Alpha-7 nicotinic receptor signaling pathway participates in the neurogenesis induced by ChAT-positive neurons in the subventricular zone

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Abstract

Choline acetyltransferase-positive (ChAT⁺) neurons within the subventricular zone (SVZ) have been shown to promote neurogenesis after stroke in mice by secreting acetylcholine (ACh); however, the mechanisms remain unclear. Receptors known to bind ACh include the nicotinic ACh receptors (nAChRs), which are present in the SVZ and have been shown to be important for cell proliferation, differentiation, and survival. In this study, we investigated the neurogenic role of the $\alpha 7$ nAChR in a mouse model of middle cerebral artery occlusion (MCAO) by using $\alpha 7$ nAChR inhibitor methyllycaconitine. Mice subjected to MCAO exhibited elevated expression of cytomembrane and nuclear fibroblast growth factor receptor 1 (FGFR1), as well as increased expression of PI3K, pAkt, doublecortin (DCX), polysialylated neuronal cell adhesion molecule (PSA-NCAM), and mammalian achaete-scute homolog 1 (Mash1). MCAO mice also had more GFAP/BrdU-positive cells and DCX-positive cells in the SVZ than did the sham-operated group. Methyllycaconitine treatment increased cytomembrane FGFR1 expression and GFAP/BrdU-positive cells, upregulated the levels of PI3K and pAkt, decreased nuclear FGFR1 expression, decreased the number of DCX-positive cells, and reduced the levels of DCX, PSA-NCAM, and Mash1 in the SVZ of MCAO mice compared with levels in vehicle-treated MCAO mice. MCAO mice treated with $\alpha 7$ nAChR agonist PNU-282987 exhibited the opposite effects. Our data show

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Conflict of interest

The authors assert no conflict of interest.

that $\alpha 7$ nAChR may decrease the proliferation of neural stem cells and promote differentiation of existing neural stem cells after stroke. These results identify a new mechanism of SVZ ChAT⁺ neuron-induced neurogenesis.

Keywords

Neurogenesis; subventricular zone; $\alpha 7$ nicotinic acetylcholine receptor; ChAT-positive neuron

Introduction

Neurogenesis promotes brain recovery after ischemic stroke [1–5]. After stroke, the neural stem/progenitor cells that express glial fibrillary acidic protein (GFAP) and nestin within the subventricular zone (SVZ) gradually differentiate into transit-amplifying cells that express mammalian achaete-scute homolog 1 (Mash1) and neuroblasts that express doublecortin (DCX) [6–8]. The newly generated neurons can migrate to the infarct area and provide neuroprotection by replacing dead neurons, integrating into the neuronal circuits, or secreting neuroprotective cytokines [9–12].

The cholinergic system plays a vital role during neurogenesis under both physiologic and pathologic conditions. Studies have shown that acetylcholine (ACh) is necessary at the onset of neurogenesis and could facilitate neuronal differentiation and integration into the existing neural network [13]. Greater numbers of cholinergic neurons and enhanced neurogenesis have been reported in neurodegenerative conditions such as Alzheimer's disease [14]. However, such studies of the cholinergic system and neurogenesis have focused mainly on hippocampus.

Recently, a group of choline acetyltransferase-positive (ChAT⁺) neurons identified in the rodent SVZ under normal conditions has revealed new insight into SVZ neurogenesis [15]. These SVZ ChAT⁺ neurons were shown to secrete ACh and induce neurogenesis under physiologic conditions, possibly by activating the fibroblast growth factor receptor (FGFR) signaling pathway. Our previous study also indicated that SVZ ChAT⁺ neurons promote neurogenesis after ischemic stroke in mice, in part via the muscarinic AChR (mAChR) signaling pathway [16]. AChRs comprise mAChRs, which are G-protein-coupled receptors, and nicotinic AChRs (nAChRs), which are ligand-gated ion channels. It has been shown that nicotinic $\alpha 7$ AChRs ($\alpha 7$ nAChRs) are present in the SVZ and are important for cell proliferation, differentiation, and survival [17, 18]. Additionally, a previous study found that $\alpha 7$ nAChR agonists might influence the distribution of FGFR1 under normal conditions [19]. To explore the mechanism of SVZ ChAT⁺ neuron-induced neurogenesis after stroke, we investigated whether $\alpha 7$ nAChR can promote neurogenesis via the $\alpha 7$ nAChR-FGFR1 pathway in a mouse model of middle cerebral artery occlusion (MCAO).

