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The GluN2B-Containing NMDA Receptor Alleviates Neuronal Apoptosis in Neonatal Hypoxic-Ischemic Encephalopathy by Activating PI3K-Akt-CREB Signaling Pathway

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Summary

Neonatal hypoxic-ischemic encephalopathy (HIE) is a disease caused by insufficient blood supply in the brain in newborns during the perinatal period. Severe HIE leads to patient death, and patients with mild HIE are at increased risk of cognitive deficits and behavioral abnormalities. The NMDA receptor is an important excitatory receptor in the central nervous system, and in adult hypoxic-ischemic injury both subtypes of the NMDA receptor play important but distinct roles. The GluN2A-containing NMDA receptor (GluN2A-NMDAR) could activate neuronal protective signaling pathway, while the GluN2B-NMDAR subtype is coupled to the apoptosis-inducing signaling pathway and leads to neuronal death. However, the expression level of GluN2B is higher in newborns than in adults, while the expression of GluN2A is lower. Therefore, it is not clear whether the roles of different NMDA receptor subtypes in HIE are consistent with those in adults. We investigated this issue in this study and found that in HIE, GluN2B plays a protective role by mediating the protective pathway through binding with PSD95, which is quite different to that in adults. The results of this study provided new theoretical support for the clinical treatment of neonatal hypoxic ischemia.

Key words

Neonatal hypoxic-ischemic encephalopathy • NMDA receptor • GluN2B • PSD95 • Apoptosis • Neuronal survival

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Introduction

Hypoxic-ischemic encephalopathy (HIE) is caused by insufficient blood supply in the brain during the perinatal period, leading to oxygen deprivation and insufficient glucose [1-2]. HIE is the main cause of neonatal morbidity and death and is one of the most common causes of long-term neurological impairment in children [3-4]. Severe neonatal patients have cognitive deficits and an increased risk of abnormal behavior, including cerebral palsy, mental retardation, epilepsy, and learning and visual impairments [2-3].

NMDA receptors are one of the main ionotropic glutamate receptors in the central nervous system [6] and are involved in a variety of neurodevelopmental, physiological, and pathological processes [7]. Under physiological conditions, NMDA receptors mediate central excitatory signal transmission and Ca^{2+} influx [8-9] and play important roles in multiple physiological processes, such as synaptic plasticity, learning, memory formation and cognition [10-11]. Under pathological conditions, including ischemia, a large amount of glutamate is released, triggering the process of excitotoxicity mediated by NMDA receptors, activating apoptosis-related signaling pathways in neurons, and regulating neuronal death [12].

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The NMDA receptor is a heterotetrametric structure and is mainly composed of the structural subunit GluN1 and the regulatory subunit GluN2 or GluN3 [6]. In the early stages of development, the expression of the GluN2B subunit is dominant, and the function of NMDA receptors is mainly mediated by GluN2B-NMDAR. However, in later stage of development, the expression of GluN2A gradually increase and finally exceed GluN2B [13]. At present, there are two main hypotheses regarding the mechanisms by which different subtypes of NMDA receptors play during hypoxia-ischemia: the “NMDA receptor location” hypothesis and the “NMDA receptor subtype” hypothesis.

The “NMDA receptor location” hypothesis suggests that stimulating NMDA receptors on the synapse will activate the survival signaling pathway [15], such as the phosphoinositide-3-kinase-protein kinase B (PI3K)-Protein kinase B (PKB, or Akt)-cAMP response element-binding protein (CREB) and extracellular signal-regulated kinase (ERK)-CREB pathways, increase the phosphorylation level of CREB. CREB regulates expression of genes that are important for neural protection, such as brain derived growth factor (BDNF), therefore phosphorylation of CREB exerts a protective role during hypoxia [14-16]. Contrary to the protective effect of NMDA receptors on synapses, extrasynaptic NMDA receptors are coupled with proapoptotic signaling pathways to dephosphorylate the ERK pathway, prevent CREB activation, increase the expression of death genes, and mediate damage [14]. The “NMDA receptor subtype” hypothesis suggests that different NMDA receptor subtypes mediate different downstream signaling molecules, thus playing different roles [11]. In mature neurons, NMDA receptors containing GluN2B are enriched outside synapses, overactivation of GluN2B-NMDAR during ischemia could lead to excitotoxicity and neuronal apoptosis, while the synaptic localized GluN2A-NMDAR mediates neuronal survival and neuroprotection [17-18].

However, neurons in the neonatal brain are still in the early stages of development; GluN2B-NMDAR is the dominant subtype on the membrane surface, and the expression of GluN2A-NMDAR is very low, which is quite different from that in adults. Are the roles of these two subtypes in neonatal hypoxic-ischemia consistent with those in mature neurons? Studies have shown that in immature neurons, GluN2B can mediate the survival of neurons [19]. However, related research is still very scarce, the conclusions of the existing research are not consistent, and there is a lack of in-depth research on related molecular mechanisms. Because of the lack of adequate understanding of the functions and molecular mechanisms of the different subtypes of NMDA receptors in HIE, HIE treatment methods based on NMDA receptors often fail to achieve good results. Thus, we hope to clarify the molecular mechanisms by which different subtypes of NMDARs affect HIE to provide theoretical support for the targeted development of clinical therapeutic drugs.

