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Effect of midazolam on the proliferation of neural stem cells isolated from rat hippocampus☆

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Abstract

In many recent studies, the inhibitory transmitter gamma-aminobutyric acid has been shown to modulate the proliferation, differentiation and survival of neural stem cells. Most general anesthetics are partial or allosteric gamma-aminobutyric acid A receptor agonists, suggesting that general anesthetics could alter the behavior of neural stem cells. The neuroprotective efficacy of general anesthetics has been recognized for decades, but their effects on the proliferation of neural stem cells have received little attention. This study investigated the potential effect of midazolam, an extensively used general anesthetic and allosteric gamma-aminobutyric acid A receptor agonist, on the proliferation of neural stem cells *in vitro* and preliminarily explored the underlying mechanism. The proliferation of neural stem cells was tested using both Cell Counting Kit 8 and bromodeoxyuridine incorporation experiments. Cell distribution analysis was performed to describe changes in the cell cycle distribution in response to midazolam. Calcium imaging was employed to explore the molecular signaling pathways activated by midazolam. Midazolam (30–90 μM) decreased the proliferation of neural stem cells *in vitro*. Pretreatment with the gamma-aminobutyric acid A receptor antagonist bicuculline or Na-K-2Cl cotransport inhibitor furosemide partially rescued this inhibition. In addition, midazolam triggered a calcium influx into neural stem cells. The suppressive effect of midazolam on the proliferation of neural stem cells can be partly attributed to the activation of gamma-aminobutyric acid A receptor. The calcium influx triggered by midazolam may be a trigger factor leading to further downstream events.

Key Words

general anesthetics; gamma-aminobutyric acid A receptor; midazolam; hippocampus; neural stem cells; proliferation; nerve injury; neural regeneration

Research Highlights

(1) Midazolam can inhibit the proliferation of neural stem cells *in vitro*. (2) Pretreatment with the gamma-aminobutyric acid A receptor antagonist bicuculline or Na-K-2Cl cotransport inhibitor furosemide can partially rescue this inhibition. (3) Midazolam can trigger a calcium influx in neural stem cells *in vitro*. (4) The inhibitory effect of midazolam on the proliferation of neural stem cells can be partly attributed to the activation of gamma-aminobutyric acid A receptors. The calcium influx caused by midazolam may be a key trigger resulting in downstream signal transduction cascades.

Abbreviations

NKCC1: Na-K-2Cl cotransporter; KCC2: K-Cl cotransporter; BrdU: bromodeoxyuridine; CCK8: Cell Counting Kit 8

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INTRODUCTION

Neural stem cells retain the potential for both proliferation (self-renewal) and differentiation. Neurogenesis from neural stem cells is an integral process that consists of proliferation and differentiation, survival of the newborn neuron and the integration into the existing neural network. Recent studies have demonstrated that neurogenesis is maintained throughout life in the subventricular zone and in the subgranular zone of the dentate gyrus, a structure critical for learning and cognition[1]. Neurotransmitter-mediated neurogenesis appears to provide a mechanism through which hippocampal network activity recruits a precise number of new neurons for the hippocampal circuitry[2-3]. Gamma-aminobutyric acid can act as a signaling molecule to influence both the proliferation and differentiation of neural and embryonic stem cells[4-6]. Midazolam is an essential anesthetic used in clinical practice that acts as a positive allosteric modulator of gamma-aminobutyric acid A receptor receptors[7]. Previous studies have demonstrated the neuroprotective efficacy of general anesthetics on the central nervous system[8-9]. However, the effects of general anesthetics on neurogenesis are unclear. In view of the role of gamma-aminobutyric acid in neural stem cell function, we postulated that midazolam might suppress the proliferation of neural stem cells.

The particular mechanism by which gamma-aminobutyric acid-mediated excitation controls multiple steps of hippocampal neurogenesis is unclear. Calcium influx may be a key trigger that connects gamma-aminobutyric acid signals to the intrinsic signaling cascade, such as NeuroD and phosphorylated cAMP response element binding protein, which are crucial to neurogenesis[5, 10]. In this study, we investigated the effect, and the underlying mechanisms, of midazolam on the proliferation of neural stem cells.

RESULTS

Identification of neural stem cells

Immunocytochemistry revealed that the majority of cells (> 70%) within neurospheres expressed nestin, a marker of neural stem cells that is absent in differentiated cells of the central nervous system (Figure 1).