Materials and Methods

Animals and Ethics Statement

Male C57BL/6 mice (11–12 weeks old, 25–30 g) were purchased from the Animal Experimental Center of Zhengzhou University. They were housed in plastic cages (6 per

cage) with free access to food and water and were maintained under temperature-, light-, and humidity-controlled conditions. All animal procedures were conducted in accordance with the Animal Care and Use Committee of Zhengzhou University. All efforts were made to minimize the number of animals used and their suffering.

Transient MCAO Model

We induced MCAO as previously described with slight modifications [20–22]. Briefly, we anesthetized mice with an intraperitoneal (i.p.) injection of 4% chloral hydrate (0.007 mL/g) and made a midline incision in the ventral side of the neck to expose the carotid arteries[23]. We carefully separated the arteries from their sheaths and adjacent vagus nerves. We made a small incision in the right common carotid artery and inserted a 6.0 nylon suture monofilament with silicone-coated tip from the right internal carotid artery to block the origin of the middle cerebral artery. The monofilament was left in place for 30 min until reperfusion. Successful MCAO was defined as more than 80% decrease in cerebral blood flow and was confirmed by laser-Doppler flowmetry (Moor Instruments, Devon, UK) [24, 25]. Sham-operated mice were subjected to the same surgical procedure except that the filament was advanced to the origin of the middle cerebral artery and then immediately withdrawn.

Treatment and Groups

The $\alpha 7$ nAChR antagonist methyllycaconitine (MLA) and agonist PNU-282987 (PNU) were dissolved in 1% DMSO in saline. Mice were randomly divided into the following six treatment groups [26]: sham-operated mice treated with 1% DMSO in saline (Sham group, $n=54$), MCAO mice treated with 1% DMSO in saline (MCAO+vehicle group, $n=60$), sham-operated mice treated with 0.1 nmol MLA (Sham+MLA group, $n=54$), MCAO mice treated with 0.1 nmol MLA (MCAO+MLA group, $n=60$), sham-operated mice treated with 0.8 nmol PNU (Sham+PNU group, $n=54$), and MCAO mice treated with 0.8 nmol PNU (MCAO +PNU group, $n=60$). Vehicle and drugs were administered via lateral ventricle (i.c.v.) injection immediately after the surgery and then daily for the next 6 days [27, 28]. All mice also received intraperitoneal (i.p.) injections of 5-bromo-2'-deoxyuridine (BrdU; 50 mg/kg, Sigma-Aldrich, St Louis, MO, USA) once daily for 7 days beginning 24 h after the surgery [16].

Drug administration via lateral ventricle injection

Briefly, mice were deeply anesthetized with an i.p. injection of 5% chloral hydrate (0.0075 mL/g) and then placed in a stereotaxic apparatus for i.c.v. injection. The skull was drilled slowly. A stainless steel cannula (0.3 mm outer diameter) was inserted into the right lateral ventricle (0.3 mm posterior, 1.1 mm lateral, 2.5 mm ventral to the bregma) for drug injection [29]. Vehicle and drugs were administered through the cannula by a micropipettor (RWD, Shenzhen, China). To ensure that drugs reached the lateral ventricle, we injected 5 μ l of ink into two mice and sacrificed them after 30 min [28, 30]. The coronal section showed that the third ventricle, fourth ventricle, and cerebral aqueduct were filled with ink. Therefore, the location of the cannula for i.c.v. injection was appropriate.