Materials and Methods

Animals

Male and female C57BL/6J mice were obtained and acclimated to the colony room for at least 2 weeks prior to mating. The colony room was maintained on a 14-hour light/10-hour dark cycle with controlled temperature and humidity and free access to food and water. The subjects were the offspring of the pairing of a female with a male. The experimental protocols were performed in accordance with the approval of the Animal Care and Use Committee of Kunming Medical University (Approval No. KMMU2014004) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Neonatal hypoxia-ischemia

Neonatal hypoxia-ischemia was induced using the classic Vannucci method with slight modifications [20]. Nine to eleven days old (P9-11) C57BL/6J mice were anesthetized by isoflurane inhalation, and the left common carotid artery was isolated from the nerve and vein and ligated at two locations with 5–0 surgical silk. The common carotid artery was then transected between the ligatures to guarantee that blood flow through the ipsilateral carotid circulation was absent throughout the experiment. The wound was sutured, and the animals were allowed to recover for 1 h in their cages. Afterwards, the animals were placed in a chamber (34 °C) and subjected to a mixture of 8 % oxygen in nitrogen for 45 min to induce hypoxia [1]. Sham control animals underwent the same surgical procedure without ligation of the artery or hypoxia. HIE and sham animals were returned to their dams. 72 h after the procedure, the mice were euthanized by isoflurane and sacrificed for the TTC staining, Hoechst staining or Western Blot. For Co-IP experiments, the mice were euthanized by 24 or 72 h after the operation.

Drug administration

Ifenprodil (specific GluN2B inhibitor, 20 mg/kg) and TCN-201 (specific GluN2A inhibitor, 10 mg/kg) were administered (intraperitoneal injection) at three time points respectively, either before hypoxia-ischemia, immediately after hypoxia or at 24 h after HIE induction. 72 h after the operation, the mice were killed and the following experiments were performed, including TTC staining, Hoechst staining or WB. The disruption peptide TAT-GluN2B-9c (30 mg/kg) or same amount of saline (negative control) were administered (intraperitoneal injection) before HIE induction. And 24 h after the surgery, the cortex tissue were extracted, and Co-IP was used to measure the interaction between GluN2B and PSD95.

TTC staining

Ten mice were used to assess infarct size. The mice were killed at 3 days after HIE induction. The brains were rapidly removed and frozen at -20 °C for 13 min. A total of six 2-mm-thick coronal sections of the brain were then cut starting at the frontal pole. This series of brain sections totaling 12 mm in thickness included the entire infarct area caused by HIE. The sections were incubated for 20 min and stained with 1 % triphenyl tetrazolium chloride (TTC) at 37 °C protected from light. Then, the sections were fixed in 4 % buffered formaldehyde solution for 24 h. Infarct areas in each section were measured using ImageJ software [21].

Hoechst staining

To detect morphological evidence of apoptosis, cell nuclei were visualized by DNA staining with the fluorescent dye Hoechst 33342. Briefly, the paraffin specimens were sliced into 5-μm sections, stained with Hoechst 33342 for 8 min in the dark, after which the slices were observed under a fluorescence microscope. Cells with brightly stained or fragmented, brightly stained nuclei were regarded as apoptotic cells [2,22].

Co-IP

The GluN2B protein complex was purified from PSD samples by immunoprecipitation using a mouse GluN2B monoclonal antibody. The specific steps are described herein. The brain tissue was added to a mixture of protease inhibitor and NP40 lysis buffer (100 mg per sample with 1 ml of lysis buffer), sheared, ultrasonicated, and incubated in a 4 °C chromatography refrigerator for 8 h. Then, 50 µl of Protein G agarose beads, 5 µl of IgG antibody and 5 µl of GluN2B antibody were mixed and incubated for 2 h and then incubated overnight with the treated tissue samples at 4 °C. The next day, the tissue samples were washed with NP40 buffer twice and boiled with 50 µl of 2× sample buffer for 5 min.

Western blot

Equal amounts of protein samples (60 µg) or equal amounts of co-IP products were used for Western blot analysis. After electrophoresis on a 12 % BIS-Tris SDS polyacrylamide gel, the proteins were transferred to a PVDF membrane, and the expression of several important proteins associated with classical signaling pathways were measured. The blots were probed with primary antibodies (GluN2A, GluN2B, Akt, p-Akt, ERK, p-ERK, PI3K, p-PI3K, CREB, p-CREB, GSK, p-GSK, Caspase3, cleaved Caspase3, PSD95, and GAPDH) overnight at 4 °C. The antibodies were bound with the appropriate secondary antibodies, and the signal was visualized with enhanced chemiluminescence. The average optical density (OD) and the area of the protein signal on the scanned radiographs were measured with ImageJ software.

Statistical analysis

All experiments were performed using four or more repeated samples. The GraphPad Prism 8 software statistical package was used to analyze the experimental data. All data were tested by the normality test and variance homogeneity test, and all data are the mean ± standard error (mean ± SEM). The t test was used for statistical analysis between two groups, and one-way ANOVA followed by Tukey's test was used for statistical analysis among multiple groups. A value of $P<0.05$ was considered to be statistically significant.

Results

HIE activated NMDA receptor coupled synaptic and extrasynaptic synaptic signaling cascade

Firstly we used the modified Vannucci method to induce hypoxic ischemia in mice aged 9-11 days. Seventy-two hours after the operation, the infarct areas of age-matched C57BL/6J mice in the HIE group, sham operation group (sham group) and normal mice (CTR group) were detected by TTC staining. The results are shown in Figure 1. Compared with those of the CTR group, the brain tissues of sham group mice showed no obvious changes and were bright red, but there were

obvious white infarcts in the brains of HIE group mice (Fig. 1A). This result shows that the HIE induction method used in this study can effectively cause cerebral hypoxic-ischemic injury in mice. To further verify the effectiveness of the HIE induction, we performed Hoechst staining on the cortex in the control group, the sham-operated group and the HIE group to examine apoptosis after HIE induction. The results showed that compared with that in the sham operation group, the fluorescence intensity in the HIE group was significantly increased, indicating that the number of apoptotic cells was significantly increased after HIE induction (Fig. 1B-C). These results further confirm that the HIE treatment in this study can effectively cause cerebral hypoxic-ischemic damage in neonatal mice.

