Neonatal Anesthesia by Ketamine in Rats Interferes with Proliferation and Differentiation of Hippocampal Neural Stem Cells and Neurocognitive Function in Adulthood via Inhibition of Notch1 Signalling Pathway

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Abstract

Objective: The proliferation and differentiation of developing neural stem cells (NSCs) have been particularly interesting targets to study ketamine-induced neurotoxicity. Our previous findings have shown that neonatal ketamine exposure inhibits the proliferation of NSCs in the hippocampal dentate gyrus (DG) and promotes neuronal differentiation. However, the potential mechanisms are poorly understood. Notch signalling pathway plays an important role in the regulation of neurogenesis. The objective of this study was to investigate whether Notch signalling pathway was involved in neurogenesis impairment and long-term neurocognitive dysfunction caused by neonatal ketamine exposure.

Methods: Postnatal day 7 (PND-7) male Sprague-Dawley (SD) rats were intraperitoneally injected with normal saline (NS) or 40 mg/kg ketamine for four consecutive times (40 mg/kg×4) at an interval of 1 h. Notch ligand Jagged1 (0.5 mg/kg) was micro-injected into the hippocampal DG with the stereotactic apparatus at 1 h before NS or ketamine administration. Lentivirus over-expressing Notch1 intracellular domain (LV-NICD1) was micro-injected into the hippocampal DG 4 days before NS or ketamine administration. Western blot was used to detect the changes of Notch1 signalling pathway related proteins in the hippocampal DG at 24 h after administration. The S-phase marker 5-bromodeoxyuridine (BrdU) was administered immediately after the treatment, then the proliferation and differentiation of NSCs in hippocampal DG were detected by using double-immunofluorescence staining at 24 h after treatment. Moreover, the changes of hippocampus-dependent spatial memory in the adult rats were tested by Morris water maze test in 2-month-old rats.

Results: Ketamine anesthesia in neonatal rats decreased the expression levels of Jagged1, Notch1, Notch1 intracellular domain (NICD1) and hairy enhancer of split 1 (Hes1), and inhibited the proliferation and astrocytic differentiation of NSCs and promoted neuronal differentiation. Neonatal ketamine exposure induced the deficit in hippocampus-dependent spatial reference memory tasks in 2-month-old rats. The micro-injection of Jagged1 or LV-NICD1 reversed the inhibitory effect of ketamine on the expression of Notch1 related proteins in the hippocampal DG, and attenuated the ketamine-induced interference of NSCs proliferation and differentiation. In addition, Morris water maze test suggested that the administration of Jagged1 or LV-NICD1 could improve cognitive function in 2-month-old rats after neonatal ketamine exposure.

Conclusions: These results suggest that neonatal exposure to ketamine in rats interferes with the proliferation and differentiation of hippocampal NSCs and impairs neurocognitive function in adulthood via inhibition of Notch1 signalling pathway. These findings contribute to further understanding of the neonatal neurotoxicity induced by ketamine and its underlying mechanisms.

Introduction

During the past few years, the number of children and infants receiving surgery under general anesthesia has been increasing worldwide. However, exposure to general anesthetics during the critical stages of

neurodevelopment has been shown to induce neurotoxicity and long-term neurocognitive dysfunction in the laboratory models[1–2]. A recent large scale clinical study has also demonstrated an association between multiple general anesthesia exposure at neonatal stage and neurobehavioral deficits during childhood[3]. The anesthesia induced neurotoxicity in children has become a major health issue to both the medical community and the public.

Ketamine is one of the most commonly used anesthetics in sedation, anesthesia and analgesia during pediatric examination and surgical operation[4–6]. The studies in rodents, nonhuman primates and clinic suggest that the prolonged and/or repeated ketamine exposure during brain developmental period may evoke neuronal apoptosis and inhibit neurogenesis, leading to neurobehavioral abnormalities in the adulthood[7–11]. Therefore, it is necessary to investigate the underlying mechanisms of ketamine-induced neurotoxicity in the neonatal rat.

The critical period of development in central nervous system (CNS) lasts from the end of pregnancy to the first 2–3 weeks after birth in rodents[12]. During the peak period, substantial neurogenesis lays the foundation for the structure and function of the brain. Neurogenesis is a complex process involving proliferation and differentiation of NSCs, neuronal migration, neuronal survival and high degree of synaptogenesis. The correct balance between NSC proliferation and differentiation is essential for the development of brain[13–14]. The hippocampal dentate gyrus (DG) is one of the most important neurogenesis regions other than the subventricular zone (SVZ), which plays a critical role in the formation of hippocampus-dependent spatial learning and memory function[15–16]. In recent years, the effect of general anesthetics on developmental neurogenesis has been highly concerned by researchers [17]. Previous studies have shown that neonatal sevoflurane/isoflurane exposure affects long-term neurocognitive function through interfering the proliferation and differentiation of hippocampal NSCs[18–19], and intravenous anesthetic ketamine/propofol induces dysregulation of the proliferation and differentiation of NSCs[20–21]. Furthermore, our previous findings demonstrates that repeated ketamine exposure in postnatal day 7 (PND-7) rats disruptes the proliferation and differentiation of developmental NSCs in the hippocampal DG and SVZ[22–23]. However, the underlying molecular mechanisms remain to be elucidated.

The Notch signalling pathway has been demonstrated to play a crucial role in the regulation of neurogenesis. Especially in different animal models, the Notch signalling pathway is regarded as a “switch” to regulate the proliferation and differentiation of NSCs[24–25]. The Notch receptor is a transmembrane protein, which is activated by its ligands, such as Jagged and Delta. Notch receptor is cleaved by presenilin-1 and the γ-secretase enzyme complex and releases the active Notch internal cellular domain (NICD). The NICD then translocates into the nucleus and further activates the transcription of downstream target genes, such as hairy enhancer of split (Hes) family members. Previous study has demonstrated that Notch signalling pathway activates targeted gene Hes expression during brain development, which in turn inhibits NSC differentiation[26–27]. In contrast, the sustained expression of Hes protein leads to cell cycle exit and NSC entry into a quiescent state[28–29].

A recent study suggests that ketamine affects the early embryonic development of NSCs via the Notch signalling pathway[30]. However, few studies to date have been performed about the regulation effect of ketamine on the development of NSCs during postnatal period and its underlying mechanisms. Based on our previous study, we determined to investigate whether neonatal repeated ketamine exposure interfered with the proliferation and differentiation of hippocampal NSCs by affecting Notch signalling pathway, thereby leading to neurocognitive dysfunction.

Materials And Methods

Animals

The experimental protocol was approved by the Institutional Animal Care and Ethics Committee of Xuzhou Medical University. All pregnant Sprague-Dawley (SD) rats (average weight 200 g) were obtained from the Laboratory Animal Centre of Xuzhou Medical University, and raised individually in a temperature-controlled (23°C-25°C) conditions on a 12 h light/dark cycle (light on at 7:00 am) with free access to food and water. The PND-7 male rats (11–14 g) were selected from pregnant rats, all of them were allowed to adapt to new environments for 1 week before the experiments began. After each part of the experiment is completed, the rat pups were returned to the mother rats for feeding.

Experimental Protocol

Experiment 1:

Ketamine (Ket) group : The PND-7 rats were administered intraperitoneally with 40 mg/kg ketamine for 4 injections at 1 h interval (40 mg/kg × 4). Normal saline (NS) group : The PND-7 rats were intraperitoneally injected with the corresponding volume of NS by the same way. Hippocampal DG tissues of each group were harvested at 24 h after NS/ketamine administration and subjected to Western blot to assess the expressions of Jagged1, Notch1, NICD1 and Hes1. Morris water maze (MWM) test was used to observe the learning and memory ability of neonatal ketamine treatment rats at 2 months old. The experimental protocol is described in Fig. 1A.

Experiment 2:

The PND-7 rats were randomly divided into four groups. NS group: Artificial cerebrospinal fluid (ACSF) was micro-injected into bilateral hippocampal DG with the stereotactic apparatus at 1 h before NS administration. Ket group: ACSF was micro-injected into bilateral hippocampal DG at 1 h before ketamine anesthesia (40 mg/kg × 4). Jagged1 + NS group: Jagged1 (Santa Cruz, 390177, diluted with ACSF) was micro-injected into bilateral hippocampal DG at 1 h before NS administration, and the single injection dose was 0.5 mg/kg. Jagged1 + Ket group: Jagged1 was micro-injected into bilateral hippocampal DG at 1 h before ketamine anesthesia (40 mg/kg × 4), and the single injection dose was 0.5 mg/kg. The protein

expressions of NICD1 and Hes1 in hippocampal DG tissues were assessed by Western blot at 24 h after each treatment. The proliferation and differentiation of NSCs in hippocampal DG were detected using double-immunofluorescence staining at 24 h after each treatment. MWM experiment was used to assess the learning and memory ability at 2 months old. The experimental protocol is described in Fig. 2A.

Experiment 3:

NS group: Lentiviral vector that expresses green fluorescent proteins (LV-GFP) alone was micro-injected into bilateral hippocampal DG at PND-3 and single injection dose was 1.5 μl, then NS injections were administered at PND-7. Ket group: LV-GFP was micro-injected into bilateral hippocampal DG at PND-3 and the single injection dose was 1.5 μl, then ketamine anesthesia (40 mg/kg × 4) were administered at PND-7. LV-NICD1 + NS group: LV-NICD1 was micro-injected into bilateral hippocampal DG at PND-3 and the single injection dose was 1.5 μl, then NS injections were administered at PND-7. LV-NICD1 + Ket group: LV-NICD1 was micro-injected into bilateral hippocampal DG at PND-3 and the single injection dose was 1.5 μl, then ketamine anesthesia (40 mg/kg×4) were administered at PND-7. The biochemical and behavioral protocol is described in Fig. 6A.

During the interval between NS/ketamine injections, the rats were placed in a transparent chamber, temperature probes were used to facilitate control of temperature at 36.5 ± 1°C using computer-controlled heater/cooler plates integrated into the floor of chamber, which would help maintain body temperature and reduce stress.

Lentivirus Production and Stereotaxic Injection

The coding sequence of NICD1 was amplified by RT-PCR and ligated into the pGC-FU plasmid (Suzhou GenePharma) to produce pGC-FU-NICD1. A lentiviral vector that expresses GFP alone (LV-GFP) was used as the control. We carried out hippocampal DG micro-injection of LV-NICD1-GFP or LV-GFP with a stereotaxic instrument (World Precision Instruments) at PND-3. Rat pups were anesthetized with isoflurane (4 % induction, 1.5 % maintenance) and placed in a stereotaxic apparatus with an adapter for neonatal rats (David Kopf, CA). A scalp incision was made in the skull surface and the bregma was exposed. The intracerebral injection was performed with a 10 μl precise syringe (World Precision Instruments, Sarasota, FL), at a location of 2.0 mm posterior to bregma on the midline, 1.8 mm left or right to the midline and 2.3 mm depth below the meninges. Two microliters of replication-incompetent lentivirus was injected at a rate of 0.5 μl/min, and the tip was left in the injection place for 2 min. All rat pups were then returned to the dam and monitored until they resumed nursing.

