Increased extrasynaptic GluN2B expression is involved in cognitive impairment after isoflurane anesthesia

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Abstract. There is increasing concern regarding the post-operative cognitive dysfunction (POCD) in the aging population, and general anesthetics are believed to be involved. Isoflurane exposure induced increased N-methyl-D-aspartic acid receptor (NMDAR) GluN2B subunit expression following anesthesia, which was accompanied by alteration of the cognitive function. However, whether isoflurane affects this expression in different subcellular compartments, and is involved in the development of POCD remains to be elucidated. The aims of the study were to investigate the effects of isoflurane on the expression of the synaptic and extrasynaptic NMDAR subunits, GluN2A and GluN2B, as well as the associated alteration of cognitive function in aged rats. The GluN2B antagonist, Ro25-6981, was given to rats exposed to isoflurane to determine the role of GluN2B in the isoflurane-induced alteration of cognitive function. The results showed that spatial learning and memory tested in the Morris water maze (MWM) was impaired at least 7 days after isoflurane exposure, and was returned to control levels 30 days thereafter. Ro25-6981 treatment can alleviate this impairment. Extrasynaptic GluN2B protein expression, but not synaptic GluN2B or GluN2A, increased significantly after isoflurane exposure compared to non-isoflurane exposure, and returned to control levels approximately 30 days thereafter. The results of the present study indicated that isoflurane induced the prolonged upregulation of extrasynaptic GluN2B expression after anesthesia and is involved in reversible cognitive impairment.

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Introduction

Postoperative cognitive dysfunction (POCD) is generally considered a subtle decline in the cognitive function that occurs in patients following surgery, for which the elderly population is at high risk. While it is believed that general anesthetics produce reversible restraining effects on the brain, it has been shown that use of anesthetics results in prolonged anesthesia-related neuropathophysiological effects after their elimination, such as neuroinflammation (1), blood-brain barrier disruption (2), neurotoxicity (3,4) or persistent depression of synaptic function (5), and are likely involved in the pathogenesis of POCD.

N-methyl-D-aspartic acid receptors (NMDARs) are a major type of excitatory neurotransmitter-gated ionotropic channels with a particularly important role in synaptic plasticity, learning and memory (6,7). NMDARs are one of the important targets of general anesthetics. Previous evidence suggested that isoflurane exposure induced a prolonged NMDAR subunit, and GluN2B upregulation after anesthetic elimination, which was accompanied by changes in cognitive function, albeit the behavioral outcomes were inconsistent (8-12). Functional NMDARs are composed of two obligatory GluN1 subunits, together with two GluN2 subunits or a combination of GluN2 and GluN3 subunits, and most central NMDARs are GluN1/GluN2 assemblies. GluN2A and GluN2B are the primary GluN2 subunits in the forebrain including the hippocampus. After maturation, most GluN2A-containing NMDA receptors are incorporated into synapses. GluN2B-containing NMDA receptors are also present in synapses, but are mainly found extrasynaptically. NMDARs with different subunits and localization trigger different signaling pathways (13), which play different roles in synaptic plasticity, learning and memory, cell survival, excitotoxicity and cell death (6,14,15).

It is not clear whether synaptic and extrasynaptic GluN2B subunits have different roles in anesthetic-induced cognitive dysfunction. Therefore, the aim of the study was to determine the effects of isoflurane on the expression of the NMDAR subunits, GluN2A and GluN2B, as well as the associated alteration of cognitive function in aged rats. The specific GluN2B antagonist, Ro25-6981, was administered to test the role of GluN2B in anesthetic-induced changes of spatial learning and memory.

Materials and methods

Animals. Sprague-Dawley rats (male, 20 months of age and weighing 500-600 g) obtained from the Dongchuang Laboratory Animal Center, Changsha (Hunan, China) were used for the experiments. The animals were housed in a temperature- and humidity-controlled room (21±20˚C, 60%) using a 12-h light/dark cycle (light on at 06:00) with food and water available *ad libitum*. The animals were given an interval of at least 14 days to adapt to their new environment prior to experiments. The animal protocol was approved by the Peking University Biomedical Ethics Committee, Experimental Animal Ethics Branch (approval no. LA 2012-38).