Midazolam inhibited neural stem cell proliferation

Cell Counting Kit 8 (CCK8) studies indicated that midazolam significantly decreased the proliferation of neural stem cells at a concentration of 30 μM and 90 μM (P < 0.05). Bromodeoxyuridine (BrdU) incorporation experiments indicated that midazolam significantly decreased the proliferation of neural stem cells at a concentration of 90 μM (P < 0.05).

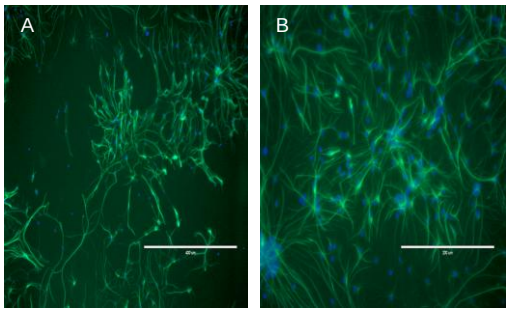

Figure 1 Fluorescence microscopy of nestin immunocytochemistry in cultured neural stem cells (scale bar is 400 μm in A and 200 μm in B).

The majority of cells within neurospheres were immunopositive for the neural stem cell-specific cytoskeletal protein nestin (green), whereas only a minority of neurospheres were negative for nestin. The nuclei were also counterstained with 4,6-diamino-2-phenylindole (blue).

Repeated administration of 30 μM midazolam significantly decreased the proliferation of neural stem cells, as revealed by the CCK8 proliferation assay. Pretreatment with the gamma-aminobutyric acid A receptor receptor antagonist bicuculline (10 μM; P < 0.05) or the Na-K-2Cl cotransport (NKCC1) inhibitor furosemide (300 μM; P < 0.05) partly rescued the depressed proliferation (Figure 2).

To confirm the repressive effect of midazolam on proliferated cells, we employed the BrdU pulse incorporation experiment. Pretreatment with midazolam did indeed significantly decrease the proportion of cells that incorporated BrdU, whereas pretreatment with 10 μM bicuculline (P < 0.05) or 300 μM furosemide (P < 0.05) reversed this effect (Figure 3, supplementary Figure 1 online).

Effect of midazolam on cell cycle distribution of neural stem cells

This experiment was performed to further understand the above results. Midazolam again decreased the proportion of neural stem cells in S phase (cells showing active DNA replication, P < 0.05), whereas pretreatment with 300 μM furosemide (P < 0.05) or 10 μM bicuculline (P < 0.05) completely or partly reversed the effect (Figure 4, and supplementary Figure 2 online).

Midazolam increased intracellular calcium concentration in neural stem cells

To explore the underlying mechanisms mediating the effect of midazolam on neural stem cells, we examined intracellular calcium metabolism using the cal-

cium-sensitive fluorescent dye fluo-3 AM. Midazolam (90 μM) evoked a rapid increase in the intracellular calcium concentration, whereas pretreatment with 10 μM bicuculline almost completely blocked the calcium influx caused by midazolam (Figure 5, supplementary Figure 3 online).

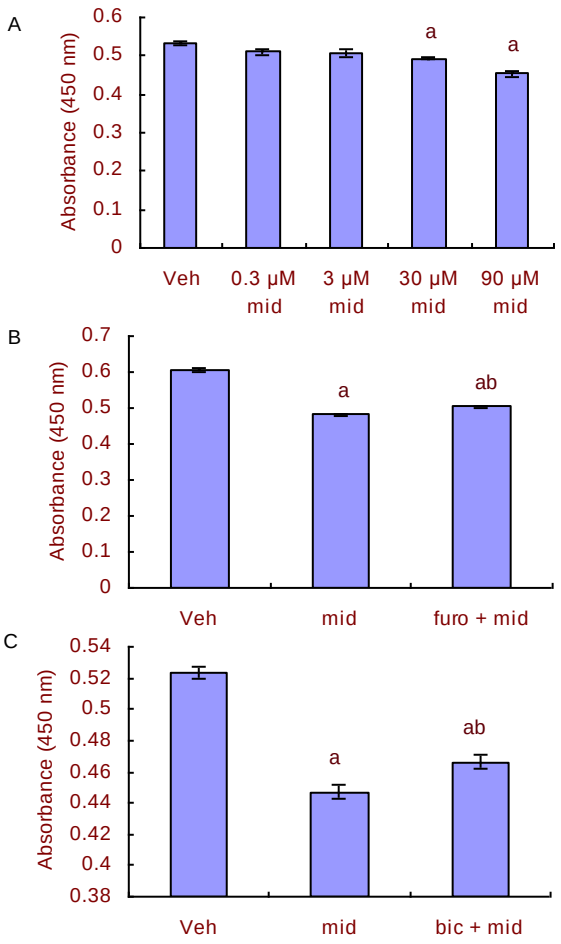

Figure 2 Cell Counting Kit 8 cell proliferation test.