Western Blot Analysis

All animals were deeply anesthetized with ketamine, and we performed a decapitation to extract hippocampus tissues of rat pups in all groups at 24 h after the end of each administration, then the hippocampal DG tissues were homogenized with lysis buffer and protease inhibitors (Beyotime, China). The lysates were placed on ice for 15 mins and centrifuged at 12000 rpm for 15 mins at 4°C. The lysates

were then resolved by 10% or 12% polyacrylamide gel and the target proteins were transferred to nitrocellulose membranes. The blots were incubated with blocking buffer for 2 h at room temperature and then incubated for 24 h at 4°C with the following primary antibodies. Rabbit anti-Jagged1 monoclonal antibody (1:1000, Cell Signaling Technology) was used to detect Jagged1 (150 kDa), rabbit anti-Notch1 monoclonal antibody (1:1000, Cell Signaling Technology) was used to detect Notch1 (120 kDa), rabbit anti-activated Notch1 polyclonal antibody (1:1000, Abcam) was used to detect NICD1 (110 kDa), mouse anti-Hes1 monoclonal antibody (1:1000, Santa Cruz) was used to detect Hes1 (30 kDa), and antibody β-actin (1:2000, Abcam) was used to detect β-actin (42 kDa). Then the membranes were incubated with appropriate secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:2000, Beyotime) or horseradish peroxidase-conjugated donkey anti-mouse antibody (1:2000, Beyotime) for 1 h. At last, the proteins were revealed with chemiluminescence detection system. The technical repetitions of Western blot for every protein were performed at least three times. All films were subjected to optical density analysis using the computer image analysis program ImageJ (National Institutes of Health, Bethesda, MD, USA). The ratio of target protein to internal reference was normalized to the control group.

Bromodeoxyuridine Injections and Immunofluorescence

Bromodeoxyuridine (BrdU, Sigma) was used to determine the proliferation and differentiation of NSCs by double-immunofluorescence. BrdU injection was performed using the method described in our previous study[22]. The rats in four groups received a single intraperitoneal injection of BrdU at a concentration of 10 mg/ml immediately after the end of each treatment. Individual injection dose was 100 mg/kg body weight.

The tissue preparation and immunofluorescence staining were conducted as described in our previous study[22]. All animals were deeply anesthetized with ketamine and then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde at 24 h after BrdU injection. The brains were removed, postfixed overnight in 4% paraformaldehyde and placed in 30% sucrose until sunk. According to the Atlas of the Developing Rat Brain and previous reports[31–32], the coronal slices of brain were cut consecutively at a thickness of 30 μm when the hippocampus was initially exposed. The fifteenth slice was taken and stored in PBS.

For double-immunofluorescence detection, DNA was denatured to expose the antigen. Incubation with 50 % formamide in PBS for 2 h at 65°C preceded 2-normal hydrochloric acid incubation for 30 mins at 45°C and 3 washes with PBS for 10 min in between each of these steps. After blocking of nonspecific epitopes with 10% donkey serum in PBS with 0.3% Triton-X for 2 h, the brain slices were incubated overnight at 4°C with the appropriate anti-BrdU mouse monoclonal primary antibody and following antibodies listed in Table 1. On the next day, the slices were washed with PBS and incubated with the suitable secondary fluorescent antibodies (Alexa 488-labeled donkey anti-rabbit or Alexafluor 594 donkey anti-mouse; 1:200; Invitrogen) for 2 h at room temperature.

Table 1
Primary antibodies

Antibody Name	Specificity	Host Species	Dilution Rates	Company
Nestin	Neural stem cells	Rabbit	1:100	Abcam
β-tubulin III	Newborn neurons	Rabbit	1:200	Abcam
GFAP	Astrocytes	Rabbit	1:200	Millipore
BrdU	Newly generated cells	Mouse	1:1000	Sigma

The hippocampal slices were observed by a skilled pathologist blinded to this research using laser scanning confocal microscope (40×, Fluoview 1000, Olympus). Single positive cells and double positive cells were manually counted by another experimenter with the help of the Cell Counter function of Image J 1.4 software (National Institutes of Health, Bethesda, MD, USA). The ratio of $Nestin^+/BrdU^+$ cells to $Nestin^+$ cells, the ratio of $\beta\text{-tubulin III}^+/BrdU^+$ cells to $BrdU^+$ cells and the ratio of $GFAP^+/BrdU^+$ cells to $BrdU^+$ cells were calculated respectively. The density of $Nestin^+/BrdU^+$ cells, $\beta\text{-tubulin III}^+/BrdU^+$ cells and $GFAP^+/BrdU^+$ cells in the hippocampal DG were also calculated respectively (n = five animals/group).

Morris Water Maze Test

After the end of treatment, the animals were raised normally until 2 months old and the spatial reference memory was tested by Morris water maze (MWM). The apparatus and behavioral procedures of MWM test have been described in previous study[22]. The behavioral test was conducted in a circular, black painted pool (180 cm diameter, 50 cm deep). The water temperature was maintained at 25 ± 1℃. An invisible platform (10 cm diameter) was submerged 1 cm below the water surface and was placed in the quadrant III, which was determined by using four starting locations (defined as Ⅰ, Ⅱ, Ⅲ and Ⅳ). There was a 90 degree angular offset between each pair of starting locations. During five consecutive training days, the experiments were conducted in a dimly lit and quiet laboratory setting, and a lamp was placed in a corner of the laboratory and the same light level was kept for both training and testing period. All animals could detect extra maze visual cues and learn how to locate the platform. The rats were trained four times per day with the different starting position being randomized for each rat. When the rat found the platform, it was allowed to stay on the platform for 30 s. If the rat did not find the platform within 120 s, the rat would be gently guided to the location and allowed to stay on the platform for 30 s, and the latency time in finding the hidden platform was recorded as 120 s. The average escape latency from the 4 trials was represented as the daily result for each rat. The swim path of each animal was tracked using computerized video system (Stoelting, USA). The latency to reach the platform was calculated for each trial and then averaged over each daily session. The probe test was conducted 24 h after the end of training phase. During the test, the platform was removed and the animals were allowed to swim freely in the pool for 60 s. The number of crossing over the previous platform and the percentage of time that animals spent in target quarter were calculated and used for the assessment of the long term memory. After the end of each trial, the rat was placed on a heater plate for 1 to 2 mins until they were dry, then they were returned to their chambers (n = nine animals/group).

Statistical analysis

The statistical analysis was conducted using SPSS software (version, 17.0; SPSS, Inc., Chicago, IL, USA) and the graphs were prepared with GraphPad Prism 5. Differences among treatment groups were evaluated by one-way ANOVA. The two-way ANOVA was used to analyze the difference of escape latency among treatment groups in the MWM test. The numerical data are presented as means ± SD. $P < 0.05$ was considered statistically significant.

Results

Neonatal Ketamine Exposure Down-Regulated the Expression of Notch Signalling Related Proteins in the Hippocampal DG

Compared with control group, injection of 40 mg/kg ketamine for 4 times significantly decreased the expression level of Jagged1 in the hippocampal DG of neonatal rats (0.7 ± 0.12 *vs.* 1 ± 0.09, $P = 0.0008$, n = six animals) (Fig. 1B). Next, we investigated the effect of ketamine on the expression levels of Notch signalling pathway receptor Notch1 and active Notch1 internal cellular domain NICD1 in the hippocampal DG. Quantification of the Western blot showed that ketamine significantly decreased the expression level of Notch1 (0.5 ± 0.1 *vs.* 1 ± 0.22, $P = 0.0005$, n = six animals) (Fig. 1C) and immunoblotting results of NICD1 revealed that the ketamine inhibited the activation of Notch1 receptor compared with the control condition (0.5 ± 0.08 *vs.* 1 ± 0.1, $P < 0.0001$, n = six animals) (Fig. 1D). Finally, we assessed the effect of ketamine on the downstream target gene of Notch signalling in the hippocampal DG tissues. The Western blot analysis suggested that neonatal ketamine exposure significantly decreased the level of Hes1 in the hippocampal DG (0.6 ± 0.1 *vs.* 1 ± 0.05, $P < 0.0001$, n = six animals) (Fig. 1E).

Considered together, neonatal exposure to 40 mg/kg ketamine for 4 injections caused a significant inhibition of Notch1 signalling pathway in the hippocampal DG, including decreased expression of Jagged1 and Notch1, combined with reduction of active Notch1 internal cellular domain and target gene Hes1.

Neonatal Ketamine Exposure Reduces the Rats' Learning and Memory Ability During Adult Stage.

During reference training period, the time that each rat spent to reach the platform showed a statistically significant difference between NS group and Ket group from training day 3 to 5 (Fig. 1G). On training day 6, the rats exposed to ketamine required a significantly greater amount of time to find the platform when compared with the control group (14.33 ± 6.76 s *vs.* 6.22 ± 2.91 s, $P = 0.0045$) (Fig. 1H). In the probe test, the number of times that each rat crossed the location of an absent platform showed a significant

decrease in Ket group compared with that in the control condition (2.0 ± 1.12 *vs.* 3.8 ± 1.56, $P = 0.0135$) (Fig. 1I). Additionally, the percentage of platform quadrant retention time was significantly reduced in the rats exposed to ketamine when compared with the control group (11.6 ± 4.20 % *vs.* 20.0 ± 4.89, $P = 0.0012$) (Fig. 1J). Considered together, these findings suggested that repeated ketamine anesthesia in neonatal rats injured hippocampal related learning and memory during adult stage.

Micro-injection of Jagged1 in the Hippocampal DG Reversed the Down-expression of Notch-related Proteins Induced by Neonatal Ketamine Exposure

Immunoblotting showed that pretreatment with Jagged1 before NS injections induced more visible bands representing NICD1 (Fig. 2B) and there was a significant difference in the NICD1 expression between NS group and Jagged1 + NS group (1 ± 0.09 *vs.* 1.3 ± 0.21, $P = 0.0367$, n = eight animals) (Fig. 2C). Analysis of Western blot showed the expression of Hes1 in Jagged1 + NS group were significantly up-regulated compared with the control condition (1.3 ± 0.26 *vs.* 1 ± 0.26, $P = 0.0236$, n = eight animals) (Fig. 2B and 2D). Next, the immunoblotting results showed that pretreatment with Jagged1 before ketamine anesthesia induced more visible bands representing NICD1 and Hes1 when compared with the Ket group (Fig. 2B). Quantification of Western blot demonstrated that the down-expressions of NICD1 and Hes1 induced by ketamine could be reversed by the micro-injection with Jagged1 in the hippocampal DG before ketamine anesthesia (NICD1: 0.4 ± 0.22 *vs.* 1 ± 0.22, $P < 0.0001$; Hes1: 0.5 ± 0.19 *vs.* 0.9 ± 0.23, $P = 0.0037$; n = eight animals) (Fig. 2B-2D).