This study aimed to examine the function of NMDA receptors in HIE. Therefore, we then tested whether HIE affects the expression of NMDA receptors in the mouse cortex. Seventy-two hours after HIE induction, cortical proteins were extracted from control and HIE mice, and western blotting was used to measure the expression of GluN2A and GluN2B. The results showed that after 72 h of HIE, the expression of GluN2A in the cortex was significantly lower than that in the control group (GluN2A expression in the HIE group was 79.7±1.0 % of that in the control group, P=0.0026, n=3), while the expression of GluN2B did not change significantly (GluN2B expression in the HIE group was 95.1±9.9 % of that in the control group, P=0.6688, n=3) (Fig. 1D-E).

Inhibiting GluN2B-NMDAR activation increased neuronal apoptosis in HIE

Then we further explored the functions of different subtypes of NMDA receptors in HIE. We examined the role of two NMDA receptor subtypes, GluN2A-NMDAR and GluN2B-NMDAR in HIE separately. We administered TCN-201 (10 mg/kg), a selective inhibitor of GluN2A and ifenprodil (20 mg/kg), a selective inhibitor of GluN2B-NMDAR, to mice by intraperitoneal injection at three time points: before, after, and 24 h after HIE induction. Seventy-two hours after HIE induction, Hoechst staining was used to detect neuronal apoptosis. The results showed that compared with the sham group, the fluorescence intensity in the HIE group was increased (P=0.001, n=11), which was consistent with the aforementioned experimental results. After administering ifenprodil, which inhibited GluN2B-NMDAR, the fluorescence intensity further increased in the HIE group, indicating that inhibiting GluN2B-NMDAR exacerbated neuronal apoptosis (B-ifen (ifenprodil administered before HIE induction)) vs. sham: P<0.001; A-ifen (ifenprodil administered immediately after HIE induction) vs. sham: P<0.001; 24 h-ifen (ifenprodil administered 24 h after HIE induction) vs. sham: P<0.001; B-ifen vs. HIE: P=0.017; A-ifen vs. HIE: P=0.035; 24 h-ifen vs. HIE: P=0.013, n=15) (Fig. 2A-C). However, there was no significant difference in fluorescence intensity between HIE group and TCN-201 treatment group (B-TCN (TCN-201 administered before HIE induction) vs. sham: P=0.007; A-TCN (TCN-201 administered immediately after HIE induction) vs. sham: P=0.016; 24 h-TCN (TCN-201 administered 24 h after HIE induction) vs. sham: P<0.001; B-TCN vs. HIE: P=0.283; A-TCN vs. HIE: P=0.946; 24 h-TCN vs. HIE: P=0.596, n=15) (Fig. 2A-C). These results show that selective inhibition of GluN2B-NMDAR can exacerbate HIE-induced neuronal apoptosis, while selective inhibition of GluN2A-NMDAR does not change HIE-induced neuronal apoptosis.

GluN2B-NMDAR played a protective role by activating the synaptic signaling pathway in HIE

To further examine the molecular mechanism of the protective effect of GluN2B-NMDAR after HIE, we first examined the activation of apoptosis-related signaling pathways coupled to NMDA receptors. We used western blotting to measure the expression and phosphorylation levels of Akt and ERK, which are key proteins in apoptosis-related signaling pathways during cerebral hypoxia [23]. Akt is coupled to synaptic NMDA receptors and its activation promotes neural survival, while ERK is coupled to extrasynaptic NMDA receptors, and its activation could activate the apoptotic pathway. The results showed that compared with the control group, in the HIE group, the phosphorylation levels of Akt and ERK decreased significantly, the expression of p-Akt in the HIE group decreased to 72.2±3.9 % of that of the control group (P=0.0193, n=3), and the expression of p-ERK decreased to 69.2±5.6 % of that of the control group (P=0.0312, n=3) (Fig. 1D-E). These results indicate that the apoptosis-related signaling pathways are activated in HIE.

Next, we tested whether GluN2B-NMDAR was involved in the activation of apoptosis-related signaling pathways in HIE. Consistent with previous experiments, we administered the GluN2A-NMDAR and GluN2B-

NMDAR inhibitors TCN-201 and ifenprodil to mice before HIE induction, immediately after HIE induction, and 24 h after HIE induction. Seventy-two hours after HIE, western blotting was used to measure the protein expression and phosphorylation levels of Akt and ERK. Consistent with the previous results, the expression levels of GluN2A, p-Akt and p-ERK decreased significantly after HIE induction (GluN2A: P=0.002, n=5; p-Akt: P=0.012, n=5; p-ERK: P=0.003, n=5) (Fig. 3A-G). However, ifenprodil administration before or immediately after HIE further downregulated the level of Akt phosphorylation (B-ifen vs. HIE: P=0.005, n=5; A-ifen vs. HIE: P=0.002) (Fig. 3A-G). In addition, the expression of Akt in mice given ifenprodil before and immediately after HIE induction was also significantly lower than that in the sham group (B-ifen vs. HIE: P=0.018, n=5; A-ifen vs. HIE: P=0.003) (Fig. 3A, D-E). We also found that ifenprodil reversed the dephosphorylation of ERK (A-ifen vs. HIE: P=0.018, n=5; 24 h-ifen vs. HIE: P=0.027, n=5) (Fig. 3A, F-G). These experimental results indicate that GluN2B-NMDAR promotes Akt phosphorylation and ERK dephosphorylation which means that due to the lack of GluN2A, GluN2B-NMDAR can activate synaptic protective signaling pathway and inhibit extrasynaptic injury-promoting signaling pathways. To further clarify the function of activated GluN2B-NMDAR in HIE, we examined the expression level of caspase 3 and cleaved caspase 3, which is a key apoptosis signaling molecule. The results showed that after inhibiting GluN2B-NMDAR, the expression of caspase3 and cleaved caspase3 increased significantly (caspase 3: B-ifen vs. HIE: P=0.007, n=5; A-ifen vs. HIE: P<0.001, n=5; 24 h-ifen vs. HIE: P=0.001, n=5; cleaved caspase 3: B-ifen vs. HIE: P=0.011, n=5; A-ifen vs. HIE: P=0.005, n=5) (Fig. 3A, H-I), which confirmed that after using ifenprodil to selectively inhibit GluN2B-NMDAR, Akt, a key protein in the neuroprotective signaling pathway, was dephosphorylated and neuronal apoptosis was exacerbated.

