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Bilateral Inhibition of γ-Aminobutyric Acid Type A Receptor Function within the Basolateral Amygdala Blocked Propofol-induced Amnesia and Activity-regulated Cytoskeletal Protein Expression Inhibition in the Hippocampus

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Background: **It has been reported that bilateral lesions of the basolateral amygdala complex (BLA) blocked propofol-induced amnesia of inhibitory avoidance (IA) training. Based on these results, the authors hypothesized that the amnesia effect of propofol was partly due to its impairment of memory formation in the hippocampus through activating the BLA γ-aminobutyric acid type A receptor function. The authors determined the changes in activity-regulated cytoskeleton–associated protein (Arc) expression to be an indicator of IA memory formation.**

Methods: **Male Sprague-Dawley rats received bilateral injection of bicuculline methiodide (10, 50, or 100 pmol/0.5 μl) or saline (0.5 μl) into the BLA. Fifteen minutes later, the rats were intraperitoneally injected with either propofol (25 mg/kg) or saline. After 5 min, the one-trial IA training was conducted. Rats intraperitoneally infused with saline served as controls and only received saline injections into the BLA. Twenty-four hours later, the IA retention latency was tested. Separate groups of rats treated the same way were killed either 30 min after IA training for hippocampal Arc mRNA measurement or after 45 min for protein level quantification.**

Results: **The largest dose of bicuculline methiodide (100 pmol) not only blocked the propofol-induced amnesia but also reversed the inhibition effect of propofol on Arc protein expression in the hippocampus ($P < 0.05$). However, the mRNA level of Arc showed no significant changes after propofol and bicuculline methiodide administration.**

Conclusions: **The amnesic effect of propofol seems to involve the modulation of Arc protein expression in the hippocampus, occurring through a network interaction with the BLA.**

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DESPITE being a well-established clinical phenomenon, the molecular and cellular mechanism of anesthetic-induced amnesia remains poorly understood.[1,2] Both postoperative cognitive dysfunction[3,4] and intraoperative awareness[5–7] have been distressing problems and continue to attract public attention; however, it is still not clear whether anesthetic drugs have anything to do with these two issues *per se*. Therefore, to communicate something about the potential of anesthetic drugs to contribute to such issues in ways we do not yet know, more work is needed to elucidate how these powerful drugs work in the brain.

Extensive evidence from animal and human studies indicates that emotionally charged events are typically better remembered than neutral ones.[8–10] The amygdala, particularly the basolateral amygdala (BLA), is a key structure in emotional processing in the brain.[11,12] It has also been reported that bilateral BLA lesions block the memory-modulating effect of diazepam, propofol, and sevoflurane.[1,13–15]

In the current study, we investigated the mechanism underlying propofol produced amnesia and the necessity for the integrity of the BLA. The study was designed on the basis of the following findings. First, the γ-aminobutyric acid–mediated (GABAergic) system within the BLA is involved in memory modulation,[16,17] whereas propofol has a predominant effect on enhancing γ-aminobutyric acid type A ($GABA_A$) receptor function.[18–20] Second, the BLA modulates memory consolidation *via* its projections into the perirhinal cortex, postrhinal cortex, and hippocampus.[21] The BLA–hippocampus interactions have attracted the most attention. Third, activity-regulated cytoskeletal protein (Arc) plays an important role in the synaptic plasticity underlying the consolidation of long-term memory. Changes in Arc expression are recognized as an indicator of such synaptic plasticity.[22,23] Alkire *et al.*[24] found in their study that amnesic doses of the inhalational anesthetics sevoflurane and desflurane could reduce hippocampal Arc protein expression in rats with inhibitory avoidance (IA) training.

Here, we proposed the hypothesis that propofol induces amnesia by increasing GABAergic activity in the BLA. To test this hypothesis, rats trained with IA were systemically given propofol with or without $GABA_A$ receptor inhibition by either BLA-injected bicuculline (a

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competitive $GABA_A$ receptor antagonist) or saline. Afterward, the long-term memory of IA and Arc expression in the hippocampus were determined at 24 h and 30 or 45 min after training, respectively.

Materials and Methods

Animals

After obtaining Institutional Animal Care and Use Committee approval (Shanghai JiaoTong University School of Medicine, Shanghai, China), 120 male Sprague-Dawley rats (250–280 g on arrival) were obtained from SLAC Laboratory Animal (Shanghai, China). They were housed individually in a temperature-controlled (22°C) colony room, with food and water available *ad libitum*. Animals were maintained throughout the experiments on a 12-h light–dark cycle (lights on at 7:00 AM). Behavioral testing was performed during the light cycle between 9:00 AM and 4:00 PM. Rats were given 1 week to acclimatize to the vivarium before surgery, and behavioral procedures began 7 days after surgery.