One week prior to anesthesia exposure, the animals were trained to swim in the Morris water maze (MWM) without a platform, four times per day for at least 3 days, and there was a 1 min interval between each 2 min of swimming. Animals with continuous thigmotactic behavior or those floating without swimming for the last 2 days were excluded from experiments.

Isoflurane exposure. The animals were randomly exposed to isoflurane (Baxter Healthcare of Puerto Rico, Guayama, Puerto Rico). Isoflurane (1.5%) with 2 l/min 100% oxygen as carrying gas (n=30) or vehicle gas (Beijing Millennium City Gas Sales Center (Beijing, China) was employed at 2 l/min 100% oxygen (n=30) for 4 h. To examine the role of GluN2B in anesthetic-induced changes of spatial learning and memory, an additional two groups (n=30, respectively) were administered the specific GluN2B antagonist, Ro25-6981 (3 mg/kg, 2 ml/kg, i.p.; Sigma-Aldrich, St. Louis, MO, USA) daily for 6 days, 24 h after exposure to isoflurane or vehicle gas.

The animals were maintained in an anesthesia chamber during isoflurane or vehicle gas exposure. At the outlet of the chamber, the concentrations of isoflurane, oxygen, and carbon dioxide in the chamber were continuously analyzed with a gas monitor (Datex-Ohmeda, Inc., Louisville, CO, USA). In a previous study, it was shown that this anesthesia protocol did not cause significant changes in blood gas or glucose (1,2).

MWM. After 24 h of treatment, 10 animals from each group were trained for MWM tests for 6 days. Another 10 animals from each group were used for the same tests after 30 days of treatment. The MWM tests were conducted by investigators blind to the group conditions as previously described (1,2). The swimming route was observed by video (Beijing Sunny Instruments Co. Ltd., Beijing, China). The time spent to locate the submerged platform by the animals (defined by the latency cut-off time point of 120 sec) and the swimming velocity were recorded. On day 6, a probe trial was performed without the platform. The percentage of time spent in the previous platform quadrant in a 120-sec period was determined.

Hippocampal tissue harvest and separation of synaptic and extrasynaptic membranes. Following anesthesia exposure, 3 animals from the isoflurane and vehicle gas exposure groups were sacrificed by decapitation at 1, 7 and 30 days post-treatment for harvesting of the hippocampus without MWM tests. The separation of synaptic and extrasynaptic fractions was performed as described by Zhang *et al* and Goebel-Goody *et al* (16,17), which was based on the principle that the postsynaptic density (PSD) protein-associated or synaptic fraction was insoluble in Triton X-100, whereas the non-PSD protein-associated or extrasynaptic fraction was soluble in Triton X-100.

Antibodies and immunoblotting. The protein extracted from the hippocampal tissue homogenate, including the total protein, PSD protein-associated (or synaptic) fraction and the non-PSD protein-associated (or extrasynaptic) fraction were used for western blot analysis to test the expression of GluN2A and GluN2B. Total protein (60 μg), as well as 40 μg protein from synaptic fraction and extrasynaptic fraction per lane were separated electrophoretically in 8% SDS-PAGE gels, and transferred to nitrocellulose membranes (Millipore, Newyork, USA). The membranes were incubated in rat polyclonal antibodies against GluN2B (1:1,000, cat no.: ab65783) and polyclonal antibodies against GluN2A (1:1,000, cat no.: ab14596) (both from Abcam, Cambridge, MA, USA). The binding of the primary antibodies was detected by fluorescently-labeled secondary antibody (1:10,000), and was visualized by scan-ning membranes in an Odyssey infrared imaging system (both from LI-COR Biosciences, Lincoln, NE, USA). For densito-metric analysis, the signal intensity was quantified as a ratio of GluN2A or 2B/actin and normalized to the values of the corresponding control animals.

Statistical analysis. Statistical analyses were performed using SPSS 20.0 for Windows (SPSS, Inc., Chicago, IL, USA). The values of latency and swimming speed in the MWM test were analyzed using two-way repeated-measures analysis of variance (ANOVA), with Bonferroni post-hoc analysis. The percentage of time spent in the previous platform quadrant and data from the western blot analysis were compared between isoflurane and control groups by one-way ANOVA using Bonferroni post-hoc analysis. Data were presented as mean ± SEM and statistical significance was set at $P<0.05$.