(A) Dose response studies indicated that mid significantly decreased the proliferation of neural stem cells at a concentration of 30 μM and 90 μM.

The repeated administration of 30 μM mid decreased the proliferation of neural stem cells. Pretreatment with 300 μM furo (B) or 10 μM bic (C) partly reversed this repressed proliferation.

Data are expressed as mean ± SEM (n = 5 wells/group in A; n = 8 wells/group in B and C). [a]P < 0.05, *vs*. veh group; [b]P < 0.05, *vs*. mid group using one-way analysis of variance followed by Newman-Keuls multiple comparison test.

mid: Midazolam; veh: vehicle; furo: furosemide; bic: bicuculline.

The presence of gamma-aminobutyric acid in culture media containing neural stem cells

Using high-performance liquid chromatography, our studies identified the presence of gamma-aminobutyric acid in culture medium containing neural stem cells (supplementary Figure 4 online). This is consistent with previous studies[4, 11-12].

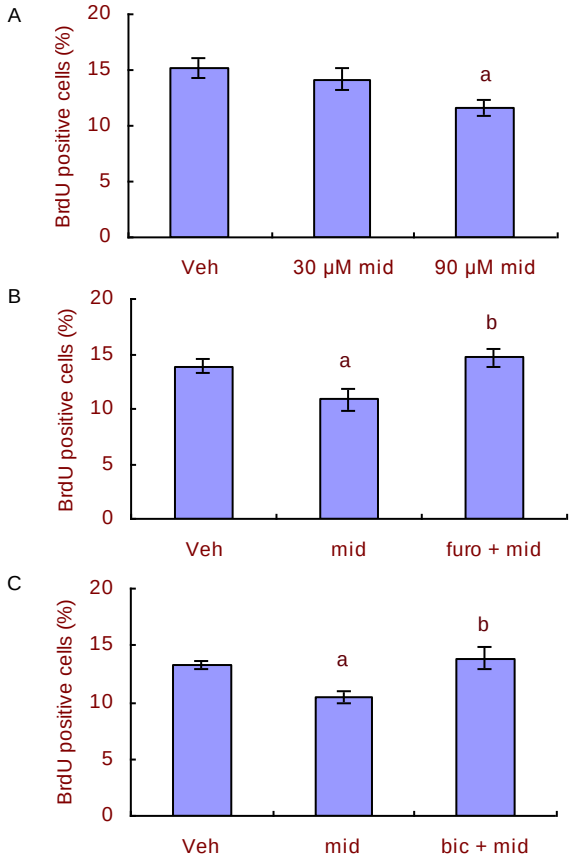

Figure 3 Bromodeoxyuridine (BrdU) incorporation studies.

Rhodamine-conjugated secondary antibody was used to label the cells incorporating BrdU. Mid significantly decreased the percentage of BrdU$^+$ neural stem cells at a concentration of 90 μM (A).

Repeated administration of 30 μM mid decreased the percentage of cells labeled with BrdU, whereas pretreatment with 300 μM furo (B) or 10 μM bic (C) reversed the repressive effect (original images can be obtained in the supplementary Figure 1 online).

Data are expressed as mean ± SEM (n = 6 wells/group). [a]P < 0.05, *vs*. veh group; [b]P < 0.05, *vs*. mid group using one-way analysis of variance followed by Newman-Keuls multiple comparison test.

mid: Midazolam; veh: vehicle; furo: furosemide; bic: bicuculline.

DISCUSSION

The neuroprotective effect of general anesthetics on the mature neural system is well known, but the effect of general anesthetics on neurogenesis has rarely been studied. Recent studies have demonstrated that gamma-aminobutyric acid can act as a signaling molecule to influence both the proliferation and differentiation of neural and embryonic stem cells[4-5].