Surgery

Rats were anesthetized for surgery with an intraperitoneal injection of 20% chloral hydrate (300 mg/kg). Bilateral guide cannulae (23 gauge) were implanted dorsal to the BLA (coordinates: anteroposterior, −2.8 mm from bregma; mediolateral, ±5.0 mm from midline; dorsoventral, −6.5 mm from skull surface; incisor bar, −3.3 mm from interaural line). After 2 days of recovery from surgery, rats were handled daily for 5 days (5 min/day).

IA Training

Rats were bilaterally intra-BLA infused with one of three doses of bicuculline methiodide (BMI; Sigma-Aldrich Corp., St. Louis, MO) (10, 50, or 100 pmol/0.5 μl) or saline (0.5 μl) through a 30-gauge microinjection needle extending 2 mm beyond the cannulae. The duration of all 0.5-μl infusions was 2 min. The injection needle remained in place for an additional 2 min to maximize diffusion of the solution. Fifteen minutes later, the rats were intraperitoneally injected with either 25 mg/kg propofol (Propofol-Fresenius; Beijing Fresenius Kabi Pharmaceutical Co. Ltd., Beijing, China) or saline. After 5 min, the IA training was conducted. The rat was placed in the illuminated compartment of an IA apparatus (Institute of Biomedical Engineering, Tianjin, China), facing away from the door. It is a trough-shaped alley (15 cm deep, 20 cm wide at the top, and 6.4 cm wide at the floor), with the illuminated compartment 30 cm long and the dark compartment 38 cm long, divided by a retractable door. When the animal turned to face the door, the door was lifted out of the way to reveal the dark–shock compartment. After the rat crossed from the illuminated to the dark compartment of the alley, the door was then closed, and a single foot shock (0.4 mA for 2 s) was delivered. The appropriate dosage and time interval of propofol used was determined by referring to the research of Alkire *et al.*[1] Rats with saline intraperitoneal injection only received saline infusion into the BLA without using BMI. Thus, the animals were divided into five treatment groups: saline–saline, saline–propofol, BMI 10–propofol, BMI 50–propofol, and BMI 100–propofol. Twenty-four hours after training, the animals were placed again in the lighted compartment of the IA alley. Latency to enter the dark compartment was considered as a measure of memory retention for the aversive stimulus.

Separate groups of rats treated the same way were used in the Arc expression analysis experiments. According to the work of McIntyre *et al.*,[22] the animals were deeply anesthetized with an overdose of chloral hydrate (600 mg/kg intraperitoneally) 30 min after IA training for hippocampus Arc messenger RNA (mRNA) measurement and 45 min for protein level quantification, respectively. After these rats were killed and their brains were removed, the hippocampal tissues were isolated with a spatula and immediately stored in liquid nitrogen.

Histology

After the long-term memory test, all of the rats were anesthetized with an overdose of chloral hydrate (600 mg/kg intraperitoneally). The animals were perfused intracardially with a 0.9% saline solution followed by 4% polyoxymethylene solution. Brains were removed from each animal, placed into a 4% polyoxymethylene solution for 2 h, and then transferred to a 20% sucrose solution overnight. Brains were sectioned into 40-μm sections using a freezing microtome and stained with thionin. Brain sections were analyzed under a light microscope to identify the location of cannula placement. Only brains that had needle tracks in the BLA and no lesions in the surrounding BLA tissue were included in the analysis.

Real-time Reverse Transcription Polymerase Chain Reaction Quantification

Total RNA was prepared from the hippocampus tissue using Trizol reagent (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. One microgram of total RNA was reversely transcribed to complementary DNA (cDNA). Real-time reverse transcription polymerase chain reaction was conducted on the high-throughput fluorescent quantitative polymerase chain reaction equipment 7900HT (Applied Biosystems, Foster City, CA), and amplification was undertaken by using an SYBR premix ExTaq Kit (TaKaRa Bio Inc., Otsu, Japan). Expression of the target gene was normalized by β-actin levels. The sequences of rat Arc and β-actin polymerase chain reaction primers were designed according to cDNA sequences reported in the GenBank

database, analyzed using the Primer 3 software (PREMIER Biosoft International, Palo Alto, CA) and synthesized at the DNA Bio Tec (Shanghai, China). Primers sequences used were as follows: sense: 5′- AGGGAGGTCTTCTAC-CGTCT-3′ and antisense: 5′-AGTGTAGTCGTAGCCAT-CAGC-3′ for Arc; sense: 5′-CTCTTCCAGCCTTCCTTC-CT-3′ and antisense: 5′-TCATCGTACTCCTGCTTGCT-3′ for β-actin.