Administration of Jagged1 Prevented Cognitive Decline Induced by Neonatal Ketamine Exposure

The typical track charts were shown in Fig. 2E. During reference training period, there was a statistical decrease in the escape latency between Jagged1 + Ket group and Ket group from training day 3 to day 5 (Fig. 2F). On training day 6, the escape latency in Jagged1 + Ket group was significantly shortened compared with the Ket group (11.51 ± 9.37 s *vs.* 33.79 ± 22.75 s, $P = 0.012$) (Fig. 2G).

In the probe test, comparison of the times that each rat crossed the previous platform showed a statistically significant increase in Jagged1 + Ket group compared with the Ket group (2.1 ± 0.83 *vs.* 0.9 ± 0.64, $P = 0.0135$) (Fig. 2H). Additionally, the time spent in the target quadrant in Jagged1 + Ket group was significantly increased compared with that in the Ket group (18.8 ± 4.19 % *vs.* 12.6 ± 3.66 %, $P = 0.0462$) (Fig. 2I). Considered together, these findings suggested that the Notch signalling pathway was involved in the hippocampus-dependent learning and memory impairment during adult stage induced by neonatal ketamine anesthesia.

Activation of Notch1 Signalling Pathway with Jagged1 Reversed the Ketamine-induced Disturbence on the Proliferation and Differentiation of Hippocampal NSCs

Typical immunofluorescence pictures of NSC proliferation in the hippocampal DG were shown in Fig. 3A. Statistical analysis indicated that the ratio of $Nestin^+/BrdU^+$ cells (11.7 ± 1.16 %) and density of $Nestin^+/BrdU^+$ cells (419 ± 57 /mm^2) in Ket group were significantly decreased compared to those in the NS group (16.1 ± 1.82 %, $P = 0.0019$; 603 ± 60 /mm^2, $P = 0.0006$) (Fig. 3B and 3C), while activation of Notch signalling by Jagged 1 in Jagged1 + NS group could significantly promote the proliferation of NSCs with increased ratio (25.0 ± 1.58 %) and density (874 ± 70 /mm^2) of $Nestin^+/BrdU^+$ cells compared with NS group (16.1 ± 1.82 %, $P < 0.0001$; 603 ± 60 /mm^2, $P < 0.0001$) (Fig. 3B and 3C). In addition, pre-treatment with Jagged1 before ketamine anesthesia in the Jagged1 + Ket group could significantly promote the proliferation of NSCs with increased ratio (16.4 ± 1.61 %) and density (694 ± 39 /mm^2) of $Nestin^+/BrdU^+$ cells compared with Ket group (11.7 ± 1.16 %, $P = 0.001$; 419 ± 57 /mm^2, $P < 0.0001$) (Fig. 3B and 3C).

Typical immunofluorescence pictures of neuronal differentiation in the hippocampal DG were shown in Fig. 4A. The Fig. 4B and 4C indicated that the percentage (19.57 ± 0.99 %) and density (671 ± 53 /mm^2) of β-tubulin $III^+/BrdU^+$ cells were significantly increased in the Ket group compared to those in the NS group (14.89 ± 1.88 %, $P < 0.0001$; 527 ± 67 /mm^2, $P = 0.001$). While activation of Notch1 signalling by Jagged 1 in the Jagged1 + NS group could significantly inhibit the neuronal differentiation of NSCs with decreased ratio (11.31 ± 0.82 %) and density (400 ± 21 /mm^2) of β-tubulin $III^+/BrdU^+$ cells compared with NS group (14.89 ± 1.88 %, $P = 0.0009$; 527 ± 67 /mm^2, $P = 0.0031$). In addition, pre-treated with Jagged1 before ketamine anesthesia could significantly decreased ratio (16.04 ± 0.51 %) and density (556 ± 36 /mm^2) of β-tubulin $III^+/BrdU^+$ cells compared with Ket group (19.57 ± 0.99 %, $P = 0.001$; 671 ± 53 /mm^2, $P = 0.0073$).

Typical immunofluorescence pictures of the astrocytic differentiation in hippocampal DG were shown in Fig. 5A. Similar to the effect of ketamine on NSC proliferation, the percentage (15.3 ± 1.49 %) and density (354 ± 40 /mm^2) of $GFAP^+/BrdU^+$ cells were significantly decreased in the Ket group compared to those in the NS group (20.31 ± 1.99 %, $P = 0.0008$; 588 ± 53 /mm^2, $P < 0.0001$) (Fig. 5B and 5C). While the activation of Notch1 signalling by Jagged 1 in the Jagged1 + NS group could significantly promote the astrocytic differentiation of NSCs with increased ratio (24.23 ± 1.89 %) and density (783 ± 34 /mm^2) of $GFAP^+/BrdU^+$ cells compared with NS group (20.31 ± 1.99 %, $P = 0.0066$; 588 ± 53 /mm^2, $P < 0.0001$) (Fig. 5B and 5C). In addition, pre-treatment with Jagged1 before ketamine anesthesia could significantly increase the ratio (19.78 ± 0.68 %) and density (546 ± 54 /mm^2) of $GFAP^+/BrdU^+$ cells compared with Ket group (15.3 ± 1.49 %, $P = 0.0021$; 354 ± 40 /mm^2, $P < 0.0001$) (Fig. 5B and 5C).

Considered together, these findings suggested that neonatal ketamine exposure interfered with the proliferation and differentiation of hippocampal NSCs by inhibiting Notch1 signalling pathway.

Activation of Notch1 Signalling Pathway by Pre-injection of LV-NICD1 in the Hippocampal DG Reversed the Down-expression of Notch-related Proteins Induced by Neonatal Ketamine Exposure

The immunoblotting results showed that pretreatment with LV-NICD1 before NS injections induced more visible bands representing NICD1 and Hes1 as compared with the control condition (Fig. 6C). Analysis of Western blot showed the expressions of NICD1 and Hes1 in LV-NICD1 + NS group were significantly up-regulated compared with the control condition (NICD1: 1.4 ± 0.12 *vs.* 1 ± 0.11, $P < 0.0001$; Hes1: 1.4 ± 0.22 *vs.* 1 ± 0.25, $P = 0.014$, n = eight animals) (Fig. 6D and 6E). Next, the immunoblotting results showed that pretreatment with LV-NICD1 before ketamine anesthesia induced more visible bands representing NICD1 and Hes1 as compared with the Ket group (Fig. 6C). Quantification of Western blot demonstrated that the down-expressions of NICD and Hes1 induced by ketamine could be reversed by the micro-injection with LV-NICD1 in the hippocampal DG before ketamine anesthesia (NICD1: 0.5 ± 0.1 *vs.* 1.1 ± 0.11, $P < 0.0001$; Hes1: 0.6 ± 0.2 *vs.* 1 ± 0.16, $P = 0.0092$, n = eight animals) (Fig. 6D and 6E).

Pretreatment with LV-NICD1 Prevented Cognitive Decline Induced by Neonatal Ketamine Exposure

The typical track charts were shown in Fig. 6F. During reference training period, the escape latency of four groups showed a downtrend from training day 3 to day 5 and the escape latency in LV-NICD1 + Ket group was statistically decreased compared with Ket group (Fig. 6G). On the training day 6, the escape latency in LV-NICD1 + Ket group was significantly shortened compared with the Ket group (7.88 ± 5.98 s *vs.* 30.93 ± 23.16 s, $P = 0.0046$) (Fig. 6H). In the probe test, the times that the rats crossed the previous platform showed a statistically significant increase in LV-NICD1 + Ket group compared with the Ket group (2.9 ± 0.83 *vs.* 1.4 ± 1.06, $P = 0.0407$) (Fig. 6I). Additionally, the time spent in the target quadrant in LV-NICD1 + Ket group was significantly increased compared with that in the Ket group (20.1 ± 7.19 % *vs.* 10.4 ± 5.23, $P = 0.0437$) (Fig. 6J). Considered together, these findings further suggested that the Notch signalling pathway was involved in the hippocampus-dependent learning and memory impairment during adult stage induced by neonatal ketamine anesthesia.

LV-NICD1 Reversed the Ketamine-induced Disturbence on the Proliferation and Differentiation of Hippocampal NSCs

Typical immunofluorescence pictures of NSC proliferation were shown in Fig. 7A. Compared with NS group, activation of Notch signalling by LV-NICD1 in LV-NICD1 + NS group could significantly promote the proliferation of NSCs with increased ratio (24.17 ± 2.07 % *vs.* 15.1 ± 1.22 %, $P < 0.0001$) and density of

Nestin$^+$/BrdU$^+$ cells (931 ± 61 /mm^2 *vs.* 614 ± 25 /mm^2, $P < 0.0001$) (Fig. 7B and 7C). In addition, pre-treatment with LV-NICD1 before ketamine anesthesia in the LV-NICD1 + Ket group could significantly promote the proliferation of NSCs compared with Ket group (ratio: 15.91 ± 1.04 % *vs.* 9.46 ± 1.48 %, $P < 0.0001$; density: 738 ± 55 /mm^2 *vs.* 438 ± 45 /mm^2, $P < 0.0001$) (Fig. 7B and 7C).

The double immunofluorescence staining of neuronal differentiation in the hippocampal DG was shown in Fig. 8A. The statistical analysis indicated that pre-treatment with LV-NICD1 in the LV-NICD1 + NS group could significantly inhibit the neuronal differentiation of NSCs compared with NS group, accompanied by decreased ratio and density of β-tubulin III$^+$/BrdU$^+$ cells (ratio: 11.39 ± 1.09 % *vs.* 14.56 ± 1.02 %, $P = 0.0072$; density: 395 ± 47 /mm^2 *vs.* 525 ± 59 /mm^2, $P = 0.0048$) (Fig. 8B and 8C). In addition, activation of Notch1 signalling by LV-NICD1 before ketamine anesthesia could significantly decrease the ratio and density of β-tubulin III$^+$/BrdU$^+$ cells compared with Ket group (ratio: 14.5 ± 1.43 % *vs.* 19.38 ± 1.59 %, $P = 0.0001$; density: 617 ± 52 /mm^2 *vs.* 774 ± 44 /mm^2, $P = 0.0009$) (Fig. 8B and 8C).