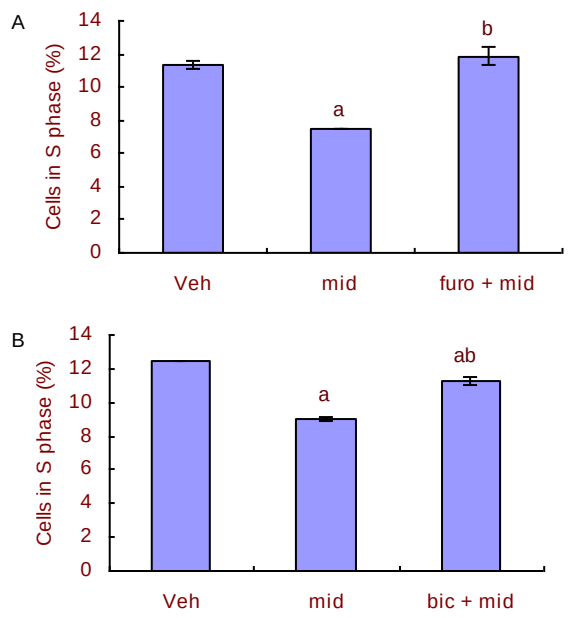

Figure 4 Cell distribution analysis. Mid (90 μM) decreased the percentage of neural stem cells in S phase, whereas pretreatment with 300 μM furo (A) or 10 μM bic (B) partly or completely reversed this repression.

15 000–20 000 events were measured for every sample. Data are expressed as mean ± SEM (n = 3). [a]P < 0.05, *vs*. veh group; [b]P < 0.05, *vs*. mid group using one-way analysis of variance followed by Newman-Keuls multiple comparison test.

mid: Midazolam; veh: vehicle; furo: furosemide; bic: bicuculline.

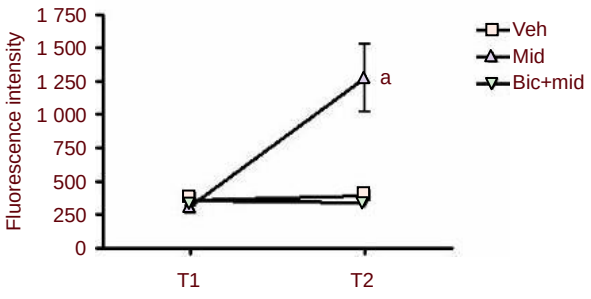

Figure 5 Calcium imaging in neural stem cells. Calcium-dependent fluorescence was elevated immediately upon administration of 90 μM mid and this effect was completely blocked by pretreatment with 10 μM bic. The images were collected at a frequency of 5 seconds per frame.

Data are expressed as mean ± SEM (n = 6). [a]P < 0.05, *vs*. veh group and bic + mid group using two-way analysis of variance. T1: Fluorescence intensity at resting state; T2: fluorescence intensity after administration; mid: midazolam; bic: bicuculline; veh: vehicle.

We therefore postulated that midazolam may suppress the proliferation of neural stem cells. In this study, CCK8 was employed to preliminarily explore the effect of clinically relevant concentrations of midazolam on the proliferation of neural stem cells.

BrdU incorporation experiments and cell cycle distribution analysis were then performed. These classic experiments detected proliferation activity and were used to confirm results and explore the underlying mechanism of midazolam action. The above experiments identified the depressive effect of midazolam on the proliferation of neural stem cells. Another anesthetic, isoflurane, has been reported to have the same effect in young rodents in several *in vivo* studies[13-14]. These studies suggested potential toxicity of general anesthetics to neurogenesis. The adult brain contains localized populations of neural stem cells. Recent studies have demonstrated that neurogenesis from neural stem cells is maintained throughout life in the subventricular zone and in the subgranular zone of the dentate gyrus, a structure critical for learning and cognition[1]. Deficient neurogenesis may influence cognition and mental health, so the effects of general anesthetics on the neurogenesis of neural stem cells warrants further study. Here, we focused on the effect of midazolam on the proliferation of neural stem cells and provided a new direction for exploring the cognitive dysfunction after general anesthesia. Midazolam caused a decrease in the proliferation of neural stem cells that could be partly reversed by the gamma-aminobutyric acid A receptor antagonist bicuculline and the NKCC1 inhibitor furosemide (a common loop diuretic), indicating that the effects of midazolam on proliferation were partly mediated by the activation of gamma-aminobutyric acid A receptor receptors. The discrepancy between the results of CCK8 and BrdU experiments might be attributed to the different index and detection sensitivity. CCK8 and BrdU experiments were applied for measurement of cell proliferation. The CCK8 experiment is based on the detection of dehydrogenase activity in viable cells and intended to detect the overall proliferation activity in the wells. BrdU incorporation experiments are based on immunocytochemistry and intended to detect the percentage of cells incorporating BrdU, which has a positive correlation with proliferation activity. Although the inhibitory effect is relatively minor in proliferation experiments, minor changes in neural stem cell pools will result in dramatic difference in neuronal output[15].

