clinical patients shown in Figure 1, we found that the expression levels of z2d-1 and GluN1 in the brains of ICH patients were significantly up-regulated. We therefore next measured the expression levels of these two proteins using Western blot in the WT ICH, z2d-1KO,
KO ICH, and gabapentin-treated WT ICH mouse groups, and found that there was almost no expression of α2δ-1 in all KO groups (Fig. 4A–C). The use of gabapentin significantly reduced the high levels of α2δ-1 and GluN1 in the WT ICH group. But the expression of GluN1 in the KO group didn’t change significantly compared with the

Figure 1. Increased α2δ-1–NMDAR complex in the brains of patients with ICH. Representative images (A) and statistical analysis (B) show the protein level of GluN1 and α2δ-1 in the brain tissues from ICH patients or non-hemorrhagic controls. (C) Relative mRNA levels of GluN1 and α2δ-1 were analyzed. Data were shown as mean ± SD and evaluated by Student’s t test. *p < 0.05 compared with control.

Figure 2. Co-immunoprecipitation analysis shows α2δ-1 physically interacts with NMDAR in brain tissues of human (two separate individuals) (A and B) and mice (three mice per sample) (C and D). IgG and input were used as negative and positive controls, respectively.
control group, indicating that the elimination of α2δ-1 prevented GluN1 from ICH challenge.

**α2δ-1 KO reduces neuronal apoptosis after ICH**

Apoptosis is an important pathological mechanism of ischemic brain injury. In order to further clarify the role of α2δ-1/GluN1 complex in ICH, we performed TUNEL assay to evaluate the level of apoptosis in each group, and found that the neuronal cell apoptosis in all ICH groups was elevated, while was significantly reduced in all KO groups. In addition, the WT ICH group treated with gabapentin also exhibited attenuated apoptosis (Fig. 5A and B), indicating that elimination of α2δ-1 significantly reduced the ICH-induced neuronal apoptosis.

**Deletion of α2δ-1 alleviates the secretion of pro-inflammatory cytokines**

Inflammatory reaction plays a very important role in the secondary injury after cerebral hemorrhage. As IL-1β and TNF-α are two main pro-inflammatory factors, we conducted an ELISA test on the levels of these two factors in each group of mice, and found that TNF-α was highly expressed in all WT ICH groups, which was restored in all KO groups and the gabapentin-treated WT ICH group (Fig. 6A). IL-1β was also highly expressed in all ICH groups, which was down-regulated in the α2δ-1 KO group and gabapentin-treated WT ICH group (Fig. 6B), indicating that the removal of α2δ-1 reduced the occurrence of ICH-induced inflammation.
Discussion

To date, ICH treatment has mainly focused on early prevention of hematoma expansion, including hemostasis and anti-hypertensive therapy, but it cannot effectively reduce the expansion of hematoma and improve the prognosis.1,2 ICH results in both primary and secondary injuries. The extravasated blood in the cerebral blood vessels increases local pressure and results in primary brain injury. Subsequent degradation of the hematoma can cause cell damage, oxidative stress, and inflammation reaction, accompanied with defects in cell death, cerebral edema, and neurological behavior, leading to secondary damage.25

The rapid accumulation of glutamate in the injured brain tissues of ICH patients leads to the constitutive activation of NMDARs, which eventually damages neurons. ICH leads to the release of a large amount of endogenous molecules, including glutamic acid, Ca2+, ROS, thrombin, heme, iron, TNF-α, etc.17,26 In the detection of clinical samples (Fig. 1A) and mouse models (Fig. 4A), we did find that the expression of GluN1 was significantly up-regulated compared with the control groups, while the expression of inflammatory factors was significantly up-regulated after ICH. These molecules are implicated in excitatory and mitotic signal transduction, which also involves NMDA receptors. Mitotic signaling initiates the cell cycle of normal cell division in neural progenitor cells and microglia, while abnormal mitotic signaling leads to toxicity, killing neurons during neurological diseases including ICH. In the past 10 years, quite a few studies have demonstrated the role of apoptosis in secondary neuronal loss in peripheral blood clots after ICH. And some anti-apoptotic drugs could effectively reduce neuronal death and improve its function.

Peripheral hematoma is also considered to be an important factor in delaying neurological deterioration after ICH.27 Brain edema is caused by the breakdown of the blood–brain barrier and the local production of penetrating active substances. Inflammatory cell mediators, complement activation, by-products of coagulation and hemolysis, for example thrombin and fibrin, and hemoglobin enter the brain and cause local and systemic inflammation. These complex cascade reactions result in neuronal apoptosis and/or damage.28 We used the TUNEL assay to detect the neuronal apoptosis of mice,
and found that ICH could significantly increase the occurrence of neuronal apoptosis compared with the control groups (Fig. 5A).