To further verify this result, we measured the expression and phosphorylation levels of PI3K and CREB, which are upstream and downstream of Akt, respectively. The results showed that the phosphorylation levels of PI3K and CREB were significantly increased after HIE induction (p-PI3K: P=0.028, n=5; p-CREB: P=0.011, n=5) (Fig. 4A-C). After administering ifenprodil to inhibit GluN2B-NMDAR, the phosphorrylation of PI3K and CREB was downregulated compared with that in the HIE group (p-PI3K: B-ifen vs. HIE: P=0.001, n=5; A-ifen vs. HIE: P<0.01, n=5; p-CREB: B-ifen vs. HIE: P=0.048, n=5; A-ifen vs. HIE: P<0.001, n=5; 24 h-ifen vs. HIE: P<0.001, n=5) (Fig. 4A-C). In contrast, after inhibiting GluN2A-NMDAR, there was no significant difference in the expression or phosphorylation levels of PI3K and CREB. We also examined the expression and phosphorylation of GS3K-beta and found no significant change (Fig. 4). These results further confirm that selective inhibition of GluN2B-NMDAR in HIE can inhibit the protective PI3K/Akt/CREB pathway in the synapse.

During HIE, the GluN2B couples to the protective signaling pathway through binding with PSD95

How does GluN2B mediate the protective pathway in the synapse in HIE? Previous studies have shown that the interaction between GluN2B and PSD95 is weakened during hypoxia-ischemia in mature neurons [24-25], but after HIE, the interaction between GluN2B and PSD95 is strengthened [26]. PSD95 is a rivet protein in the postsynaptic compact area. Therefore, whether PSD95 mediates GluN2B and synaptic protection signaling pathways in HIE is unclear. To examine this, we first verified the effect of HIE on the interaction between GluN2B and PSD95 through immunoprecipitation experiments. Consistent with previous studies, we found that PSD95 interactions with GluN2B increased to 239.4±48.6 % of that in the control group (P=0.045, n=5) at 24 h after HIE and 217.5±26.3 % at 72 h after HIE (P=0.011, n=5) (Fig. 5A-B). These results confirmed that after HIE, the interaction between GluN2B and PSD95 was significantly higher than that in the control group for a long time.

To clarify whether the GluN2B-coupled protective signaling pathway depends on interactions with PSD95, we used the polypeptide TAT-GluN2B-9c, which can specifically block the interaction between GluN2B and PSD95 [26]. In the polypeptide treatment group, mice were given the TAT-GluN2B-9c polypeptide (30 mg/kg) dissolved in normal saline by intraperitoneal injection 24 h before the operation. TAT-GluN2B-9c is an amino acid sequence consisting of 9 polypeptides at the C-terminus of the GluN2B protein that are responsible for the interaction with PSD95. At the N-terminus, the transmembrane peptide TAT is connected to help the peptide cross the cell membrane and enter the cell. The mice in the control group were given the same volume of normal saline 24 h before the

operation. Twenty-four hours after the operation, mouse cortical tissue was collected, and the interaction between GluN2B and PSD95 was measured by co-IP. The results showed that compared with that in the HIE group, the amount of PSD95 interacting with GluN2B in the brain tissue in the polypeptide treatment group was significantly reduced (P=0.018, n=4), but there was no significant difference from that in the sham group (P=0.268, n=4) (Fig. 5C-D). The interaction between GluN2B and PSD95 was not impaired in mice in the saline control group. These results suggest that TAT-GluN2B-9c effectively inhibits the binding of GluN2B and PSD95 induced by HIE.

Inhibition of GluN2B-NMDAR or interrupting the interaction of GluN2B and PSD95 intensifies the neuronal death caused by HIE

Next, we examined whether TAT-GluN2B-9c could block the protective effect mediated by GluN2B-NMDAR after HIE. We first measured the expression levels of Akt, PI3K, and CREB. The results showed that compared with those in the HIE group and the saline control group, the phosphorylation levels of Akt, PI3K, and CREB in the polypeptide group were significantly decreased (p-Akt: P=0.0233, n=5; p-PI3K: P=0.0026, n=5; p-CREB: 0.0292, n=5) (Fig. 6A-G). These results indicate that the use of the TAT-GluN2B-9c polypeptide interferes with the interaction between GluN2B and PSD95 and effectively blocks the activation of the protective signaling pathway. Then we determined whether TAT-GluN2B-9c could also intensify the neuronal death after HIE by Hoechst staining. The results indicated that after applying TAT-GluN2B-9c immediately after the HIE surgery will increase the number of damaged neurons (compared to sham: HIE: 129.8±6.4 %, n=10; HIE+TAT-GluN2B-9c: 157.5±8.4 %, n=10). Compared to HIE group, the TAT-GluN2B-9c significantly intensified the neuronal death (P<0.01, n=10) (Fig. 6I-H). Overall, these results suggested that inhibition of GluN2B-NMDAR or interrupting the interaction of GluN2B and PSD95 intensifies the neuronal death after HIE.