It has been identified in many studies that calcium influx plays a key role in initiating the gamma-aminobutyric acid-induced phenotype[16]. There are two principle transporters that modulate the intracellular chloride concentration of neural stem cells-the NKCC1 and the K-Cl cotransporter (KCC2). NKCC1 imports chloride and increases the intracellular chloride concentration, whereas KCC2 promotes chloride efflux. Compared with the

expression of KCC2, the expression of NKCC1 is relatively higher in neural stem cells, whereas the expression pattern is reversed during differentiation into mature neurons. Sequential expression of the furosemide-sensitive Na-K-2Cl transporter NKCC1 and the K^+-coupled Cl^- transporter KCC2 (a Cl^- exporter) is believed to underlie the shift from depolarizing to hyperpolarizing gamma-aminobutyric acid A receptor-mediated postsynaptic potentials during neuronal maturation[17-19]. This highly expressed NKCC1 enables chloride efflux through gamma-aminobutyric acid A receptor receptors and concomitant depolarization in response to gamma-aminobutyric acidergic input[20]. Calcium influx through voltage-gated calcium channels is induced when the membrane is depolarized above a threshold level, and elevated intracellular calcium triggers a series of downstream signal transduction events, including the phosphorylation of cAMP response element binding protein at ser-133[6, 21]. In our study, bicuculline blocked calcium influx by inhibiting gamma-aminobutyric acid A receptor-mediated depolarization in neural stem cells and bicuculline. Furosemide partly or completely rescued the effect of midazolam on neural stem cells. These results support the hypothesis that midazolam-induced effects are at least partly mediated by the gamma-aminobutyric acid A receptor receptor.

It is interesting that we could measure the gamma-aminobutyric acid A receptor receptor-dependent calcium influx in response to 30 and 90 μM midazolam in the absence of added extracellular gamma-aminobutyric acid. But evidence from many studies, using high-performance liquid chromatography, has shown the presence of gamma-aminobutyric acid and components necessary for the production and release of gamma-aminobutyric acid in neural stem cells[4, 22-23]. These results indicate that midazolam, like other benzodiazepines, can reduce the threshold gamma-aminobutyric acid A receptor concentration for receptor activation.

The relationship between calcium and neurogenesis of neural stem cells has been reviewed[16]. Intracellular calcium initiates multiple downstream signal transduction cascades that are involved in neurogenesis, such as the phosphorylation of cAMP response element binding protein on Ser-133 and the expression of NeuroD. The importance of cAMP response element binding protein in the proliferation and neurogenesis of neural stem cells has been confirmed in many studies[4-5, 24]. Although we identified the depressive effect of midazolam on the proliferation of neural stem cells, these results should be interpreted conservatively. The process of neurogenesis consists of self-renewal of neural stem cells (proliferation), differentiation, survival of newborn immature neurons and integration into existing neuronal networks. Further studies are required to systemically evaluate the effects of midazolam on neurogenesis of these neural stem cells.

Midazolam is also known to bind intracellular receptors on mitochondria, referred to “peripheral benzodiazepine receptors”[25]. Peripheral benzodiazepine receptors are now known as translocator protein (TSPO; 18 kDa)[26], which was identified as being intimately involved in neurogenesis[27].

In summary, our results revealed the repressive effect of midazolam on the proliferation of neural stem cells in the hippocampus *in vitro*. This repression was potentially rescued by bicuculline or furosemide. Furthermore, a calcium-dependent mechanism may be involved in gamma-aminobutyric acid A receptor receptor-mediated neurogenesis.

MATERIALS AND METHODS

Design

This is an *in vitro* cytological comparative experiment.

Time and setting

These experiments were performed in Beijing Institute of Pharmacology and Toxicology, China, from March 2009 to April 2011.