Typical immunofluorescence pictures of astrocytic differentiation in the hippocampal DG were shown in Fig. 9A. The ratio and density of GFAP$^+$/BrdU$^+$ cells were significantly increased in the LV-NICD1 + NS group compared to those in the NS group (ratio: 26.13 ± 0.97 % *vs.* 21.13 ± 1.13 %, $P < 0.0001$; density: 828 ± 82 /mm^2 *vs.* 593 ± 34 /mm^2, $P < 0.0001$) (Fig. 9B and 9C). In addition, activation of Notch1 signalling by LV-NICD1 in the LV-NICD1 + Ket group could significantly promote the astrocytic differentiation of NSCs with increased ratio and density of GFAP$^+$/BrdU$^+$ cells compared with Ket group (ratio: 21.58 ± 1.17 % *vs.* 15.07 ± 1.21 %, $P < 0.0001$; density: 536 ± 28 /mm^2 *vs.* 370 ± 44 /mm^2, $P = 0.0006$) (Fig. 9B and 9C).

These findings further indicated that Notch1 signalling pathway was involved in the disturbence on the proliferation and differentiation of NSCs induced by the neonatal ketamine exposure.

Discussion

Compelling evidence in animal models have suggested that general anesthesia has unequivocal link to the change of developmental brain and persistent impairment in neurocognitive function. Recent large scale randomized controlled clinical trials confirm that the healthy infants received general anesthesia for less than 1 h is not associated with neurocognitive impairments in later childhood[33–34]. However, there is a lack of studies to clarify the potential impacts of longer exposure or multiple exposure to general anesthetics. The U.S. Food and Drug Administration (FDA) had issued that developmental neurotoxicity induced by multiple exposure or exposure several hours to anesthetics remains incompletely understood and the underlying mechanism required to be further investigated[35].

Neurogenesis occurs on a large scale of brain from a critical period of embryonic to postnatal stage, which includes NSC proliferation and differentiation. Neurogenesis in the certain brain is especially active throughout life in two distinct areas: the SVZ and hippocampal DG[16]. The disturbence of postnatal

neurogenesis in the DG may lead to persistent impairments in learning, memory, and behaviors[36–37]. Previous findings have revealed that inhalation/intravenous anesthetics could affect the proliferation and differentiation of developmental hippocampal NSCs and lead to the long-term neurocognitive dysfunction[18–21]. On the basis of our precedent findings, the purpose of present study was to explore the mechanism of Notch signalling pathway in the role of ketamine exposure interfering with the neurogenesis of hippocampal dentate gyrus and damaging the learning and memory function in adulthood, and to provide a strong experimental basis for further understanding the neurotoxicity of ketamine in the neonatal period.

Based on Western blot analysis, 40 mg/kg ketamine anesthesia for 4 times in neonatal rats specifically inhibited the Notch signalling pathway, accompanied by a significant reduction in Jagged1, Notch1, NICD1 and Hes1 levels 24 h after the anesthesia in the hippocampal DG tissues. In addition, the same ketamine anesthesia in neonatal rats induced the long-term neurocognitive dysfunction at 2 months old. Given that Notch signalling pathway is closely relevant to the regulation of normal neurogenesis, we further investigated whether the ketamine-induced impairment of neurogenesis and neurocognitive function could be ameliorated by activating Notch1 signalling pathway.

The Notch1 receptor is a transmembrane protein, which can be activated by its ligand Jagged1. After interaction with ligand of the Jagged1, Notch1 receptor is cleaved by presenilin-1 and the γ-secretase enzyme complex, then a series of cleavages release the Notch1 intracellular domain (NICD1), which translocates into the nucleus where it induces transcription of Notch1 downstream target genes such as hairy enhancer of split (Hes) family member. In the present study, Jagged1 and LV-NICD1 were micro-injected into the bilateral hippocampal DG. Jagged1 can activate Notch1 signalling through extracellular pathway[38] and overexpression of NICD1 can activate Notch1 signalling through intracellular pathway[39]. Therefore, we choose two methods (Jagged1 or LV-NICD1) to activate Notch1 signalling through extracellular and intracellular pathway respectively. It was showed that the decreased expression of NICD1 and Hes1 induced by ketamine could be reversed by both Jagged1 and LV-NICD1 pretreatment.

The vast majority of NSCs are in a mitotically active condition in the developing brain, therefore, the balance between mitosis and quiescence of NSCs is crucial to maintain the neuron production and regeneration[40–41]. In recent years, postnatal neurogenesis is a particularly interesting filed in neuroscience to study the anesthetic induced neurotoxicity. Our previous study has revealed that neonatal ketamine exposure significantly inhibits NSC proliferation and astrocytic differentiation, meanwhile markedly enhances neuronal differentiation[22]. However, the potential mechanism has not been clarified. Notch signalling pathway is regarded as a “switch” to determine the NSC fate[25]. Although it has been demonstrated that up-regulation of NICD1 promotes the proliferation of NSCs in the postnatal hippocampal DG, and γ-secretase inhibitor or genetic ablation of Notch1 promotes cell cycle exit and the neuronal differentiation[42], the role of Notch1 signalling pathway in the disturbence of NSC proliferation and differentiation caused by neonatal ketamine exposure has not been clearly investigated. According to the methodology in our previous study[22], we performed the double staining to analyze the ratio of NSC

proliferation and differentiation and the density of double positive cells in the hippocampal DG, and discovered the increase of NSC proliferation and astrocytic differentiation combined with the inhibition of neuronal differentiation in rats receiving Jagged1 or LV-NICD1 treatment compared with those in control groups. These results were mainly consistent with the findings in the previous research. The Hes family genes are essential effectors of Notch signalling pathway and negative regulators of neuronal differentiation, and upregulation of Hes1 could contribute to an decrease of neuronal population[43–44]. This study confirmed that the down expression of Notch targeted gene Hes1 induced by ketamine could be reversed by the Jagged1 or LV-NICD1 treatment. In addition, the disturbence of NSC proliferation and differentiation caused by ketamine could be specifically reversed by pre-activating the Notch1 signalling pathway with Jagged1 or overexpression of NICD1 in the hippocampal DG. All of the above findings suggested that neonatal ketamine exposure interfered with the proliferation and differentiation of NSCs in the hippocampal DG by inhibiting the Notch1-Hes1 signalling pathway.

A growing body of evidence strongly suggested that postnatal neurogenesis in the hippocampal DG may be highly susceptible to various physiological and pathological stimulation[37]. The damage of postnatal neurogenesis in the hippocampal DG has been associated with cognitive dysfunction in rodent models, such as the impairment in spatial learning, memory retention, memory retrieval. Our previous study had revealed that multiple exposure to clinically relevant dose of ketamine may interfere with hippocampal neurogenesis and long-term neurocognitive function in PND-7 rats. In this study, Jagged1 or LV-NICD1 was confirmed to ameliorate ketamine-induced long-term neurocognitive dysfunction, indicating that the proliferation and differentiation of neural stem cells regulated by Notch signaling pathway played a critical role in maintaining normal cognitive function.

Neurogenesis is a complicated process that includes not only NSC proliferation, neuronal and astrocytic differentiation but also the migration of newborn neurons and the formation of neuronal circuit. The appropriate number of newborn neurons in the developing brain is crucial for their migration and establishment of synaptic function[45]. Increased differentiation of NSCs into neurons during development stage may be detrimental to the formation of normal neural circuits. Our previous study indicated that ketamine interfered with the neurogenesis in hippocampal DG in PND-7 rats. Although ketamine promoted the neuronal differentiation of NSCs, the migration of newborn neurons in the GCL of hippocampal DG was markedly inhibited by ketamine[22]. In the present study, although neonatal exposure to ketamine markedly increased the differentiation of NSCs into neurons, the neurocognitive performance was significantly impaired in adulthood.

The Notch signalling pathway has been proved to be activated in the regulation of adult neurogenesis in the animal stroke model, including the promotion of NSC proliferation and acceleration of neuronal differentiation[46]. The present conclusions are not completely consistent with the previous findings partly because that the NSCs in the neonatal hippocampal DG have different biological characteristics compared with that in adult hippocampal neurogenesis[47]. During brain growth spurt (BGS), the peak levels of dentate gyrus neurogenesis in rodents last from the end of pregnancy to the first two weeks after

birth, then with a change to a lower rate during adult stage[48]. The present study revealed that multiple ketamine exposure during neonatal stage influenced the balance between the activity and quiescence of NSCs, and also influenced the quantity and percentage of neurons/astrocytes in the developing hippocampal DG by inhibiting Notch1-Hes1 signalling pathway. However, the majority of NSCs in the adult hippocampal DG were kept in an inactive state. The promotion effects on the proliferation and neuronal differentiation of NSCs induced by stroke through activating the Notch1 signalling pathway should be taken as a protective mechanism to compensate the loss of neurons.

There are several limitations in this study. Although the present results showed neonatal anesthesia by ketamine in rats interfered with the proliferation and differentiation of hippocampal NSCs via inhibition of notch1 signalling pathway, it will be better to obtain the additional results in vitro. In addition, the effects of neonatal ketamine anesthesia on the migration of newborn neurons and the formation of neuronal circuit remain to be further investigated. Finally, although the present study demonstrated that neonatal exposure to ketamine could impair neurocognitive function in adulthood, its effect on hippocampal synaptic plasticity such as the growth of dendrites and dendritic spines as well as long-term potentiation (LTP) remain to be clarified.

In conclusion, the present study indicates that neonatal exposure to ketamine in rats interferes with the proliferation and differentiation of hippocampal NSCs and impairs neurocognitive function in adulthood via inhibition of Notch1 signalling pathway. These findings contribute to further understanding of the neonatal neurotoxicity induced by general anesthesia and its underlying mechanisms.