Results

Isoflurane exposure induces reversible spatial learning and memory impairment. We used the MWM test to investigate whether isoflurane affects spatial learning and memory. Fig. 1 shows the results of these experiments in the first week (Fig. 1A-C) and 30 days (Fig. 1D-F) after anesthesia. During the first week after treatment, the time required to locate the platform (latency) in the spatial acquisition training was significantly affected by isoflurane treatment compared to the control group (Fig. 1A; $P<0.01$). A probe trial was conducted to evaluate reference memory at the end of learning. The time

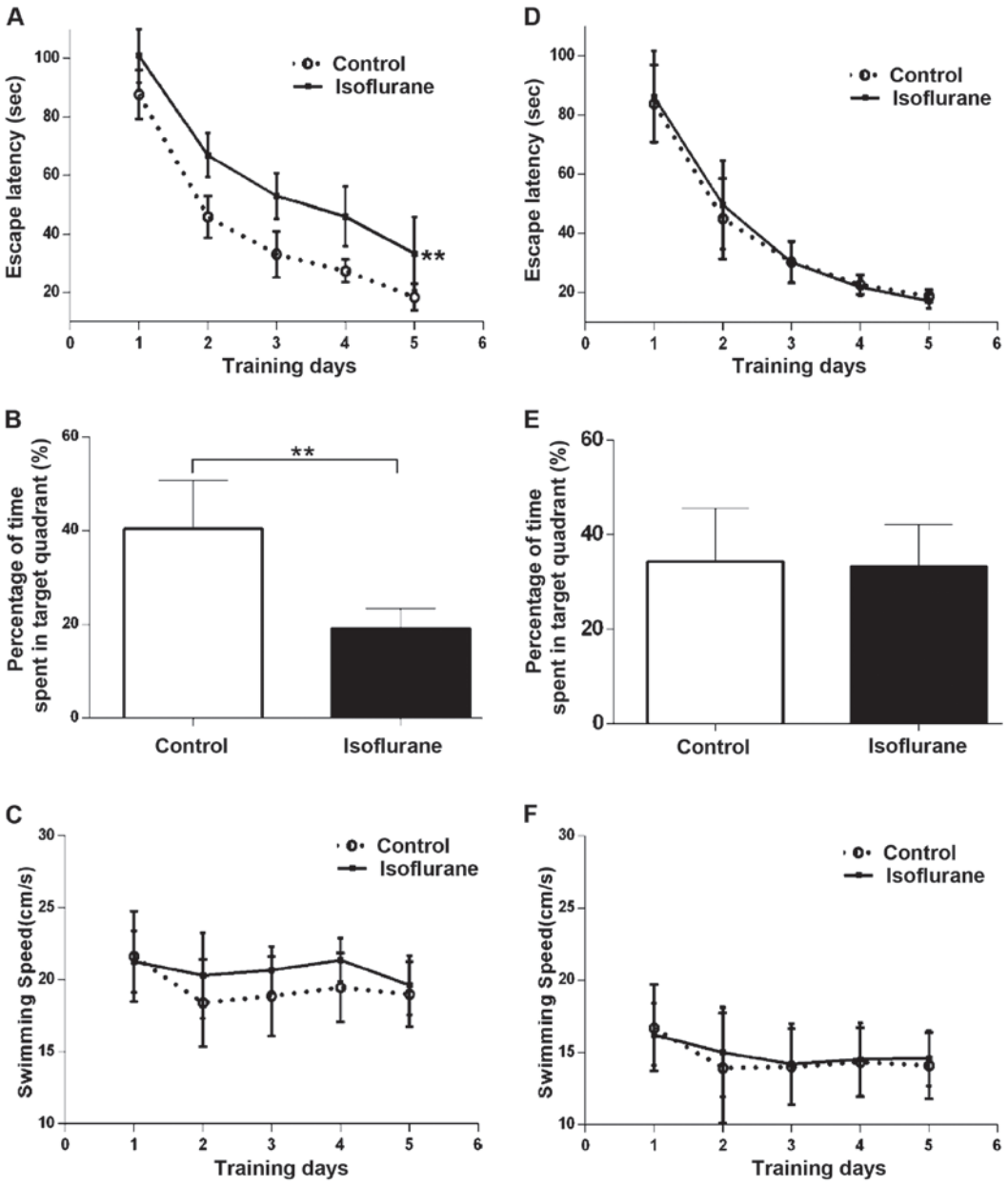

Figure 1. Effect of isoflurane on the spatial memory function in the MWM task during the (A-C) first week and (D-F) 30 days after treatment. (A and D) Escape latency, (B and E) percentage of time spent in target quadrant (%), and (C and F) swimming speed. Results are shown as means ± SEM (n=10). **P<0.01. MWM, Morris water maze.