Western Blot Analysis

Ten mice from each group were sacrificed for Western blot analysis on day 7 after MCAO as described previously [31–33]. Briefly, mice were deeply anesthetized with an i.p. injection of 4% chloral hydrate. Then the brain was carefully dissected out, and the right SVZ was dissected from the frontal slice extending between the crossing of the anterior commissure and the rostral opening of the third ventricle as previously described [34–36]. We used the Membrane and Nuclear Protein Extraction Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. For the membrane protein extraction, we digested the tissue with solution A and PMSF for 15 min and centrifuged it at 700g for 10 min to remove the nuclei and unbroken cells [37, 38]. We centrifuged supernatant again at 14,000g for 30 min to obtain cytosolic proteins. The remaining precipitate was digested by solution B and centrifuged at 14000g for 5 min. The supernatant contained the membrane protein. For the nuclear protein extraction, we digested the tissue with solutions A, B, and PMSF at 4°C for 15 min and centrifuged it at 1,500g for 5 min [39, 40]. The supernatant contained partial cytosolic proteins. The pellet was digested again and centrifuged to obtain the rest of the cytosolic proteins. The remaining pellet was digested by nuclear protein extraction reagent and centrifuged at 16,000g for 10 min. The supernatant contained nuclear protein. Both membranous and nuclear fractions of the SVZ tissue were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to separate the proteins, which were then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA)[41]. Membranes were blocked with 5% bovine serum albumin (wt/vol) in Tris-buffered saline-0.1% Tween 20 (TBST) for 1 h at room temperature and incubated with one of the following primary antibodies: anti-FGFR1 (1:1000, Abcam, Cambridge, MA, USA), anti-phosphoinositide 3-kinase (PI3K; 1:1000, Abcam), anti-phospho-Akt (pAkt; 1:1000, Abcam), anti-Mash 1 (1:1000, Abcam), anti-DCX (1 µg/mL, Abcam), rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2000, Sangon Biotech, Shanghai, China), or anti-Histone H3 (1:1000, Cell Signaling Technology, Danvers, MA, USA) [42, 43]. After four washes in TBST, membranes were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (1:2000, Sangon Biotech) for 2 h at room temperature. Protein bands were visualized by enhanced chemiluminescence ECL kit (CWBIO, Beijing, China), and target proteins were normalized against GAPDH and Histone H3. An investigator blinded to the animal group quantified the optical density of the protein bands using Gel Analysis V 2.02 software (Clinx Science Instruments, Shanghai, China).

Immunofluorescence Analysis

Twelve mice from each group were sacrificed for immunofluorescence analysis on day 7 after MCAO as described previously [26, 44]. After being deeply anesthetized, all mice were perfused transcardially with saline and 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS). Then brains were dissected out, post-fixed in 4% paraformaldehyde, dehydrated in 30% sucrose/0.01 M PBS, and finally serially cut into 20-µm-thick floating sections every 480 µm by cryoultramicrotomy (CM1100, Leica Biosystems, Germany). Tissue sections were stored in antifreeze buffer in 24-well plates at –20°C for later use. Seven or eight serial brain sections of each mouse were incubated in PBST (0.1% Triton X-100 in 0.01 M PBS) for 20 min, blocked in 1% bovine serum albumin solution for 30 min, and incubated at room temperature for 2 h or 4°C overnight with anti-FGFR1 (5 µg/mL,

Abcam), anti-DCX antibody (1:50, Santa Cruz Biotech, Dallas, TX, USA), anti-BrdU antibody (1:250, Abcam), or anti-GFAP antibody (1:1000, Abcam). Then sections were washed in 0.01 M PBS three times and incubated with goat anti-rabbit IgG H&L (FITC; 1:1000, Abcam), goat anti-rat IgG H&L (Alexa Fluor 594; 1:200, Abcam), or donkey anti-goat IgG-FITC (1:100, Santa Cruz) for 1 h at room temperature. After three 5-min washes with 0.01 M PBS, the sections were mounted on cover slips with a drop of mounting medium that contained 1.5 µg/mL 4',6-diamidino-2-phenylindole (DAPI; Santa Cruz). An investigator blinded to animal treatment analyzed all stained sections under a fluorescence microscope (ZEISS Scope A1, ZEISS, Germany)

Evaluation of infarct volume

Six mice were sacrificed on day 7 after MCAO for Nissl staining as we have reported previously [16, 45, 46]. Brain sections were obtained as described for immunofluorescence and placed on slides. Sections were stained in Nissl staining solution (Beyotime) for 7 min and washed in ethanol for 2 min according to the manufacturer's protocol. All stained sections were analyzed under a fluorescence microscope (ZEISS Stemi 2000-CS, ZEISS, Germany) by an investigator blinded to treatment group. The infarct volume was determined by the following formula: (contralateral cortex area – ipsilateral non-ischemic cortex area) / contralateral cortex area.