Materials

Newborn Sprague-Dawley rats were purchased from Beijing Institute of Pharmacology and Toxicology (license No. SCXK (JUN) 2009-004), and were used within 12 hours of birth. All efforts were made to minimize the number of animals used and to alleviate suffering. All procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[28].

Methods

Culture of neural stem cells

Rat pups were disinfected with 75% ethanol and decapitated. The isolated hippocampi were diced and triturated using fire-polished Pasteur pipettes. The harvested cells were re-suspended and cultured at 2×10^5 cells/mL in Dulbecco’s Modified Eagle’s Medium (DMEM/F12; Gibco, Carlsbad, CA, USA) supplemented with epidermal growth factor (20 ng/mL; Peprotech, Rocky Hill, NJ, USA), basic fibroblast growth factor (20 ng/mL, Peprotech), and 2% B27 (Gibco). The cells were passaged every 5–7 days, and half volumes of media were replaced every 2–3 days. This method of neural stem cell culture is generally accepted and resulted in high-purity neural stem

cells[12, 29].

Identification of neural stem cells

For immunocytochemistry, neurospheres were plated onto poly-l-ornithine-coated (Sigma-Aldrich, St. Louis, MO, USA) dishes and cultured for 48 hours. The concise steps for immunocytochemistry included fixation with 4% paraformaldehyde and antigen blocking with PBS solution containing 0.2% Triton X-100 and 1% bovine serum albumin. Primary antibody binding (mouse monoclone to nestin: 1:200, Abcam, Cambridge, UK; 4°C, overnight) then occurred followed by incubation with fluoresceine isothiocyanate-conjugated secondary antibody at room temperature for 1 hour (anti-mouse, 1:200; Jackson, West Grove, PA, USA). Cells were counterstained with 2-(4-amidinophenyl)- 6-indolecarbamidine dihydrochloride (Sigma-Aldrich) to visualize nuclei.

Intervention

The isolated cells grew and accumulated into neurospheres containing tens to hundreds of cells after 1–2 weeks in culture. To uniformly distribute the same concentration of cells in every well, the neurospheres were centrifuged and dissociated into single cells with Accutase® (Sigma-Aldrich) and were plated in 96-well plates at 3×10^5 cells/mL. Firstly, a dose response study (0.3, 3, 30, 90 μM, 5 wells/group) with midazolam was performed to preliminarily test the effect of midazolam. Midazolam was a gift from Jiangsu Nhwa Pharmaceutical Co., Ltd., China (purity > 99%). The pH of both normal saline and midazolam solution were calibrated to about 3.5 using 1 N HCl and sterilized with 0.22 μm PALL filter. Normal saline and/or dimethyl sulfoxide (vehicle group), 30 μM midazolam hydrochloride, 10 μM bicuculline (Sigma-Aldrich) + 30 μM midazolam hydrochloride, or 300 μM furosemide (Sigma-Aldrich) + 30 μM midazolam hydrochloride were then administered to the corresponding wells. The concentrations of midazolam, bicuculline and furosemide used in our study were determined according to previous research and our preliminary experiments[9, 19, 30-31]. The concentration of midazolam that was mainly tested in our research (90 μM) was considered as a clinically relevant concentration as described previously[9, 19, 30-32].

CCK8 cell proliferation test

To test the effect of midazolam on the proliferation of neural stem cells, we used the CCK8 (Dojindo, Mashikimachi, Kamimashiki-gun Kumamoto, Japan) following control and midazolam treatments. Calibration wells were set to subtract baseline absorbance. We added 100 μL of medium containing detached cells to each well (8 wells/group) and 12–24 hours later, midazolam or vehicle was added three times to the corresponding wells at 2-hour intervals to increase the concentration to the desired final value (90 μM). Bicuculline or furosemide was added to each corresponding well 1 hour before the administration of midazolam. After 24 to 48 hours of treatment, 10 μL of CCK8 reagent was added to each well (except for the calibration wells) and 4–5 hours later, the absorbance was measured (at 450 nm) by a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