spent in the platform area by the rats in the isoflurane group was shorter than that in the control group (Fig. 1B; P<0.01). Isoflurane treatment had no effect on swimming speed compared to the control group (Fig. 1C; P>0.05). Rats from each group not receiving MWM test during the first week post-anesthesia underwent the same procedures of MWM test 30 days after the treatment. We found that there were no differences in escape latency and percentage of time spent in the target quadrant between the isoflurane and control groups (Fig. 1D and E; P>0.05). The swimming speed between the two groups showed no difference during the first week and 30 days after treatment (Fig. 1F; P>0.05). These results indicated that the spatial learning and memory in MWM test was impaired at least 1 week following isoflurane exposure, but recovered to the control level 30 days after the treatment.

GluN2 subunit expression in the hippocampus increases following isoflurane exposure. Rats in the isoflurane and control groups were decapitated at 1, 7 and 30 days post-treatment to collect the hippocampus without MWM tests. The hippocampal NMDARs GluN2A and GluN2B subunit protein expression is shown in Fig. 2. The synaptic GluN2A levels showed no difference between isoflurane and control groups at 1, 7 and 30 days following treatment (Fig. 2A-C; P>0.05, between isoflurane and control groups). The levels of total GluN2B protein in the hippocampus increased 24 h after the isoflurane exposure compared with the control group (Fig. 2D; P<0.05, between the isoflurane and control groups). At 7 days after treatment, the GluN2B levels in the isoflurane group were higher than those in the control group although there was no statistical significance (Fig. 2D;