Evaluation of cerebral edema, neurologic function, and body weight

Cerebral edema was reflected by the brain water content, which was calculated as described previously [47, 48]. We sacrificed six mice per group to assess brain water content at day 7. The two hemispheres were separated and weighed to obtain the wet weight. The ipsilateral hemisphere was dried for 24 h and weighed again to obtain the dry weight [49]. Edema = (wet weight – dry weight) / wet weight × 100% [50].

The modified neurological severity score (mNSS) was used to assess the neurologic defects of mice (n=6 per group), as we have published previously [24]. An investigator blinded to group and treatment tested the mice on days 1, 7, 14, 21, and 28 after surgery. The mNSS measures motor function, gait, sensory function, balance, and reflex in a series of tests, each with its own scoring range [51]. Mice are given a score of 0 for normal function and a maximum score for no response or inability to perform the task. In the motor function test, mice were suspended by the tail and graded on flexion of forelimb or hindlimb and deviation of the head from the vertical axis. Gait was observed when mice were placed on a flat plane. Sensory tests included the placing test (visual and tactile test) and proprioceptive test. In the placing test, mice were stimulated on the forelimb with a needle. In the proprioceptive test, the paw was pushed against the table edge to stimulate limb muscles. Forelimb retraction indicated normal function in both tests. For evaluation of balance, mice were assessed for their ability to walk on a slim, round beam for 60 s without grasping the beam or falling from it. The reflex function test measured corneal, pinna, and startle reflexes. Normal function included blinking in response to a touch of the cornea with cotton, head shaking in response to a touch of the internal auditory canal, and a motor response to a loud sound. Points were also given for the presence of seizures, myodystony, or myoclonus. The total score range was 0 to 22, with higher scores indicating greater neurologic deficit.

Body weight change at each time point was calculated as (body weight – pre-surgical body weight)/pre-surgical body weight $\times$ 100%.

Statistical analysis

Statistical analysis was carried out with SPSS version 13.0. All results are expressed as mean $\pm$ SD. We used repeated measures ANOVA followed by the least significant difference (LSD) test to determine changes in mNSS and body weight among different groups. We used a t-test or one-way ANOVA followed by the LSD test to analyze differences in data from immunofluorescence and Western blot analysis. $p < 0.05$ was considered statistically significant.

Results

Blocking $\alpha 7$ nAChR Increases Brain Damage in Mice After MCAO

To evaluate the protective effects of $\alpha 7$ nAChR in brain of ischemic mice, we injected $\alpha 7$ nAChR agonist or antagonist into the lateral ventricle. Quantification indicated that mice from the MCAO+MLA group had larger infarct volumes ($44.9 \pm 7.0\%$) on day 7 after MCAO than did mice from the MCAO+vehicle group ($32.8 \pm 4.7\%$) or MCAO+PNU group ($22.6 \pm 5.8\%$; Fig. 1a, b). Mice that received $\alpha 7$ nAChR antagonist methyllycaconitine had higher brain water content ($90 \pm 6.1\%$) than the vehicle-treated group ($83.5 \pm 6.0\%$) and exhibited more severe neurologic deficits (Fig. 1c, d). Mice administered the $\alpha 7$ nAChR agonist PNU-282987 had less brain water content ($76.9 \pm 5.31\%$) and performed better in neurologic deficit tests than did the vehicle group (Fig. 1c, d). The MCAO+vehicle, MCAO+MLA, and MCAO+PNU groups showed no statistical difference in body weight changes (Fig. 1e).

The $\alpha 7$ nAChR Signaling Pathway Alters Distribution of FGFR1 in the SVZ

The function of FGFR1 differs depending on whether it is expressed in the cytomembrane or nucleus. We found that FGFR1 protein expression was significantly elevated in both locations after MCAO compared with that in the sham group (Fig. 2a–d). Methyllycaconitine increased the cytomembrane FGFR1 and decreased the nuclear FGFR1 in the MCAO and sham mice (Fig. 2a–d). PNU-282987 decreased the cytomembrane FGFR1 and increased the nuclear FGFR1 in both groups (Fig. 2a–d).