Discussion

When hypoxia-ischemia occurs in the central nervous system, NMDA receptors mediate excitotoxicity and participate in the pathological process of brain injury. In mature neurons, the GluN2A-NMDAR subtype is the dominant subtype and is expressed on synapses. GluN2A is coupled to the synaptic protective signaling pathway in neurons to inhibit the occurrence of neuronal apoptosis. The GluN2B-NMDAR subtype is mainly distributed outside the synapse, and it is coupled to the apoptosis-inducing signaling pathway and promotes neuronal death. Therefore, it is currently believed that when hypoxia-ischemia occurs, the fate of neurons is regulated by the combined effects of these two NMDA receptors [10].

However, the expression of NMDA receptor subtypes changes dynamically during development. In the early stages of development, the expression of GluN2A-NMDAR is extremely low, and the dominant subtype at this time is GluN2B-NMDAR. Notably, previous studies have suggested that after HIE, the expression of GluN2A is further downregulated. Our experimental results also showed that at 72 h after HIE, the expression of GluN2A decreased by nearly 30 % (Fig. 3). Therefore, when neonatal hypoxia-ischemia occurs, can GluN2A-NMDAR still play a protective role? Our results showed that injection of the selective GluN2A-NMDAR inhibitor TCN-201 [27-28] neither aggravated nor inhibited HIE-induced neuronal apoptosis, suggesting that GluN2A-NMDAR does not participate in the pathology of HIE in the early stages of development. This effect may be due to its expression level being too low.

In mature neurons, GluN2B-NMDAR, which is distributed outside the synapse, is believed to mediate the death of neurons through the proapoptotic pathway. Furthermore, according to this functional characteristic, the clinical use of the selective GluN2B-NMDAR inhibitor ifenprodil can achieve neuroprotection against hypoxic-ischemic injury such as stroke [29-30]. Therefore, we examined whether ifenprodil also has a better protective effect on HIE. In addition, to identify the effective time window of the effect of ifenprodil, we used three administration time points: before HIE induction, immediately after HIE, and 24 h after HIE. Among them, administration before HIE induction was used to test whether preventive administration has a better protective effect than administration after injury. The time points immediately after HIE induction and 24 h later were used to determine whether the administration time affects the effect of drug intervention. However, the experimental results showed that when ifenprodil was administered to mice, the level of brain damage was further increased. The Hoechst staining results showed that compared with those in the HIE group, mice in the ifenprodil administration group had increased cortical neuronal apoptosis.

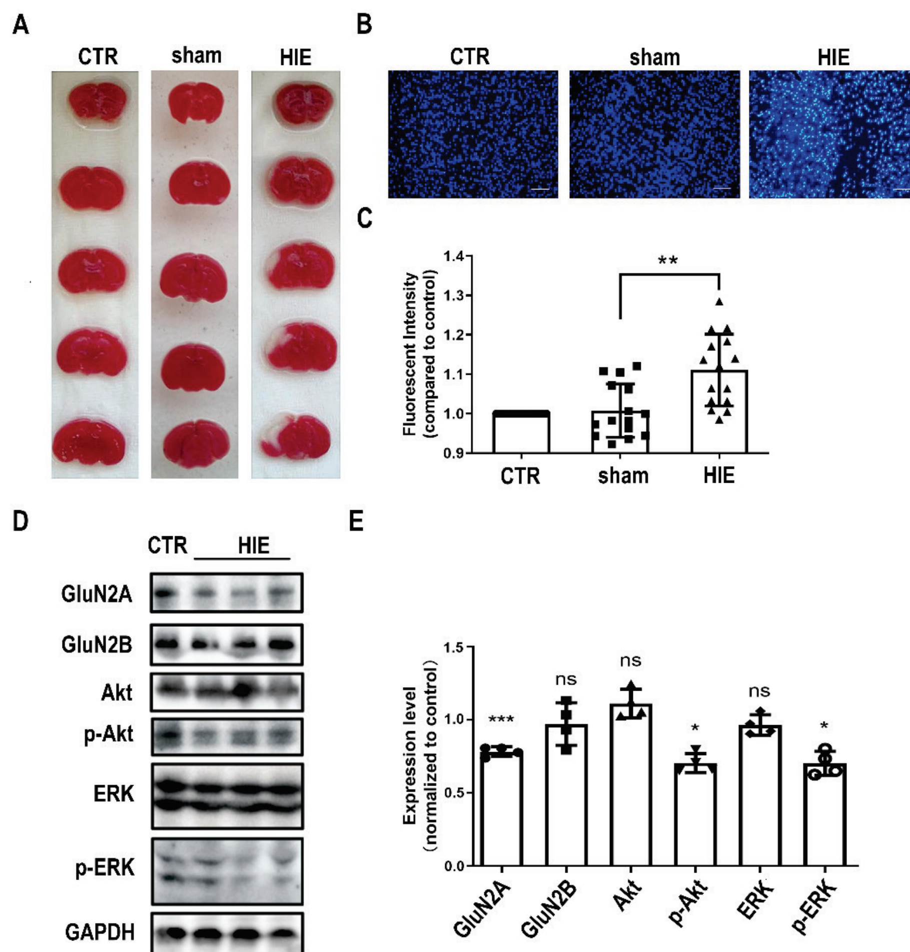

Fig. 1. The Akt and ERK signaling pathway were inhibited in HIE. (**A**) Seventy-two hours after surgery, cerebral infarction area was detected by TTC staining for HIE and sham group mice, and CTR group mice with the same age. (**B**) Coronal section of the brain was performed 72 h after surgery in HIE and sham group mice, and the CTR group mice of same age. Apoptosis of cortical neurons was detected by Hoechst staining. Scale represents 100 μm. (**C**) The statistical analysis of Hoechst staining. Compared with the sham group, the fluorescence intensity increased significantly in HIE group (** $P<0.01$, n=15). (**D**) The cortical protein of HIE and CTR mice of the same age was extracted and measured by Western Blot to detect GluN2A, GluN2B, Akt, p-Akt, ERK, p-ERK expression levels. (**E**) Statistical analysis of Western Blot results. Compared with the control group, the expression level of GluN2A, p-Akt and p-ERK decreased significantly (* $P<0.05$, ** $P<0.01$, n=3).