Western Blotting

The tissues were homogenated in a protein extraction reagent containing inhibitors (to 1 ml protein extraction reagent, add 5 μl protease inhibitor cocktail, 5 μl PMSF, and 5 μl phosphatase cocktail; Kangchen Bio-Tech, Shanghai, China). Protein concentrations were determined by the Bradford method.

Ten micrograms of protein from each sample was loaded on a 10% SDS-polyacrylamide gel (Bio-Rad Laboratories Inc., Hercules, CA) and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA) by electroblotting. Membranes were blocked with 5% nonfat milk for 2 h at room temperature and then incubated overnight with 1:200 of primary antibodies (Anti-Arc; Santa Cruz Biotechnology Inc., Santa Cruz, CA) in blocking buffer at 4°C. After washing the blots with Tris-buffered saline with Tween 20 (1 × Tris-buffered saline containing 0.1% Tween 20), the membranes were incubated with horseradish peroxidase–labeled secondary antibodies (1:1,000, Santa Cruz Biotechnology Inc.). The immunoreactive bands were detected using an enhanced chemiluminescence detection kit (PerkinElmer, Waltham, MA) following the manufacturer's instructions. Afterward, blots were directly reprobed with antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000; Kangchen Bio-Tech) for control loading. Last, the bands were semiquantified by densitometric analysis with Leica Qwin Fresenius Kabi Pharmaceutical Corp. software (Leica Microsystems Ltd., Heerbrugg, Switzerland).

The cannula location for rats used in the Arc expression analysis experiments was macroscopically detected. After the brains were rapidly removed, a group-blind detector cut the brain along the needle tracks and quickly detected the position of the tracks. The judgment was made based on the depth of the track, the brain tissue structures around the track, and the symmetry of the tracks between the two hemispheres. Only those with needle tracks macroscopically judged to be in the BLA were used for analysis.

Statistical Analysis

Statistical analysis was performed using the SPSS 11.5 software package (SPSS, Chicago, IL), and the normality of the data were evaluated. All values are given as mean and SD. The data among groups were analyzed by a one-way analysis of variance. Significant values were subsequently verified by Dunnett *post hoc* tests with the saline–saline group as the "control" category. For all tests, differences were considered significant at the 5% level ($P < 0.05$).

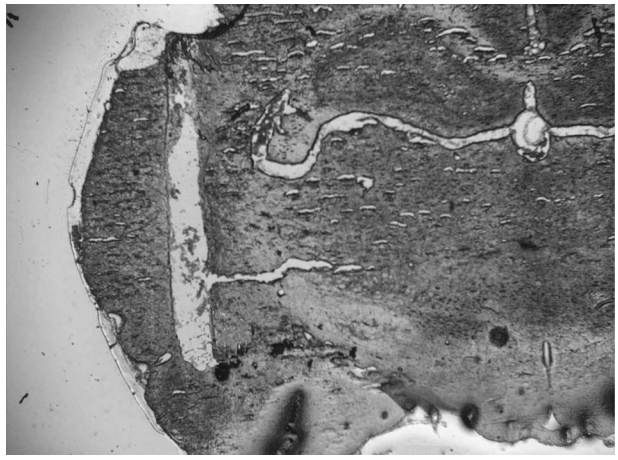

Fig. 1. Photomicrographs of representative needle tracks terminating in the basolateral amygdala.

Results

Twenty-eight of the rats were excluded because of the wrong location of the cannula, and another 17 were rejected for being too sedated with propofol to complete the IA training. Figure 1 showed representative needle tracks in the BLA.

As shown in figure 2, animals in the saline–saline group (n = 9) had a mean retention latency of 36.22 ± 25.71 s. Animals in the saline–propofol group (n = 9), BMI 10–propofol group (n = 8), and BMI 50–propofol group (n = 7) had amnesia, with retention latencies of 11.67 ± 6.25, 12.38 ± 4.24, and 11.71 ± 8.08 s, respectively ($P = 0.009$, 0.014, and 0.015 *vs.* animals in the saline–saline group, respectively). However, the BMI 100–propofol group (n = 9) had an average retention latency of 42.11 ± 25.47 s, which was comparable with that of the saline–saline group ($P = 0.956$).