Methyllycaconitine Increases Neural Stem Cell Proliferation

Double immunofluorescence staining showed that the percentage of cells positive for GFAP and BrdU, indicative of neural stem cells, was higher in the MCAO+vehicle group ($55.7 \pm 9.0\%$) than in the Sham+vehicle group ($33.6 \pm 4.3\%$; Fig. 3a, b). Blocking $\alpha 7$ nAChR signaling with methyllycaconitine significantly increased the percentage of GFAP- and BrdU-positive cells in MCAO and sham mice (MCAO+MLA, $65.0 \pm 10.2\%$; Sham+MLA, $45 \pm 4.9\%$) compared with that in the MCAO+vehicle and Sham+vehicle groups, respectively (Fig. 3a, b). Both MCAO and sham mice that received PNU-282987 had lower percentages of GFAP- and BrdU-positive cells (MCAO+PNU, $35.3 \pm 5.5\%$; Sham+PNU, $23.4 \pm 4.6\%$). Additionally, on day 7, expression levels of PI3K and pAkt, proteins downstream of the cytomembrane FGFR1 signaling pathway [52], were higher in the SVZ of

methyllycaconitine-treated MCAO mice than in that of the vehicle-treated or PNU-282987-treated MCAO mice (Fig. 3c–e).

The $\alpha 7$ nAChR Signaling Pathway Increases Neurogenesis in Mice After MCAO

To evaluate the effects of $\alpha 7$ nAChR on neurogenesis, we measured changes in DCX, PSA-NCAM, and Mash1 expression in the SVZ. Mice from the MCAO+vehicle group had more DCX-positive cells ($52.3 \pm 4.8\%$) and higher expression of DCX, PSA-NCAM, and Mash1 in the SVZ on day 7 after MCAO, but this stroke-induced neurogenesis was significantly abolished by methyllycaconitine treatment ($40.0 \pm 3.1\%$; Fig. 4a–f). MCAO mice that received $\alpha 7$ nAChR agonist PNU-282987 exhibited more DCX-positive cells ($63.1 \pm 4.5\%$) and higher expression of DCX, PSA-NCAM, and Mash1 than did mice in the MCAO+vehicle group. A similar increase was observed in PNU-282987-treated sham mice ($50.1 \pm 3.5\%$) compared to that in vehicle-treated sham mice ($39.8 \pm 4.4\%$; Fig. 4a–f).

Discussion

In this study, we showed that the $\alpha 7$ nAChR signaling pathway promotes SVZ neurogenesis by altering the distribution of FGFR1 after MCAO in mice. Moreover, our previous study showed that the activity of SVZ ChAT⁺ neurons is relatively increased after stroke [16]. Knock-out of ChAT⁺ neurons blocked SVZ neurogenesis, and adjacent cholinergic neurons had no visible effect on SVZ neurogenesis, indicating that ChAT⁺ neurons are essential for SVZ neurogenesis [15]. Together, these findings partially reveal the mechanisms by which the newly found SVZ ChAT⁺ neurons promote neurogenesis after stroke.

Cytomembrane FGFR1 and nuclear FGFR1 have differing functions in neurogenesis. FGFR1, which spans the cytomembrane, belongs to the receptor tyrosine kinase family. Studies have shown that activating cytomembrane FGFR1 can promote neural stem/progenitor cell proliferation via the PI3K-Akt- β -catenin signaling pathway or the Akt-mammalian target of rapamycin complex 1 signaling pathway, whereas nuclear FGFR1 signaling targets neurogenic and neuronal differentiation genes such as neuroD1, neuroligin, and Stat3 to promote neuronal differentiation and migration [53–56]. In this study, we found that after stroke, both cytomembrane FGFR1 and nuclear FGFR1 were activated, along with increases in expression of PI3K, pAkt, DCX, PSA-NCAM, and Mash1, and proliferation of DCX-positive and GFAP/BrdU-labeled cells in the SVZ.