Declarations

Acknowledgments

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Figures

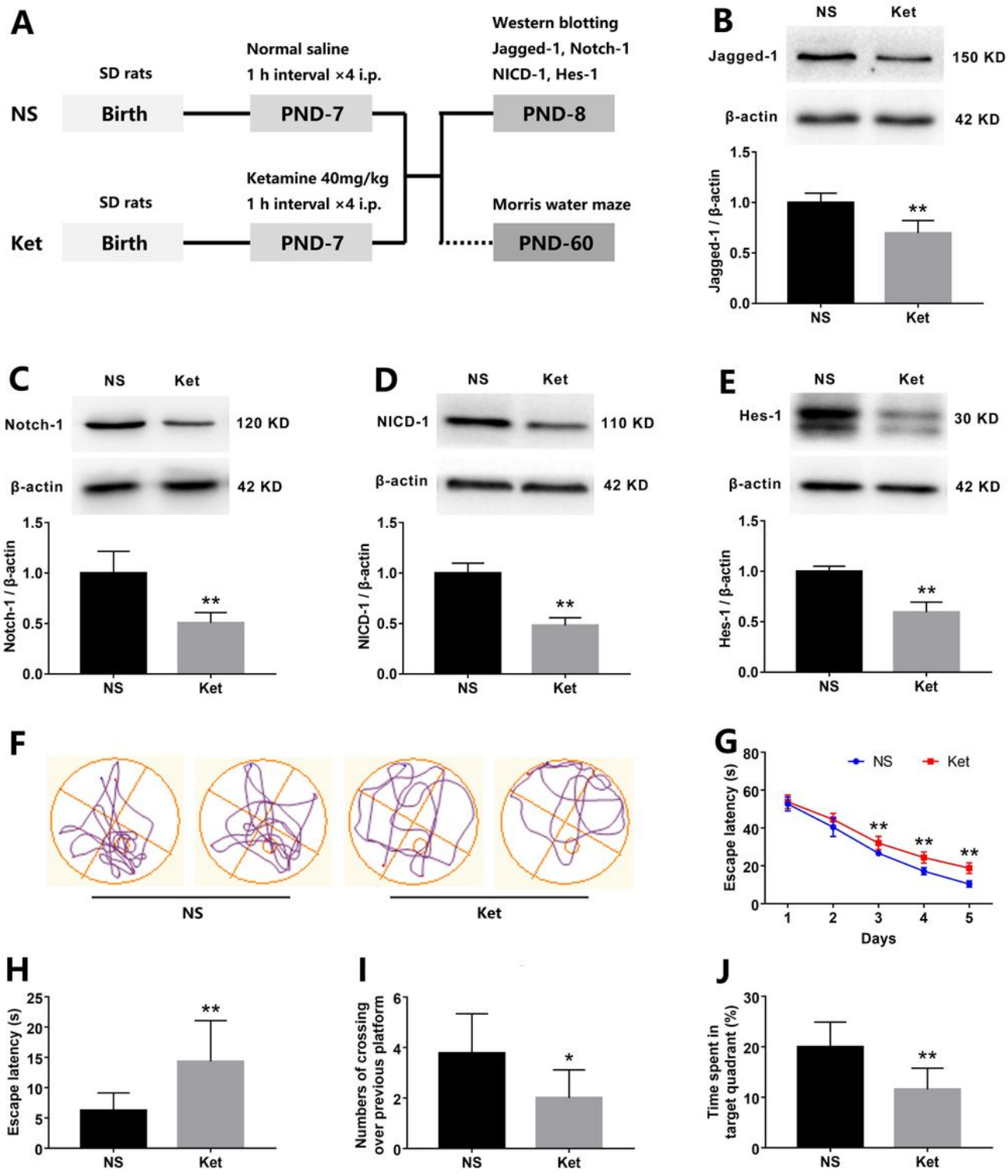
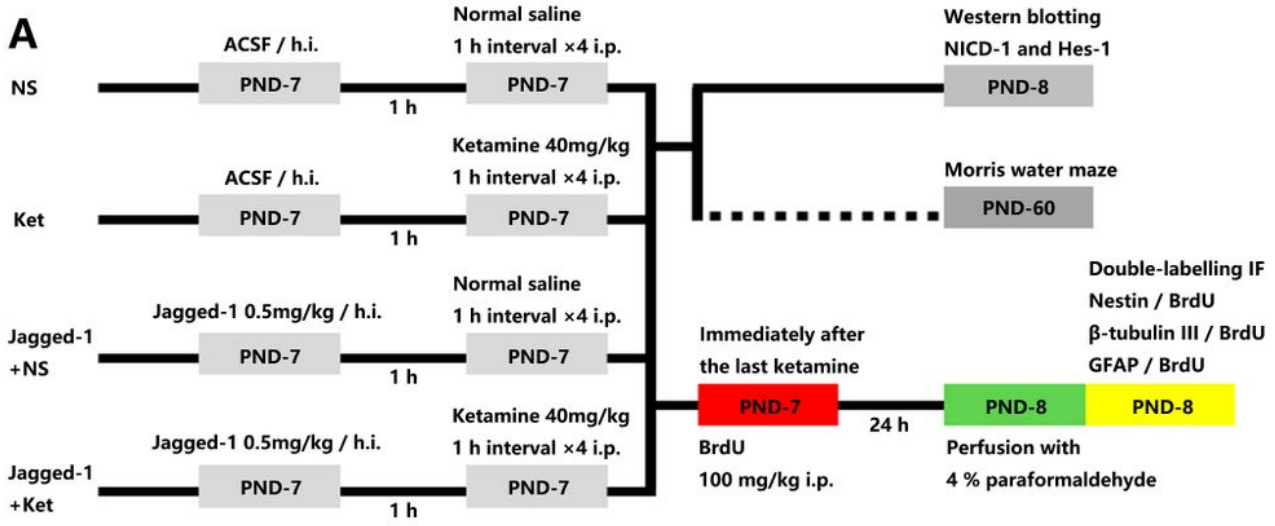
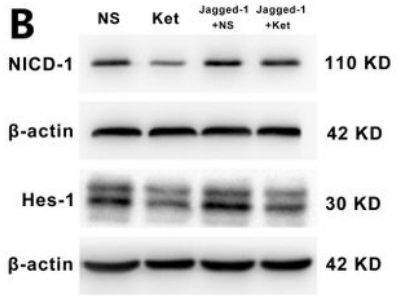


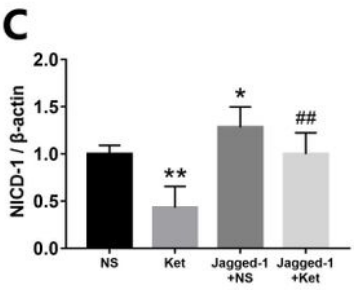
Figure 1

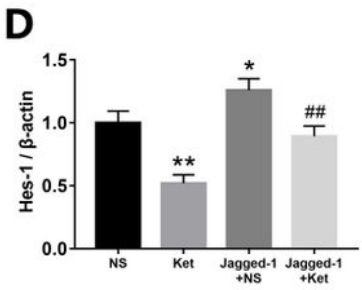
Neonatal anesthesia by ketamine inhibited the Notch1 signaling and impaired neurocognitive function of rats in adulthood. (A) Experimental protocol. Western blot was performed 24 h after each treatment. Morris water maze (MWM) test was performed at two months old. Multiple exposures to ketamine (4 injections of 40 mg/kg ketamine at 1 h intervals) decreased the expression of Jagged1 (B), Notch1 (C), NICD1 (D) and Hes1 (E) in the hippocampal DG of PND-7 rats by Western blot analysis. Neonatal

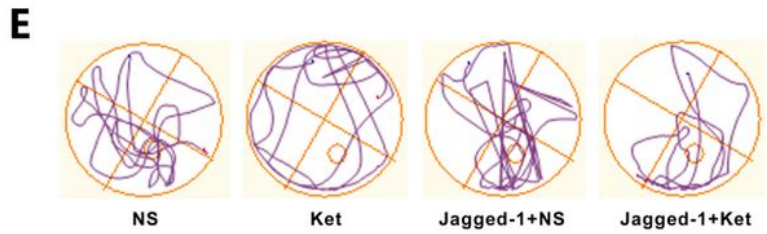
exposures to ketamine induced spatial learning and memory impairment in rats during the adulthood by Morris water maze (MWM) test. (F) Representative swimming paths of space exploration were exhibited. Neonatal ketamine anesthesia significantly prolonged the escape latency of rats in adulthood (G and H). Both the number of crossing over the previous platform site within 60 s (I) and the percentage of time spent in target quadrant (J) were significantly reduced in ketamine group than those in NS group. Data in the immunoblotting analysis are presented as mean ± SD (n=6). Data in the behavioral research are presented as mean ± SD (n=9). i.p., intraperitoneally. *P < 0.05, **P < 0.01, vs NS group.

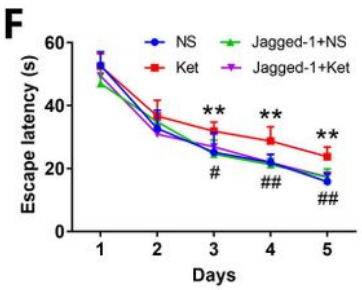


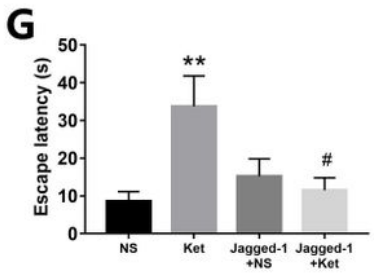


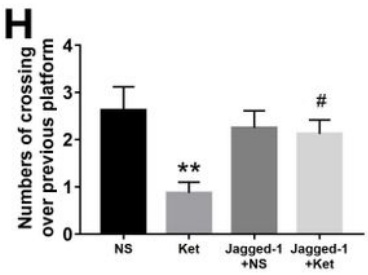












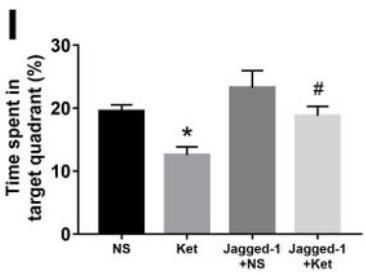


Figure 2

Effect of Jagged1 pretreatment on the expression of NICD1 and Hes1 in the hippocampal DG of rats exposed to ketamine at PND 7 and the spatial learning and memory in the adulthood. (A) Experimental protocol. Jagged1/ACSF was injected into the hippocampal DG 1 h before each treatment. The rats in four groups received a single intraperitoneal injection of 100 mg/kg BrdU immediately after the end of each treatment. Western blot and immunofluorescence staining were performed 24 h after each treatment. Morris water maze (MWM) test was performed at two months old. Pretreatment with Jagged1 significantly prevented the decrease of NICD1 (B and C) and Hes1 (B and D) in hippocampal DG induced by neonatal ketamine anesthesia by Western blot analysis. The impaired spatial learning and memory in adulthood induced by neonatal exposures to ketamine was markedly improved by pretreatment with Jagged1 in Morris water maze (MWM) test. (E) Representative swimming paths of space exploration. (F and G) Escape latency of rats. (H) The number of crossing over the previous platform site within 60 s. (I) The percentage of time spent in target quadrant. Data are presented as mean ± SD (n=8). h.i., hippocampal injection; i.p., intraperitoneally; IF, immunofluorescence. *P < 0.05, **P < 0.01, vs NS group; #P < 0.05, ##P < 0.01, vs Ket group.