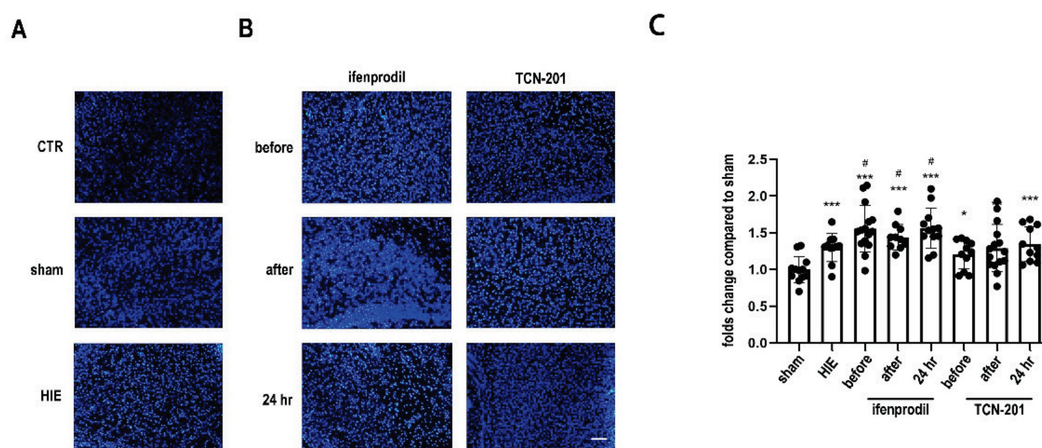

Fig. 2. Inhibition of GluN2B-NMDAR activation after HIE exacerbates neuronal apoptosis. (**A**) Coronal section of the brain was performed 72 h after surgery in HIE and sham group mice, and the CTR group mice of same age. Apoptosis of cortical neurons was detected by Hoechst staining. Scale represents 100 μm. (**B**) Selective inhibitors of GluN2A-NMDAR (TCN-201) and GluN2B-NMDAR (ifenprodil) were administrated before, after immediately and 24 h after surgery. Apoptosis was detected by Hoechst staining 72 h after HIE induction, Scale represents 50 μm. (**C**) Statistical analysis of the results resulting of (B) * indicates significant difference compared to the sham group, Differences (* $P<0.05$, ** $P<0.01$, *** $P<0.005$, n=11-15); # indicates significant difference compared to HIE group (# $P<0.05$, n=11-15).

Fig. 3. Both synaptic and extrasynaptic signaling pathways were activated by GluN2B-NMDAR. (**A**) Selective inhibitors of GluN2A-NMDAR (TCN-201) and GluN2B-NMDAR (ifenprodil) were administrated before, after immediately and 24 h after surgery. Cerebral proteins in sham, HIE and inhibitor treated group mice were extracted 72 h after surgery. The expression of GluN2A, GluN2B, Akt, p-Akt, ERK, p-ERK, caspase 3 and cleaved caspase 3 were measured by western blot. (**B-I**) The statistical analysis of (A). * indicates that compared to the sham group, Differences (* P<0.05, ** P<0.01, n=5); # indicates a difference compared to HIE group (# P<0.05, ## P<0.01, n=5).