To determine the effect of the $\alpha 7$ nAChR signaling pathway during stroke-induced neurogenesis, we treated MCAO mice with $\alpha 7$ nAChR antagonist methyllycaconitine or agonist PNU-282987. We found that both compounds significantly altered the distribution of FGFR1 in the SVZ. Mice that received methyllycaconitine had higher levels of cytomembrane FGFR1 and lower levels of nuclear FGFR1 than did the vehicle-treated MCAO mice (Fig. 2a–d). Conversely, PNU-282987-treated mice exhibited lower levels of cytomembrane FGFR1 and higher levels of nuclear FGFR1. In the SVZ, methyllycaconitine also led to significant increases in PI3K/pAkt expression and GFAP/BrdU-positive cells, significant decreases in the expression of DCX, PSA-NCAM, and Mash1, and significant reductions in the number of DCX-positive cells. PNU-282987, in contrast, led to significant decreases in PI3K/pAkt expression and GFAP/BrdU-positive cells, significant increases in

the expression of DCX, PSA-NCAM, and Mash1, and increases in the number of DCX-positive cells. These results indicate that $\alpha 7$ nAChR plays a vital role in the regulation of cytomembrane and nuclear FGFR1 distribution after MCAO. $\alpha 7$ nAChR may promote the transfer of cytomembrane FGFR1 to the nucleus and increase the nuclear-FGFR1 signaling pathway.

As mentioned above, our previous study showed that ChAT⁺ neurons in the SVZ can promote neurogenesis after ischemic stroke in mice. Together with our findings that the neurogenic effects of SVZ ChAT⁺ neurons can be partially blocked with an mAChR inhibitor, the evidence suggests that nAChR may participate in SVZ ChAT⁺ neuron-promoted neurogenesis after stroke. In our study, $\alpha 7$ nAChR activation decreased the cytomembrane FGFR1 signaling pathway and downregulated neural stem cell proliferation. However, we and others have shown that cholinergic activity of the ischemic brain is increased after stroke. Increased $\alpha 7$ nAChR activity should reduce proliferation, but that is contrary to the enhanced proliferation we observed in the MCAO group. One possible explanation may be that activated M1 AChR augments cell proliferation to neutralize the negative effect of $\alpha 7$ nAChR [57]. Therefore, the balance between M1 AChR and $\alpha 7$ AChR deserves more research.

The mechanisms by which $\alpha 7$ nAChR alters FGFR1 expression remain uncertain. Previous studies have suggested possible “crosstalk” between the $\alpha 7$ nAChR and FGFR1 signaling pathways. For example, researchers have found that the src family is essential for the activity of FGFR1 and an important downstream messenger of FGFR1 signaling during neural stem/progenitor cell proliferation. Further, the src family also participates in the neuroprotection of $\alpha 7$ nAChR in ischemic injury [34, 58, 59]. These results show that the src family may be a “cross-messenger” of $\alpha 7$ nAChRs and the FGFR1 signaling pathway.

In conclusion, this study showed that the $\alpha 7$ nAChR signaling pathway plays a vital positive role in stroke-induced SVZ neurogenesis after MCAO in mice. These results are important to reveal the mechanisms by which the newly found SVZ ChAT⁺ neurons promote neurogenesis after stroke.

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Fig. 1. Blocking $\alpha 7$ nAChR increases brain damage in mice after MCAO. (a, b) Nissl staining of brain slices from mice in each group on day 7 after MCAO. Quantification showed that the infarct volume was greater in the MCAO+MLA group and less in the MCAO+PNU group than in the MCAO+vehicle group (* $p < 0.05$ vs. MCAO+vehicle group). $n = 6$ per group. All values are mean $\pm$ SD. (c) Brain water content was greater in MCAO mice treated with methyllycaconitine (MLA) and less in MCAO mice treated with PNU-282987 (PNU) than in MCAO mice treated with vehicle (* $p < 0.05$ vs. the corresponding sham group; † $p < 0.05$ vs.

MCAO+vehicle group). $n = 6$ per group. (d) We used the modified neurological severity score (mNSS) to assess neurologic deficits of mice from each group. MLA-treated MCAO mice showed worse performance in the mNSS tests than did the vehicle-treated MCAO mice ($*p < 0.05$), whereas PNU-treated MCAO mice performed better than the vehicle-treated MCAO mice ($*p < 0.05$). (e) Weight changes in the MCAO+vehicle, MCAO+MLA, and MCAO+PNU mice were not significantly different.