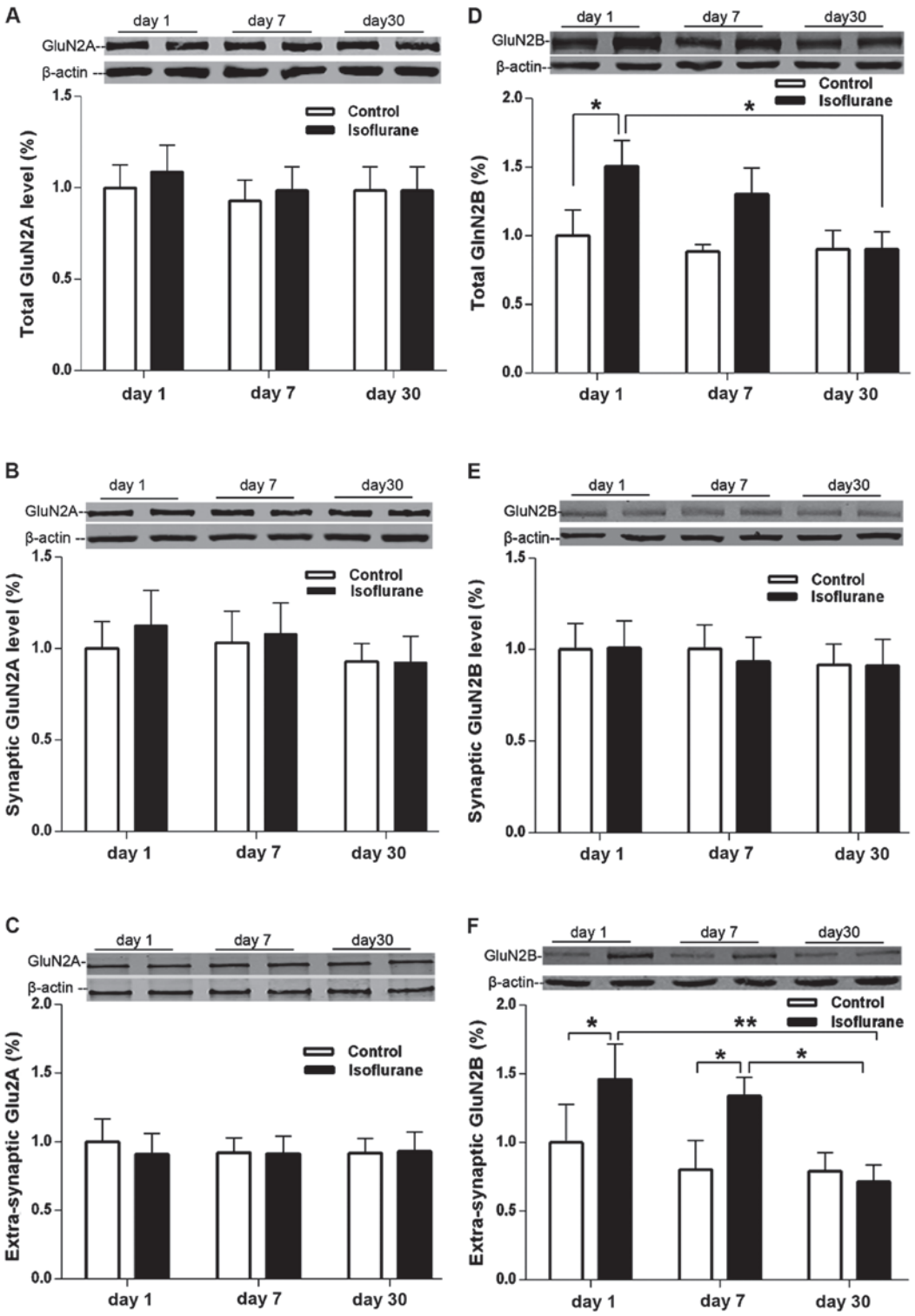

Figure 2. Hippocampal NMDARs GluN2A and GluN2B subunit protein expression. Immunoblots against (A-C) GluN2A, (D-F) GluN2B in isoflurane- and vehicle-treated rats at 1, 7 and 30 days post-treatment. *P<0.05, **P<0.01. Total Glu2B subunit and extrasynaptic GluN2B were upregulated at 1 and 7 days after isoflurane treatment, and returned to the control levels at 30 days after treatment, whereas the Glu2A and intrasynaptic GluN2B protein levels show no differences between the isoflurane and control groups.

P>0.05), and they returned to the control levels after 30 days following the isoflurane exposure (Fig. 2D; P>0.05 between the isoflurane and control groups). The levels of extrasynaptic GluN2B in the hippocampus also increased 24 h following isoflurane exposure compared with the control group (Fig. 2F; P<0.05), and remained higher than that in the control group at 7 days after treatment (Fig. 2F, p<0.05). However, the levels also returned to the control levels 30 days after the treatment (Fig. 2F, P>0.05 between the isoflurane and control groups). The synaptic levels of GluN2B showed no difference between the isoflurane and control groups at 1, 7 or 30 days after treatment (Fig. 2E, P>0.05).