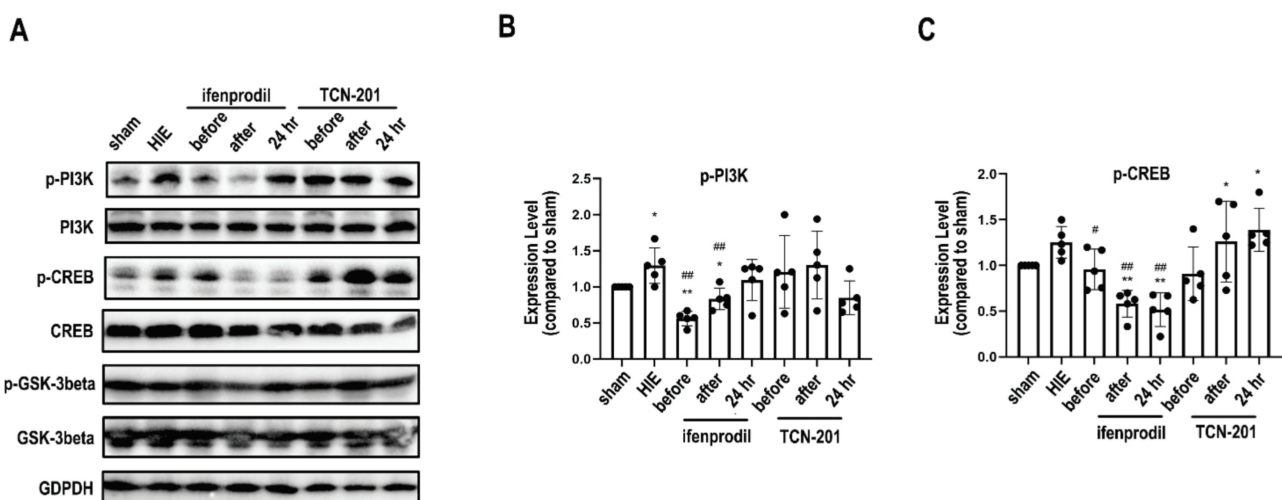

Fig. 4. GluN2B-NMDAR activates the neuroprotective signaling pathways after HIE. (**A**) Selective inhibitors of GluN2A-NMDAR (TCN-201) and GluN2B-NMDAR (ifenprodil) were administrated before, after immediately and 24 h after surgery. Cerebral proteins in sham, HIE and inhibitor treated group mice were extracted 72 h after surgery. The expression of PI3K, p-PI3K, CREB, p-CREB, GSK-3beta, p-GSK-3beta were measured by western blot. (**B-C**) Statistical analysis of the p-PI3K and p-CREB expression. * indicates that compared to the sham group, Differences (* P<0.05, ** P<0.01, n=5); # indicates a difference compared to HIE group (# P<0.05, n=5).

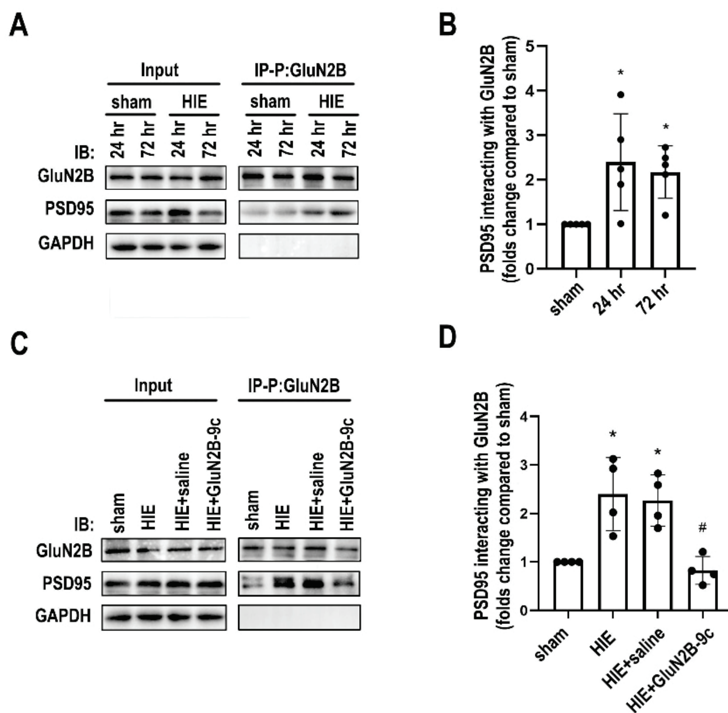

Fig. 5. HIE increases interaction of GluN2B and PSD95. (**A**) The cortical tissue was extracted at 24 and 72 h after surgery in sham and HIE group mice, and the interaction of GluN2B and PSD95 was examined by co-immunoprecipitation experiment. The specific antibodies targeting GluN2B was used for precipitation, and the expression of PSD95 and GluN2B was measured by Western blot, while GAPDH was used as negative control. (**B**) Statistical analysis of (A) graphs. 24 and 72 h after HIE induction, the level of PSD95 binding with GluN2B was significantly increased. * indicates a significant difference compared to the sham group, * P<0.5, n=5. (**C**) The mice were given intraperitoneal injection of TAT-GluN2B-9c (HIE+GluN2B-9c) or the same volume of saline (HIE+saline) 24 h before the HIE surgery. Cortical tissue was extracted 24 h after surgery to detect the interaction between GluN2B and PSD95. (**D**) statistical analysis of (C). Compared to HIE group, the interaction between GluN2B and PSD95 in was significantly reduced after TAT-GluN2B-9c treatment and showed no difference with sham group. * indicates a significant difference compared to the sham group (* P<0.5, n=5). # represents a significant difference compared to HIE group (* P<0.5, # P<0.5, n=5).