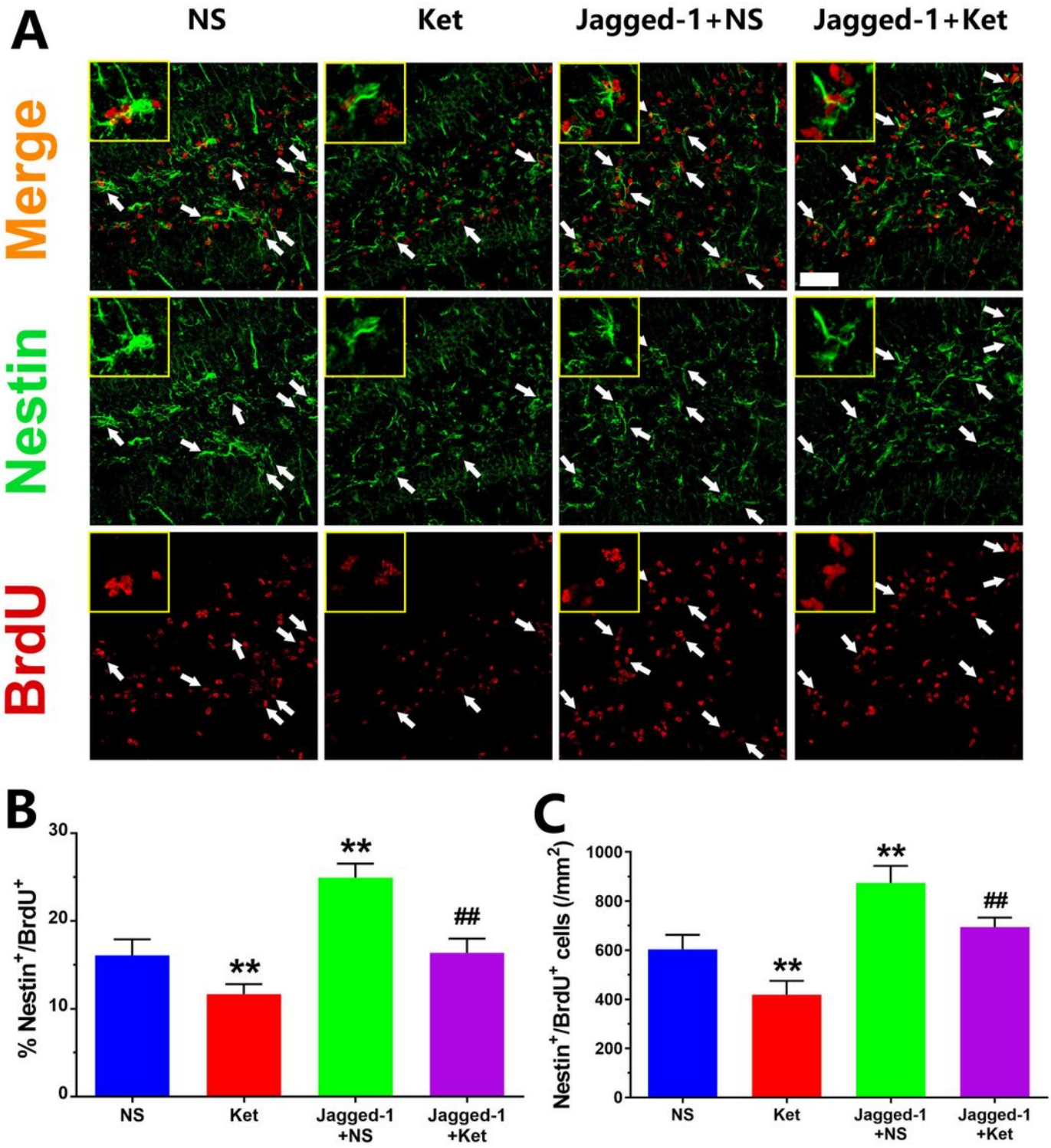


Figure 3

Effect of Jagged1 pretreatment on the NSC proliferation in the hippocampal DG of neonatal rats exposed to ketamine. (A) The proliferation of NSCs were labeled with primary antibodies against Nestin (green) and BrdU (red). The immunoreactive cells were visualized using the laser scanning confocal microscope (magnification: × 400; the scale bar is 50 μm). The white arrows pointed to Nestin/BrdU double-labeled cells. (B) The ratio of Nestin+/BrdU+ cells to Nestin+ cells in the DG. (C) The density of Nestin+/BrdU+

cells in the DG. Data are presented as the mean ± SD (n=5). **P < 0.01, vs NS group; ##P < 0.01, vs Ket group.

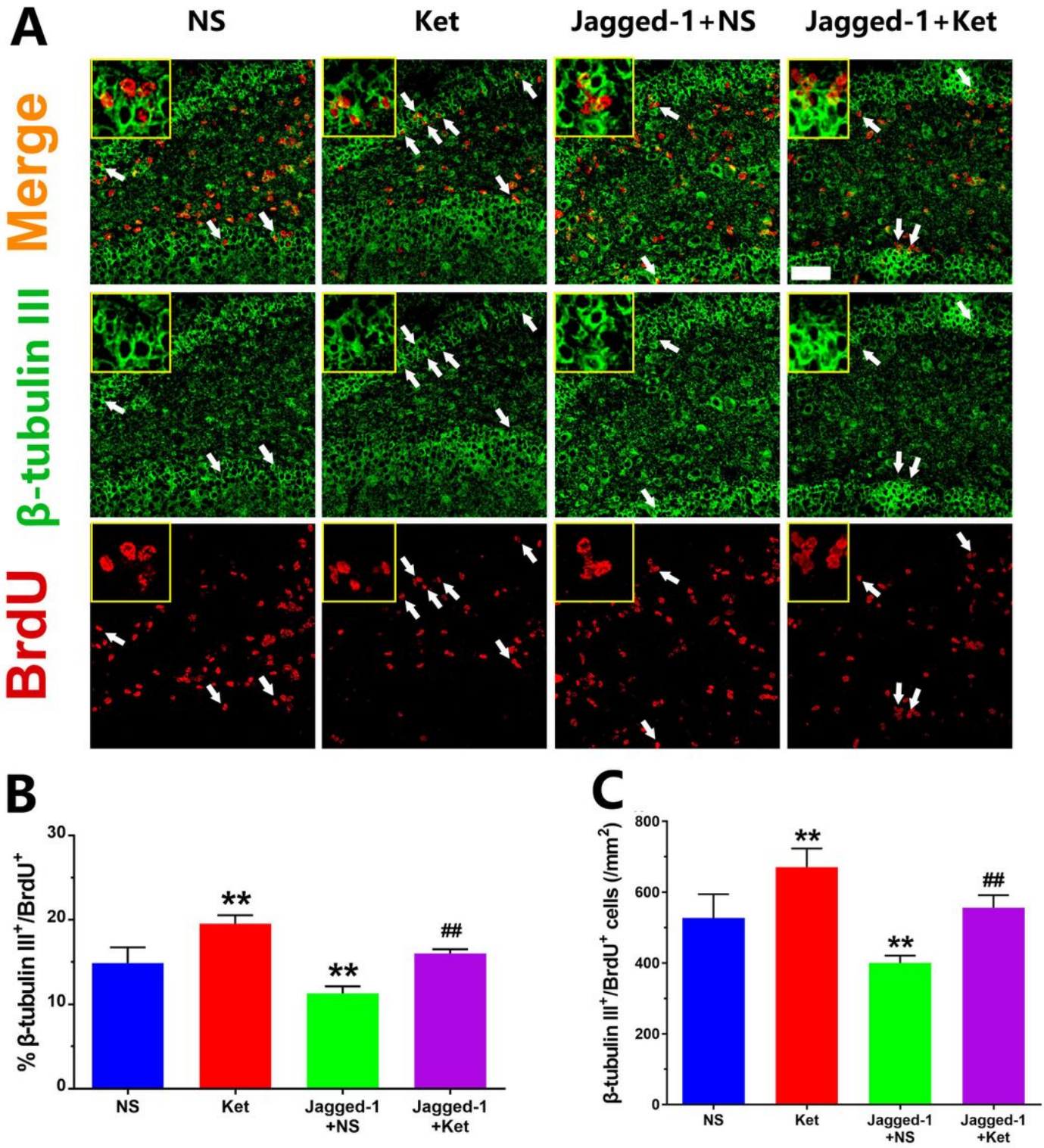


Figure 4

Effect of Jagged1 pretreatment on the neuronal differentiation in the hippocampal DG of neonatal rats exposed to ketamine. (A) The neuronal differentiation of NSCs was labeled with primary antibodies against β-tubulin III (green) and BrdU (red). The immunoreactive cells were visualized using the laser

scanning confocal microscope (magnification: × 400; the scale bar is 50 μm). The white arrows pointed to β-tubulin III/BrdU double-labeled cells. (B) The ratio of β-tubulin III+/BrdU+ cells to BrdU+ cells in the DG. (C) The density of β-tubulin III+/BrdU+ cells in the DG. Data are presented as the mean ± SD (n=5). **P < 0.01, vs NS group; ##P < 0.01, vs Ket group.