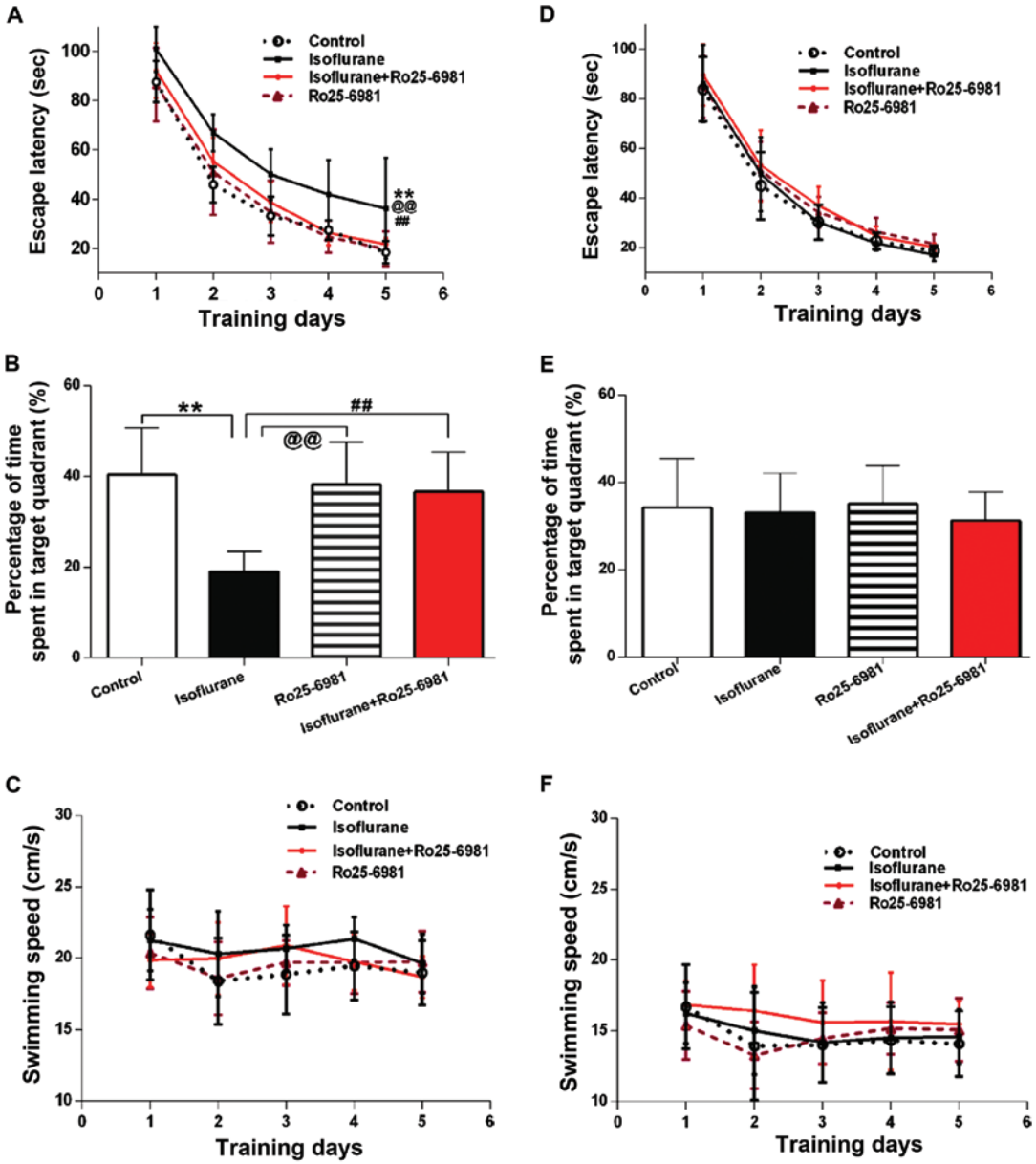

Figure 3. Ro25-6981 alleviated isoflurane-induced impairment of spatial memory function in the MWM task during the first week (A-C) and 30 days (D-F) after treatment. Escape latency (A, $p<0.01$; D, $p>0.05$); Percentage of time spent in target quadrant (%) (B, $p<0.01$; E, $p>0.05$); Swimming speed (C and F, $p>0.05$). Results are presented as means ± SEM (n=10). $^{**}p<0.01$, between isoflurane group vs. control group; $^{@@}p<0.01$ between isoflurane group vs. isoflurane + Ro25-6981 group; $^{\#\#}p<0.01$, between isoflurane vs. Ro25-6981 group. MWM, Morris water maze.

GluN2B-specific antagonist Ro25-6981 alleviates spatial learning and memory impairment induced by isoflurane exposure. During the first week after treatment, Ro25-6981 significantly reduced the latency of rats exposed to isoflurane in spatial acquisition training (Fig. 3A; $P<0.05$ between the isoflurane and isoflurane + Ro25-6981 groups), and the time spent in the platform area in the probe trial was prolonged by Ro25-6981, compared to the rats treated only with isoflurane (Fig. 3B, $P<0.01$ between the isoflurane and isoflurane + Ro25-6981 groups). However, there were no differences in the two aforementioned outcomes in the four groups 30 days after treatment (Fig. 3D and E; $P>0.05$). No differences in swimming speed in the four groups were identified ($P>0.05$). No differences in swimming speed in the four groups were identified (Fig. 3C and F; $P>0.05$).

The latency during the acquisition training was not affected by Ro25-6981 alone, when compared with the control levels during the first week and 30 days thereafter (Fig. 3A and D; $P>0.05$). The time spent in the platform area in the probe trial was not different between the control and Ro25-6981 groups during the 7 or 30 days after treatment (Fig. 3B and E; $P>0.05$).