Fig. 2.

The distribution of membrane FGFR1 (mFGFR1) and nuclear FGFR1 (nFGFR1) changed after methyllycaconitine (MLA) and PNU-282987 (PNU) application. (a) Immunofluorescence analysis showed that the expression of both mFGFR1 and nFGFR1 in the SVZ increased on day 7 after MCAO. MCAO+MLA mice exhibited greater mFGFR1 expression than did the MCAO+vehicle group as demonstrated by superimposition with DAPI. MCAO+PNU mice exhibited greater nFGFR1 expression than did the MCAO +vehicle group as demonstrated by superimposition with DAPI. (b) Western blot analysis of

mFGFR1 and nFGFR1 in the SVZ on day 7 after MCAO. (c–d) Quantification showed that mice from the MCAO+MLA group had significantly lower nFGFR1 expression and higher mFGFR1 expression than did the MCAO+vehicle group; mice from the MCAO+PNU group had significantly higher nFGFR1 expression and lower mFGFR1 expression than did the MCAO+vehicle group. Differences were found between Sham+MLA and Sham+PNU groups. $n = 12$ per group. All values are mean $\pm$ SD. * $p < 0.05$ vs. the corresponding sham group; $^{\dagger}p < 0.05$ vs. MCAO+vehicle group; $^{\#}p < 0.05$ vs. Sham+MLA group.

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Fig. 3.

Blocking $\alpha 7$ nAChR promotes neural stem cell proliferation by upregulating the PI3K-pAkt signaling pathway. (a) Double staining of GFAP/BrdU-positive cells in the SVZ on day 7 after MCAO. (b) Quantification showed that in MCAO mice, treatment with methyllycaconitine (MLA) increased the number of GFAP/BrdU-positive cells, whereas treatment with PNU-282987 (PNU) decreased the number of GFAP/BrdU-positive cells ($*p < 0.05$ vs. the corresponding sham group; $†p < 0.05$ vs. MCAO+vehicle group). Similar effects were found in sham mice treated with MLA and PNU-282987 ($§p < 0.05$ vs. Sham

+vehicle group). $n = 12$ per group. (c) Western blot analysis of PI3K and pAkt in the SVZ on day 7 after MCAO. (d–e) Quantification showed that expression of proliferation-related proteins PI3K and pAkt was elevated in the MCAO+MLA group and reduced in the MCAO+PNU group compared with that in the MCAO+vehicle group ($*p < 0.05$ vs. the corresponding sham group; $^{\dagger}p < 0.05$ vs. MCAO+vehicle group). Differences were also found between Sham+MLA and Sham+PNU groups ($^{\#}p < 0.05$ vs. Sham+MLA group). $n = 10$ per group. All values are mean $\pm$ SD.

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Fig. 4. Blocking $\alpha 7$ nAChR inhibits neural stem cell differentiation by downregulating nFGFR1. (a) Immunofluorescence staining of DCX-positive cells in the SVZ on day 7 after MCAO. (b) Quantification showed that MCAO mice treated with methyllycaconitine (MLA) had less DCX-positive cells than did mice in the MCAO+vehicle group; MCAO mice treated with PNU-282987 (PNU) had more DCX-positive cells than did mice in the MCAO+vehicle group (* $p < 0.05$ vs. the corresponding Sham group; † $p < 0.05$ vs. MCAO+vehicle group). Similar effects were found in sham mice treated with MLA and PNU-282987 (§ $p < 0.05$ vs.

Sham+vehicle group). $n = 12$ per group. (c) Western blot analysis of DCX, Mash1, and PSA-NCAM in the SVZ on day 7 after MCAO. (d–f) Quantification showed that expression levels of DCX, Mash1, and PSA-NCAM were decreased in the MCAO+MLA group but elevated in the MCAO+PNU group compared with those in the MCAO+vehicle group ($*p < 0.05$ vs. the corresponding Sham group; $^{\dagger}p < 0.05$ vs. MCAO+vehicle group). Differences were also found between Sham+MLA and Sham+PNU groups ($^{\#}p < 0.05$ vs. Sham+MLA group). $n = 10$ per group. All values are mean $\pm$ SD.

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