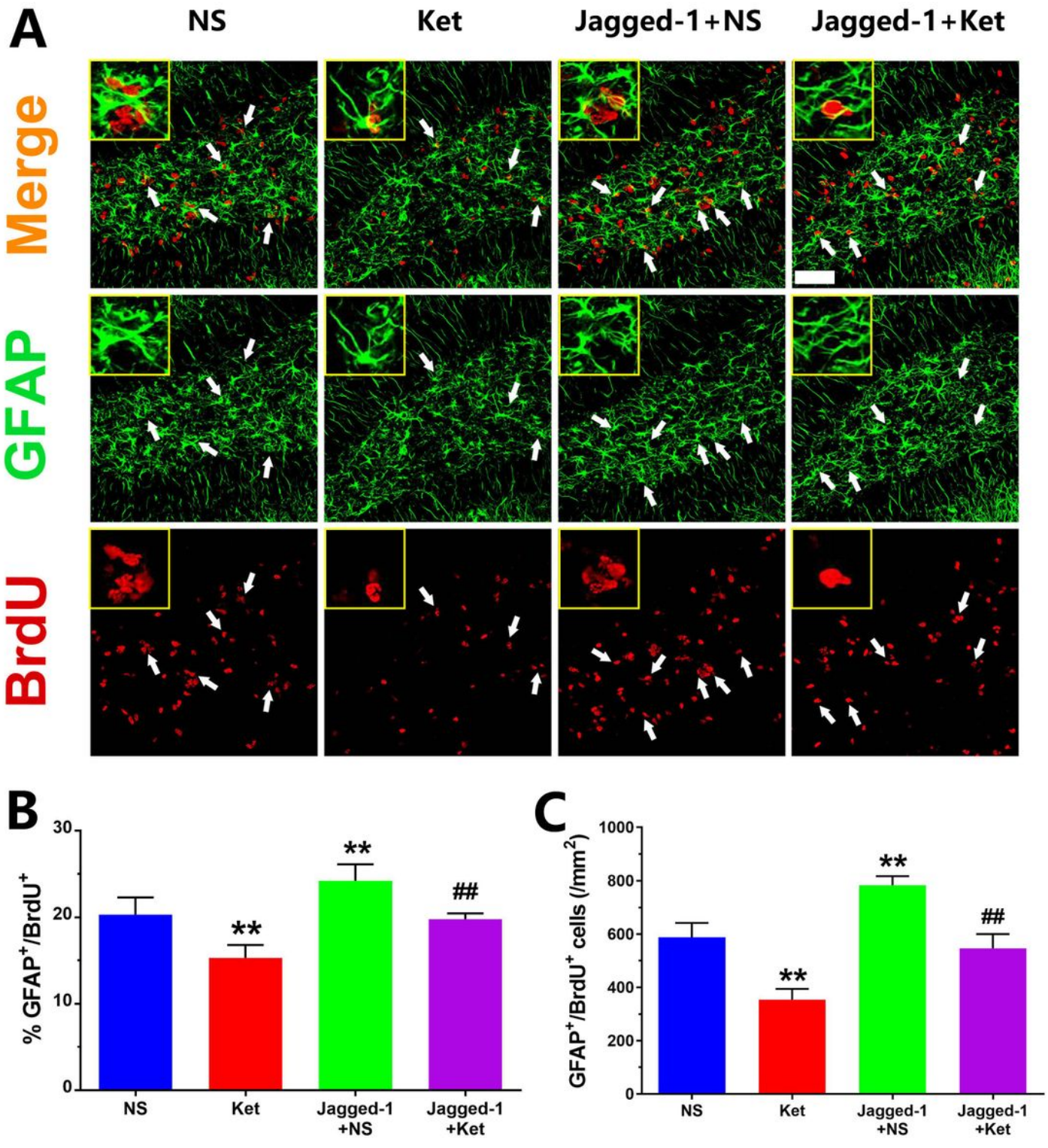


Figure 5

Effect of Jagged1 pretreatment on the astrocytic differentiation in the hippocampal DG of neonatal rats exposed to ketamine. (A) The astrocytic differentiation of NSCs was labeled with primary antibodies against GFAP (green) and BrdU (red). The immunoreactive cells were visualized using the laser scanning confocal microscope (magnification: × 400; the scale bar is 50 μm). The white arrows pointed to GFAP/BrdU double-labeled cells. (B) The ratio of GFAP+/BrdU+ cells to BrdU+ cells in the DG. (C) The density of GFAP+/BrdU+ cells in the DG. Data are presented as the mean ± SD (n=5). **P < 0.01, vs NS group; ##P < 0.01, vs Ket group.

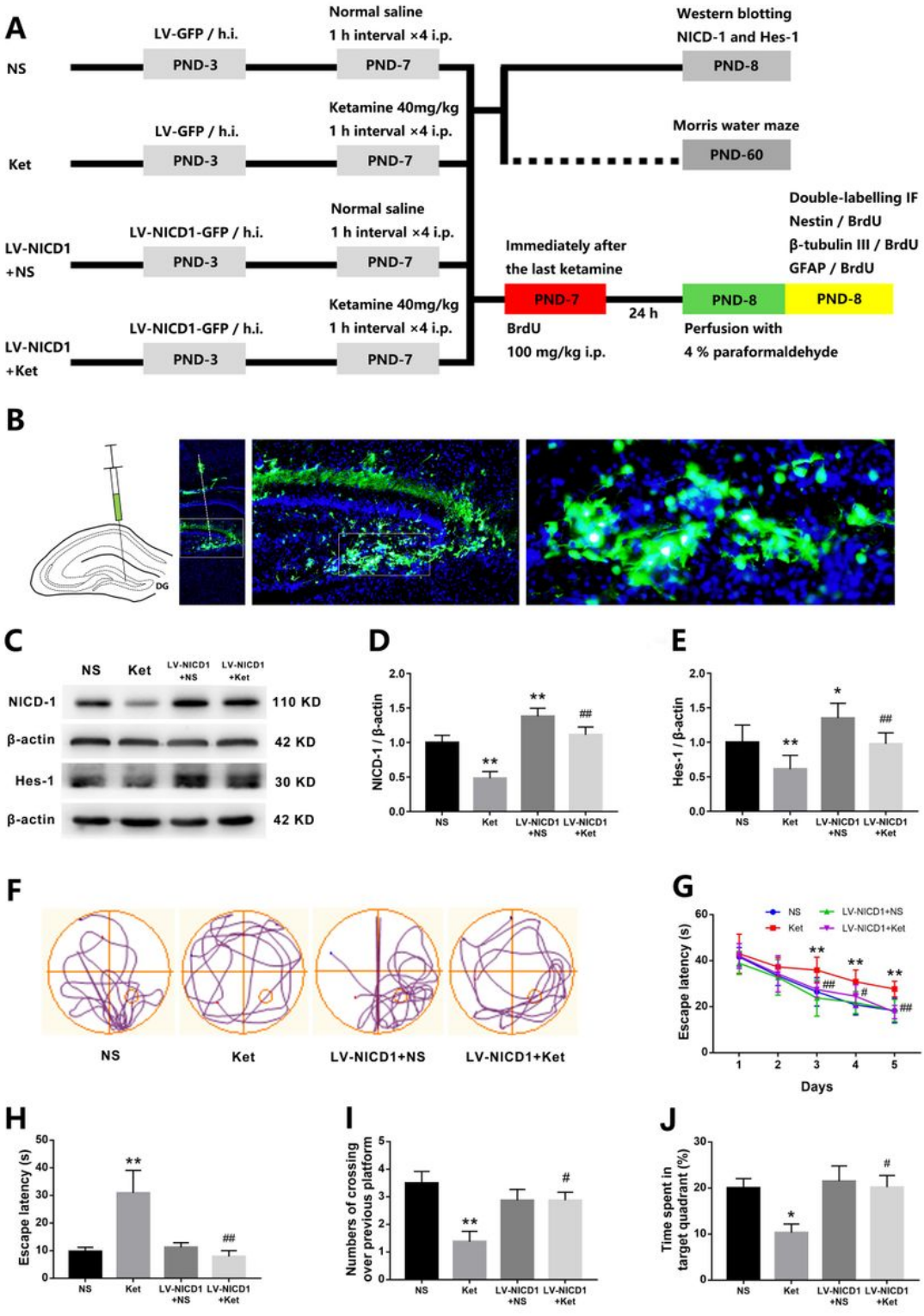


Figure 6

Effect of LV-NICD1 pretreatment on the expression of NICD1 and Hes1 in the hippocampal DG of rats exposed to ketamine at PND 7 and the spatial learning and memory in adulthood. (A) Experimental protocol. LV-GFP or LV-NICD1 was injected into the hippocampal DG 4 days before NS/ketamine treatment. The rats in four groups received a single intraperitoneal injection of 100 mg/kg BrdU immediately after the end of each treatment. Western blot and immunofluorescence staining were performed 24 h after each treatment. Morris water maze (MWM) test was performed at two months old. (B) The needle path of micro-injection in the hippocampal DG and typical immunofluorescence image of lentivirus transfection. Overexpression by LV-NICD1 significantly prevented the decrease of NICD1 (C and D) and Hes1 (C and E) in hippocampal DG induced by neonatal ketamine anesthesia by Western blot analysis. The impaired spatial learning and memory in adulthood induced by neonatal exposures to ketamine was markedly improved by overexpression of NICD1 in MWM test. (F) Representative swimming paths of space exploration. (G and H) Escape latency of rats. (I) The number of crossing over the previous platform site within 60 s. (J) The percentage of time spent in target quadrant. Data are presented as mean ± SD (n=8). h.i., hippocampal injection; i.p., intraperitoneally; IF, immunofluorescence. *P < 0.05, **P < 0.01, vs NS group; #P < 0.05, ##P < 0.01, vs Ket group.

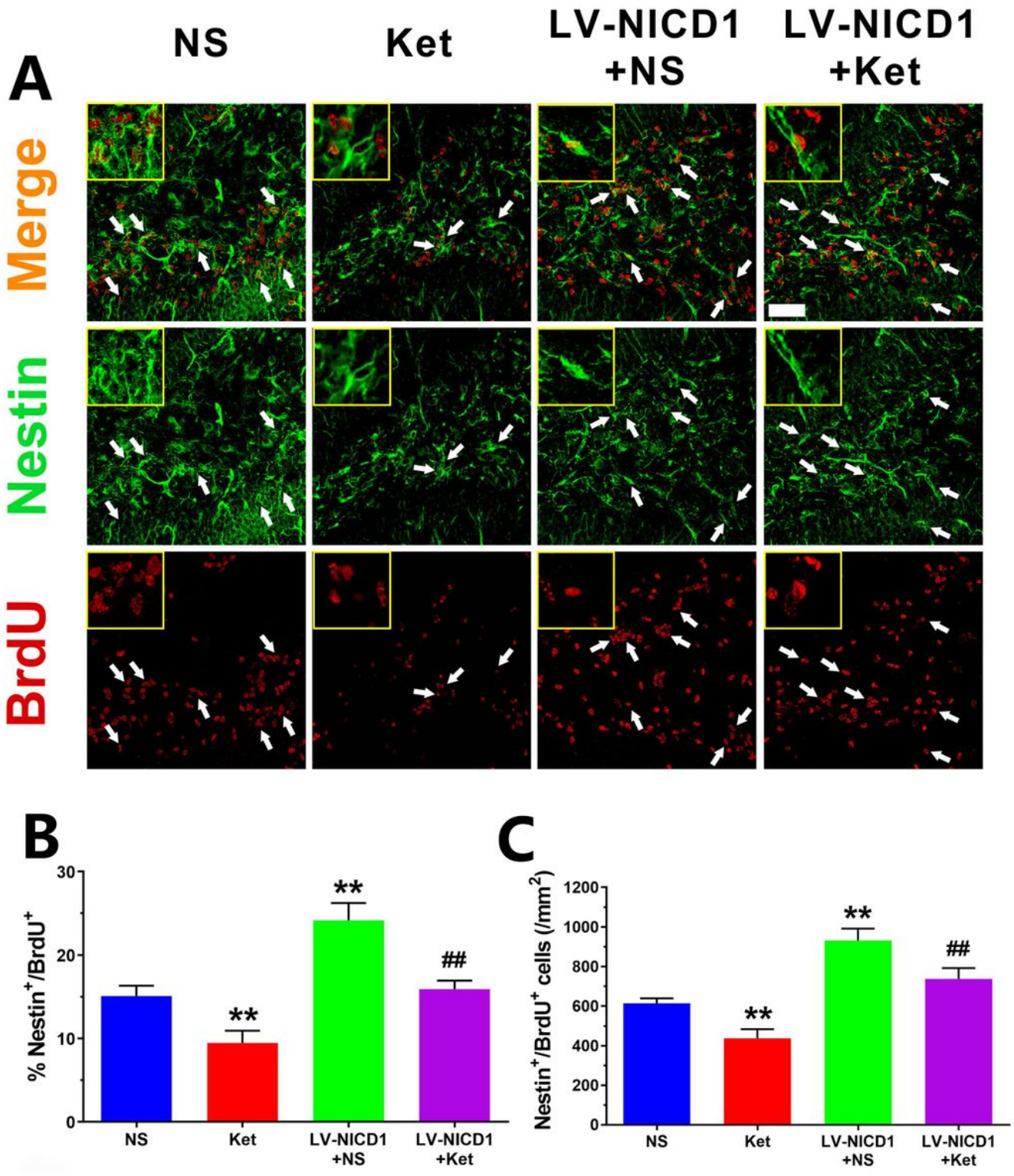


Figure 7

Effect of LV-NICD1 pretreatment on the NSC proliferation in the hippocampal DG of neonatal rats exposed to ketamine. (A) The proliferation of NSCs were labeled with primary antibodies against Nestin (green) and BrdU (red). The immunoreactive cells were visualized using the laser scanning confocal microscope (magnification: × 400; the scale bar is 50 μm). The white arrows pointed to Nestin/BrdU double-labeled cells. (B) The ratio of Nestin+/BrdU+ cells to Nestin+ cells in the DG. (C) The density of