Discussion

POCD has become a more common phenomenon in the aging population, albeit its mechanism remains unclear. Anesthetics may partially consitute the underlying problem. The majority of investigations pertaining to anesthetic-induced cognitive dysfunction were focused on neurotoxicity and cell death, and the majority of supporting evidence stemmed from

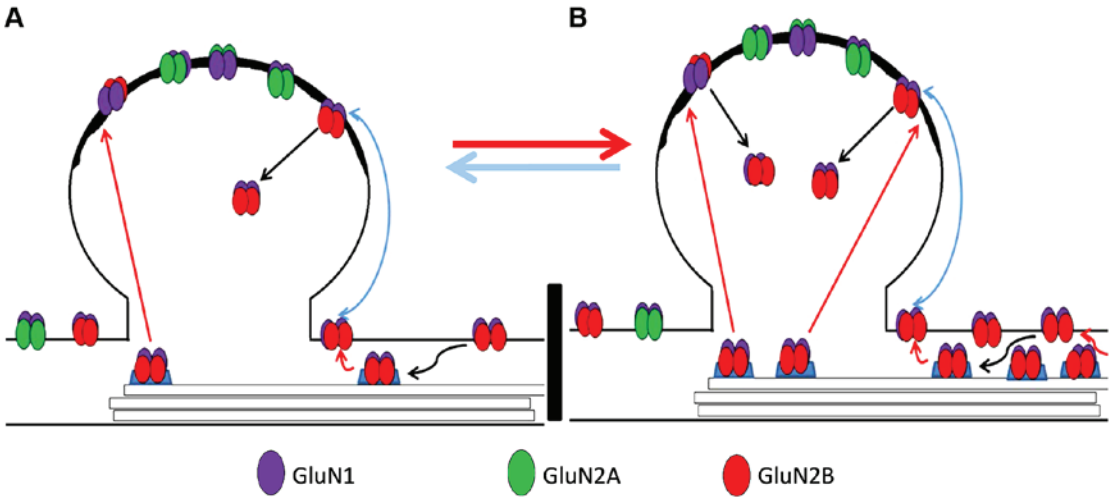

Figure 4. Isoflurane-induced upregulation of extrasynaptic GluN2B. Isoflurane-induced expression of (A and B) GluN2B pre- and post-isoflurane exposure, respectively, combined with curtailing the synaptic GluN2B location in aged animals by modification, such as dephosphorylation with CDK5, may result in the upregulation of extrasynaptic GluN2B without changes in synaptic GluN2B levels and lead to inhibition of the synaptic function.

cultured cells and the developing brain (18,19). Although there are clinical studies that support that age is a risk factor for cognitive dysfunction, there is little evidence to show that the aforementioned neurotoxicity and cell death are involved in cognitive dysfunction in animal models on aging (3,20). On the other hand, epidemiological evidence has demonstrated that the prevalence rate of POCD has decreased over time, suggesting that POCD in most patients may be temporary and self-limiting (21-23). Stratmann *et al* identified that aged rats exposed to isoflurane showed no evidence of ongoing cell death, impaired hippocampal neurogenesis, or long-term cognitive impairment (24). Cognitive impairment by anesthetics in the aging brain manifest certain temporary characteristics of Alzheimer's disease, such as elevated Aβ production and τ hyperphosphorylation (25), which caused synaptic dysfunction without obvious neural loss at the early phase of this disease (26-30). Evidence suggests that inhibitory ionotropic receptors, α-5 subunit containing γ aminobutyric acid-A receptors (α-5GABAARs) can be upregulated and maintain a persistent inhibitory current for 1 week even after a single dosage of etomidate (5). Given the aforementioned evidence, it is reasonable to hypothesize that delayed inhibitory effects in neural activity secondary to anesthetic exposure, even after their elimination, may partially account for cognitive dysfunction, in addition to cell death.