BrdU pulse incorporation and immunocytochemistry

Neurospheres were detached by Accutase® and cultured in 35 mm culture plates coated with 0.01% poly-L-ornithine solution. The cells were treated as above, according to the treatment groups. Twenty-four to forty-eight hours later, 2-hour BrdU (Sigma-Aldrich, 10 μM) incorporation was followed by fixation in 4% paraformaldehyde. Immunocytochemistry mainly consisted of five steps: (1) exposure of the DNA to 2 N HCl; (2) blocking the sample to prevent non-specific binding with PBS solution containing 0.2% Triton X-100 and 1% bovine serum albumin; (3) BrdU labeling with primary antibody (rat monoclonal to BrdU, 1:200, Abcam) at 4°C overnight; (4) staining the samples with a secondary antibody (rhodamine-labelled anti-rat IgG, 1:200 Jackson); and (5) counterstaining with 4,6-diamino-2-phenyl indole. The images (anti-nestin and BrdU) were collected using an EVOS fluorescence microscope (Advanced Microscopy Group, Bothell, WA, USA). The percentage of $BrdU^+$ cells was recorded in four randomized images (objective lens 40 ×) for each sample. The sum of $BrdU^+$/sum of 4,6-diamino-2-phenyl indole was considered as the percentage of $BrdU^+$ cells in each sample. The immunocytochemistry counting was performed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Bethesda, MD, USA), and the experimenters who performed the cell counting were blinded to the treatment history.

Cell cycle distribution analysis

Neurospheres were treated as above. For cell cycle distribution analysis, 24 to 48 hours after treatment, neurospheres were dispersed and fixed with 70% alcohol at 4°C overnight. Fixed cells were stained with propidium iodide (Sigma-Aldrich) solution and sent to cell cycle distribution analysis using FACScaliber (BD Corporation, San Jose, CA, USA). ModfitLT V3.0 software (BD Corporation) was used to analyze cell cycle distribution. About 15 000 events were recorded for every sample.

Calcium imaging

To measure possible calcium influx in response to midazolam, we used the calcium-sensitive dye fluo-3 AM[33]. The detached cells were plated in 35 mm confocal

dishes (Nest, Wuxi, Jiangsu, China), coated with 0.01% poly-l-ornithine for 6–8 hours followed by loading with 2.5–5.0 μM fluo-3 AM (Dojindo) for 45 minutes at 37°C in the dark. We then washed out the extracellular fluo-3 AM twice with 2 mL D-Hanks solution and replaced it with artificial cerebrospinal fluid (CSF; 124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose). Images of cells were then collected under a laser confocal microscope (Carl Zeiss LSM510 beta, Oberkochen, Germany, excitation wavelength = 488 nm). Neural stem cells were perfused with artificial CSF, artificial CSF containing midazolam hydrochloride (30 and 90 μM) or artificial CSF containing bicuculline (10 μM) 15 minutes prior to midazolam. Fluorescence intensity in response to treatments was recorded. High potassium saline (30 mM) was also administered as a positive control.

High-performance liquid chromatography

High-performance liquid chromatography was performed to identify the presence of gamma-aminobutyric acid in the culture media. The media from neural stem cells was collected and samples (20 μL) were injected by an autosampler into an ODS-C18 (10 μm, 150 mm × 4.6 mm). The mobile phase for gamma-aminobutyric acid separation consisted of 10% acetonitrile, 90% water and 0.1% phosphoric acid. This experiment was performed on Agilent 1100 (Agilent Technologies, Inc., Santa Clara, CA, USA). At least three independent experiments were undertaken for each study.

Statistical analysis

All statistics were calculated using Graphpad Prism v4.0 software (La Jolla, CA, USA) on a personal computer. The experiments among three groups were compared using one-way analysis of variance followed by Newman-Keuls multiple comparison test. Fluorescence intensity of calcium imaging was analyzed using two-way analysis of variance (treatments and time as two factors). Data are represented as mean ± SEM. Differences between groups were considered statistically significant at $P < 0.05$.

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Author contributions: Sanjun Zhao and Yajing Zhu designed and conducted the study, as well as analyzed the data, and wrote the manuscript. Rui Xue conducted the study and analyzed the data. Yunfeng Li designed the study. Hui Lu analyzed the data. Weidong Mi designed the study and wrote the manuscript. All authors have seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

Conflicts of interest: None declared.

Ethical approval: Ethical approval for this study (protocol number: 2009-05) was provided by the Institutional Animal Care and Use Committee of the General Hospital of Chinese PLA, Beijing, China.

Supplementary information: Supplementary data associated with this article can be found in the online version by visiting www.nrronline.org.

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