Nestin+/BrdU+ cells in the DG. Data are presented as the mean ± SD (n=5). **P < 0.01, vs NS group; ##P < 0.01, vs Ket group.

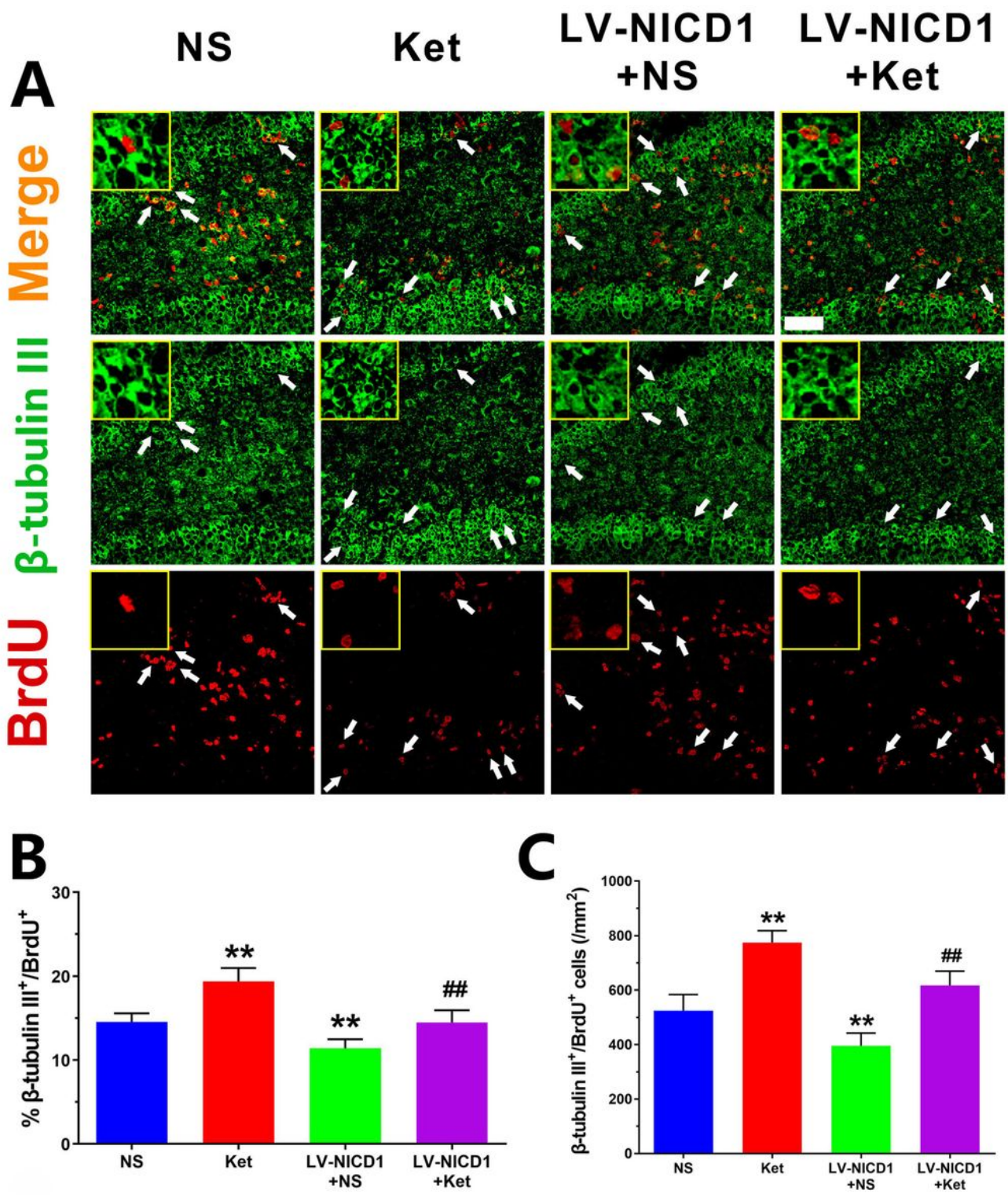


Figure 8

Effect of LV-NICD1 pretreatment on the neuronal differentiation in the hippocampal DG of neonatal rats exposed to ketamine. (A) The neuronal differentiation of NSCs was labeled with primary antibodies against β-tubulin III (green) and BrdU (red). The immunoreactive cells were visualized using the laser

scanning confocal microscope (magnification: × 400; the scale bar is 50 μm). The white arrows pointed to β-tubulin III/BrdU double-labeled cells. (B) The ratio of β-tubulin III+/BrdU+ cells to BrdU+ cells in the DG. (C) The density of β-tubulin III+/BrdU+ cells in the DG. Data are presented as the mean ± SD (n=5). **P < 0.01, vs NS group; ##P < 0.01, vs Ket group.

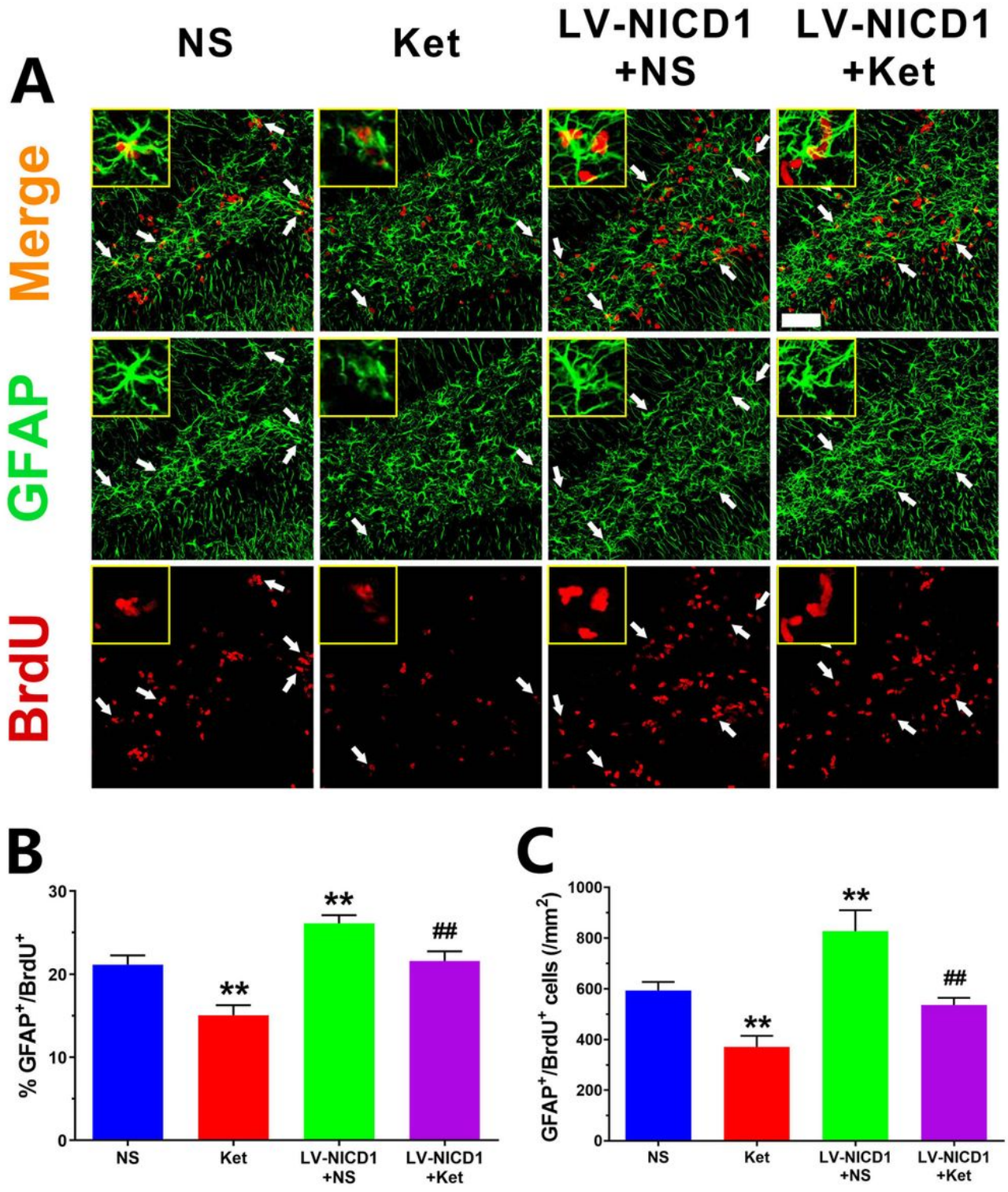


Figure 9

Effect of LV-NICD1 pretreatment on the astrocytic differentiation in the hippocampal DG of neonatal rats exposed to ketamine. (A) The astrocytic differentiation of NSCs was labeled with primary antibodies against GFAP (green) and BrdU (red). The immunoreactive cells were visualized using the laser scanning confocal microscope (magnification: × 400; the scale bar is 50 μm). The white arrows pointed to GFAP/BrdU double-labeled cells. (B) The ratio of GFAP+/BrdU+ cells to BrdU+ cells in the DG. (C) The density of GFAP+/BrdU+ cells in the DG. Data are presented as the mean ± SD (n=5). **P < 0.01, vs NS group; ##P < 0.01, vs Ket group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- data.pdf