NMDARs are a target of general anesthetics, and play an important role in synaptic plasticity, learning and memory (31). Previous findings have shown that the effects on NMDARs by isoflurane can be sustained beyond the elimination of anesthetics and are involved in changes in cognitive function, albeit the behavioral outcomes were inconsistent (8-12). Our results demonstrate spatial learning and memory in aged rats tested in the MWM. These rats showed impairment of these parameters for at least 1 week following isoflurane exposure, and returned to the control levels 30 days later, suggesting that isoflurane did not induce permanent damage to the central nervous system. We also found that isoflurane exposure for 4 h may induce prolonged changes in GluN2B expression levels for at least 1 week following isoflurane treatment. It is a common pharmacological phenomenon that chronic treatment with receptor antagonists can lead to an increased density of receptors. Evidence suggests that acute (8,9,12) and chronic (32) treatment with anesthetics targeting NMDARs results in the upregulation of NMDA receptors as assessed by the levels of GluN2B subunit protein and mRNA. Since GluN2B-containing receptors are highly sensitive to isoflurane compared to GluN2A-containing receptors (33), the finding that only GluN2B was upregulated in the hippocampus, whereas GluN2A was not altered, can be attributed to the high affinity of isoflurane for GluN2B-containing receptors, which allows for the specific upregulation of GluN2B expression in the hippocampus following acute isoflurane exposure.

Furthermore, we found that extrasynaptic GluN2B protein expression, but not synaptic GluN2B, increased significantly after 4 h of isoflurane exposure compared to non-isoflurane exposure, and treatment with the GluN2B antagonist Ro25-6981 was able to alleviate this impairment. Since the majority of GluN2A is incorporated into synapses, and GluN2B is mainly found extrasynaptically, these outcomes indicate that extrasynaptic GluN2B subunit upregulation may be involved in isoflurane-induced cognitive dysfunction.

GluN2B subunits are particularly important for plasticity, learning and memory, and their location in synaptic and extra-synaptic compartments play different roles (6,14,16,17,34-36). Elevations in the synaptic GluN2B subunit levels and GluN2B-containing NMDARs are involved in spatial learning tasks and hippocampal long-term potentiation (LTP) enhancement (36,37), whereas intrasynaptic GluN2B reduction is accompanied by cognitive impairment (16,38). On the other hand, activating extrasynaptic GluN2B can facilitate hippocampal long-term depression (LTD) and inhibit LTP (34,35). Therefore, increasing extrasynaptic GluN2B expression by isoflurane exposure may produce inhibitory effects on synaptic transmission and cause loss of cognitive function.

The molecular machinery regulating the sub-cellular localization of GluN2B subunits remains to be elucidated. Evidence suggests that anesthetics and aging can disturb

these mechanisms. For example, it has been recognized that phosphorylation of GluN2B is important for the regulation of such processes (39), which can be influenced by aging and exposure to anesthetics (40-42). Increasing levels of CDK5 with age are capable of regulating the binding of Src to PSD-95, leading to dephosphorylation of tyrosine-1472 of GluN2B at synapses and its endocytosis, and reducing levels of synaptic GluN2B, but does not affect extrasynaptic GluN2B levels (43,44). Therefore, isoflurane-induced upregulation of GluN2B, combined with increased CDK5 in aging rats, which curtails the synaptic location of GluN2B by dephosphorylation of tyrosine-1472, or by directly phosphorylating GluN2B at Serine-1116, decreasing its synaptic expression (45) may explain our result that intrasynaptic GluN2B expression showed no changes, while there was upregulation of total and extrasynaptic GluN2B. The schematic in Fig. 4 outlines the process. The animal model used by Rammes *et al* (8) was an adult model and may have had a relatively lower activity of CDK5 and lower GluN2B endocytosis compared to aged animals, and consequently, the upregulation of total GluN2B may lead to increased synaptic GluN2B and improved cognitive function.

Although previous findings have shown that systemic or intracerebral administration of NMDAR antagonists affects learning and memory performance on various tasks, including spatial learning and memory test in MWM (46-48), our findings showed that rats treated with Ro25-6981 alone have no changes in spatial learning and memory compared with the control animals. However, rats exposed to isoflurane and Ro25-6981 present improved cognitive function compared to the isoflurane group. Possible explanations include that intrasynaptic GluN2B-containing NMDA receptors are not sensitive to the small dose of Ro25-6981 used in our investigation under 'normal' physiological conditions, or the latter cannot pass through blood-brain barrier without isoflurane (2). A higher dose of Ro25-6981 may interfere with NMDARs function severely and lead to cognitive impairment (8,48).

In conclusion, the present data suggest that isoflurane may induce reversible cognitive impairment, and isoflurane-induced sustained upregulation of extrasynaptic GluN2B, not synaptic GluN2B, after anesthesia, may be involved in this cognitive impairment. The precise mechanisms for general anesthetic-mediated modulation of the subcellular localization of GluN2B subunits and their role in cognitive function remain to be elucidated.

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