Altered ion channel function and expression are characteristic of neuropathic pain. VGCCs are indispensable for membrane excitability and nerve transmission. 29,30

Figure 5. a2δ-1 KO reduces neuronal apoptosis after ICH. (A) Apoptotic neurons in the perihematoma brain region 3 day after ICH were examined by co-staining of TUNEL (red), NeuN (green), and DAPI (blue). Scale bar = 30 μm. (B) Quantitative analyses of apoptotic neurons in different groups. n = 6 per group. Data were shown as mean ± SD and evaluated by one-way ANOVA followed by the Tukey post hoc test. *p < 0.05 compared with WT group, #p < 0.05 compared with WT + ICH group.

Figure 6. Deletion of a2δ-1 alleviates the secretion of pro-inflammatory cytokine. TNF-α (A) and IL-1β (B) in the perihematoma brain region on day 3 after ICH were detected by ELISA. n = 6 per group. Data were shown as mean ± SD and evaluated by one-way ANOVA followed by the Tukey post hoc test. *p < 0.05 compared with WT group, #p < 0.05 compared with WT + ICH group.

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Altered ion channel function and expression are characteristic of neuropathic pain. VGCCs are indispensable for membrane excitability and nerve transmission. 29,30

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Role of α2δ-1/NMDA Receptor in Brain Injury

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α2δ-1 is a family of VGCC-related subunits anchored by glycosylphosphatidylinositol and is the target of the potent neuropathic analgesics gabapentin and pregabalin. Studies have found that synaptic VGCCs configured with α2δ-1 drive exocytosis through extracellular metal ion-dependent adhesion sites (MIDAS), which is a conserved amino acid group in the predicted von Willebrand A domain of α2δ-1.α2δ-1 is reported to act on NMDARs, thereby affecting the development of neuropathic pain. Consistently, we found that α2δ-1 expression was significantly increased in clinical samples and mouse models (Figs. 1A and 4A). Overexpression of Cacna2d1 enhances the postsynaptic and presynaptic NMDAR activities of the spinal dorsal horn neurons, thereby causing pain hypersensitivity. By contrast, KO or knockdown of Cacna2d1 increases nerve activity and increases synaptic NMDAR activity. The interaction between α2δ-1 and NMDAR promotes surface transport and synaptic targeting of NMDAR. Gabapentin or α2δ-1C terminal interfering peptide can normalize NMDAR synaptic targeting and enhance activity due to nerve damage. Therefore, α2δ-1 is an NMDAR-interacting protein that increases NMDAR synaptic transmission during neuropathic pain. Gabapentin compounds can reduce neuropathic pain by inhibiting the forward transport of the α2δ-1/NMDAR complex. However, the role of the α2δ-1/NMDA receptor complex in nerve injury after cerebral hemorrhage is unknown. In our experiments, the brain water content and lesion volume of ICH mice were restored significantly (Fig. 3C and D), and the ICH-induced apoptosis was also significantly reduced (Fig. 5A). Similarly, KO of α2δ-1 also reduced the levels of inflammatory factors caused by ICH (Fig. 6a and B), and the behavioral experiments in mice also showed that nerve function recovered well (Fig. 3A and B). These results have shown that the reduction of α2δ-1 does have a good alleviating effect on secondary injury caused by ICH, and is likely to serve as a promising therapeutic target.

Conclusion

We hereby report that α2δ-1 KO reduces cerebral hematoma in ICH mice, and also exhibits a significant regulatory effect on the secondary neuronal apoptosis and inflammation, which restores the nerve functions of ICH mice. The lack of α2δ-1 is an interesting research focus for the treatment of secondary injury after ICH, and our current results provide a theoretical basis for the clinical treatment of ICH.

Conflict of Interest

All authors declare that they have no conflict of interest.

Authors’ Contributions

Jingchen Li, Guoqiang Song, Qianxu Jin, Liqiang Liu, Liang Yang, Yuanyu Wang, Xuesong Zhang, and Zongmao Zhao performed the experiments, analyzed, and interpreted the data. Jingchen Li and Zongmao Zhao were the major contributors in writing the manuscript. All authors read and approved the final manuscript.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Demographics of patients with and without ICH.