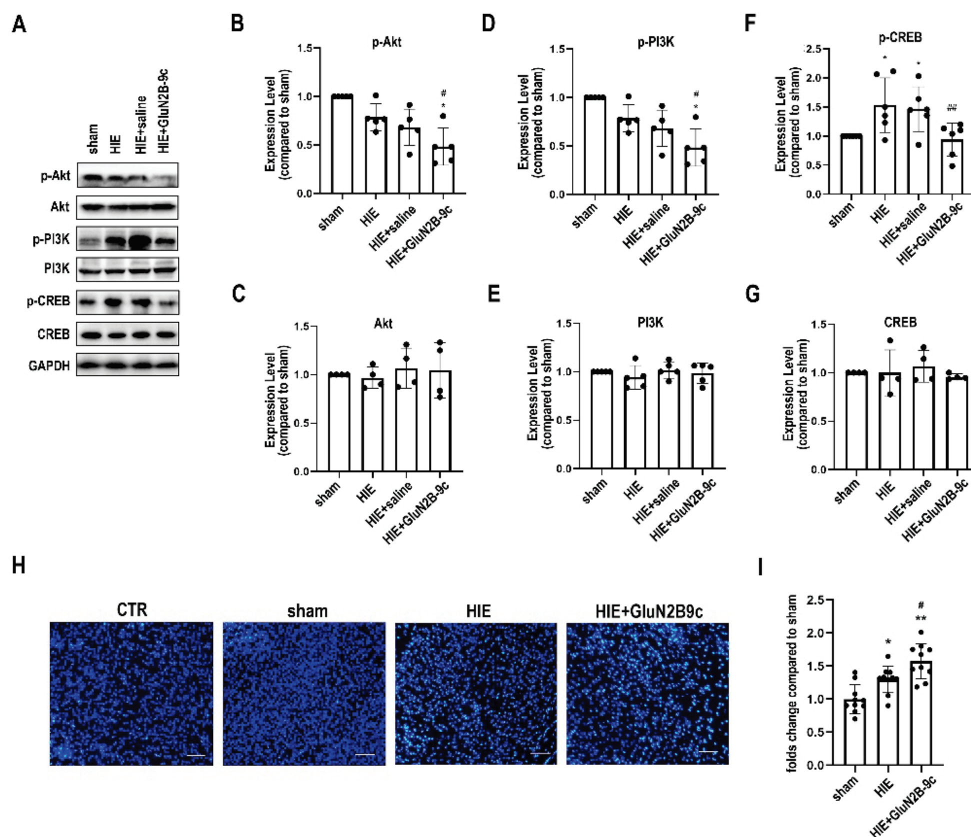

Fig. 6. GluN2B-NMDAR activates protective signaling pathway through binding with PSD 95. (**A**) TAT-GluN2B-9c (HIE+GluN2B-9c) or equivalent volume of saline (HIE+saline) were administered intraperitoneally 24 h before the surgery. Cortical tissue was extracted to detect Akt, PI3K, CREB and phosphorylation level of these proteins 72 h after surgery. (**B-G**) The statistical analysis of (A). Compared to HIE group, the expression of Akt PI3K and CREB in TAT-GluN2B-9c treatment group did not change significantly, but their phosphorylation was significantly down-regulated. * indicates significant difference compared to the sham group (* P<0.05, ** P<0.01, n=5). # indicates a significant difference compared to HIE group (# P<0.05, n=5). (**H**) Coronal section of the brain was performed 72 h after surgery in sham, HIE group mice, TAT-GluN2B-9c treated mice, and the CTR group mice of same age. Apoptosis of cortical neurons was detected by Hoechst staining. Scale represents 100 μm. (**I**) Statistical analysis of (H). * indicates significant difference compared to the sham group (* P<0.05, ** P<0.01, n=10). # indicates a significant difference compared to HIE group (# P<0.05, n=10).

This result first shows that ifenprodil does not protect against HIE at the cellular level and exacerbates damage. Second, this result also shows that the role of GluN2B-NMDAR in the pathological process of neonatal hypoxic-ischemia is not the same as that of adult hypoxic-ischemia.

Previous studies have shown that if the NMDA receptor plays a protective role, it needs to activate the protective signaling pathway in the synapse, which is mainly mediated by GluN2A-NMDAR [11]. However, our findings showed that when hypoxia-ischemia occurs early in development, GluN2B but not GluN2A mediates and activates the protective signaling pathway. In mice with HIE, blocking GluN2B-NMDAR with ifenprodil significantly reduced the phosphorylation levels of PI3K, Akt, and CREB in the protective signaling pathway, indicating that the protective signaling pathway can be inhibited by selective inhibitors of GluN2B-NMDAR. In addition, we also found that the expression of caspase 3 and its degradation products was increased, indicating that the caspase 3-dependent apoptotic response was strengthened. Consistent with this finding, the Hoechst results also showed that neuronal apoptosis damage was intensified. In summary, these results suggest a specific protective role of GluN2B-NMDAR in immature neurons, which is different from its role in adult hypoxia-ischemia.

Why is there such a difference? What is the protective mechanism of GluN2B in immature neurons? We further studied these issues. Consistent with previously reported studies [1], we found that the interaction of GluN2B and PSD95 was increased after hypoxia-ischemia in newborn mice. Because PSD95 is a scaffolding protein which is responsible for locating receptors to the synapse and coupling receptor subunits and downstream signaling pathways, it might mediate the interaction of GluN2B and synaptic protective signaling pathways. Therefore, we used the polypeptide TAT-GluN2B-9c to block the interaction between GluN2B and PSD95. Results show that the synaptic protective signaling pathway in mice given the peptide was also inhibited after HIE. These results are highly consistent with the effect of ifenprodil blockade of GluN2B-NMDAR. This finding shows that during HIE, GluN2B couples to the protective pathway in the synapse by interacting with PSD95.

Due to the dynamic changes in the expression of GluN2A and GluN2B during development, the expression of NMDA receptor subtypes in early development is very different from that in adulthood, which leads to inconsistent functions of NMDA receptor subtypes in early development and adulthood. The resulting question is whether the drugs used to treat adult hypoxic ischemia are still effective for neonatal HIE. Based on our experimental results, in neonatal HIE, drugs that target GluN2B may actually exacerbate injury. There are many drugs that target NMDA receptors to treat hypoxia and ischemia in adults, including the typical noncompetitive NMDA receptor antagonist MK801 and memantine [31], the selective inhibitor of GluN2B-containing NMDA receptor ifenprodil [32], and TAT-GluN2B9c [33]. Our research results showed that GluN2B has a robust protective effect on neonatal HIE, and both ifenprodil and TAT-GluN2B9c inhibits the GluN2B coupled protective signaling pathway. Therefore, future studies are required to determine whether and to what level can these drugs treat neonatal HIE clinically requires further examination.

In addition, this study also showed that in the early developmental stage after hypoxia-ischemia, the expression of GluN2A was further downregulated. Therefore, it is worth studying that whether and how does HIE affect the developmental expression of GluN2A, and will the expression defects of GluN2A caused by HIE affect later behavioral cognition? We hypothesize that after HIE, a further decline in GluN2A expression is likely to cause long-term neurological dysfunction in children.

Conflict of Interest

There is no conflict of interest.

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