Separate groups of rats were trained in IA, given pretraining drug infusions, and killed either 30 min later for Arc mRNA level measurement or 45 min later for protein level quantification, as described in the Materials and Methods. The rats were divided into three groups. During 25 mg/kg propofol administration, they received saline or 100 pmol BMI intra-BLA injection, respectively. The rats in the control group were injected with saline both intra-BLA and intraperitoneally.

No significant differences were seen among the three groups in the mRNA level of Arc detected by real-time reverse transcription polymerase chain reaction (fig. 3), whereas the protein levels of Arc were remarkably different. As shown in figure 4, propofol remarkably reduced Arc expression when compared with the control group (saline–saline group), whereas BMI admin-

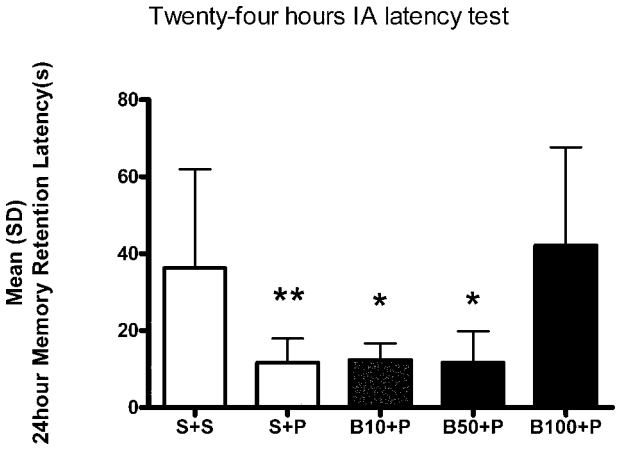

Fig. 2. Statistical difference of the 24-h inhibitory avoidance memory retention performance was compared among the five groups. This finding implicated that infusion of the γ-aminobutyric acid–mediated antagonist bicuculline methiodide into the basolateral amygdala (BLA) before inhibitory avoidance training reversed the amnesia effect of systemically administration of propofol in a dose-dependent way. S + S = rats received saline both intra-BLA and intraperitoneally; S + P = rats received saline intra-BLA and 25 mg/kg propofol intraperitoneally; B10 + P = rats received 10 pmol bicuculline methiodide intra-BLA and 25 mg/kg propofol intraperitoneally; B50 + P = rats received 50 pmol bicuculline methiodide intra-BLA and 25 mg/kg propofol intraperitoneally; B100 + P = rats received 100 pmol bicuculline methiodide intra-BLA and 25 mg/kg propofol intraperitoneally. n = 7–9 for each group. * $P < 0.05$, ** $P < 0.01$ compared with the control (S + S) group.

istration into the BLA increased Arc expression. There was no statistical difference of Arc protein level between the BMI 100–propofol group and the control group.

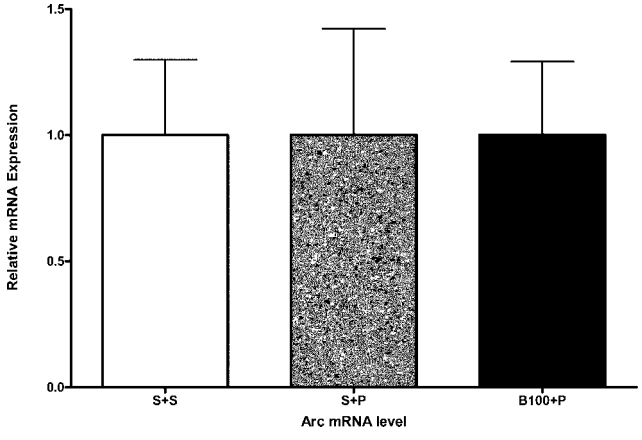

Fig. 3. Quantitative reverse transcription polymerase chain reaction analysis of relative activity-regulated cytoskeleton–associated protein (Arc) messenger RNA (mRNA) levels in the hippocampus. The result showed that propofol (25 mg/kg) intraperitoneal administration neither alone nor together with bicuculline methiodide (100 pmol) intra–basolateral amygdala (BLA) injection changed Arc transcription level. S + S = rats received saline both intra-BLA and intraperitoneally; S + P = rats received saline intra-BLA and 25 mg/kg propofol intraperitoneally; B100 + P = rats received 100 pmol bicuculline methiodide intra-BLA and 25 mg/kg propofol. n = 6 for each group.

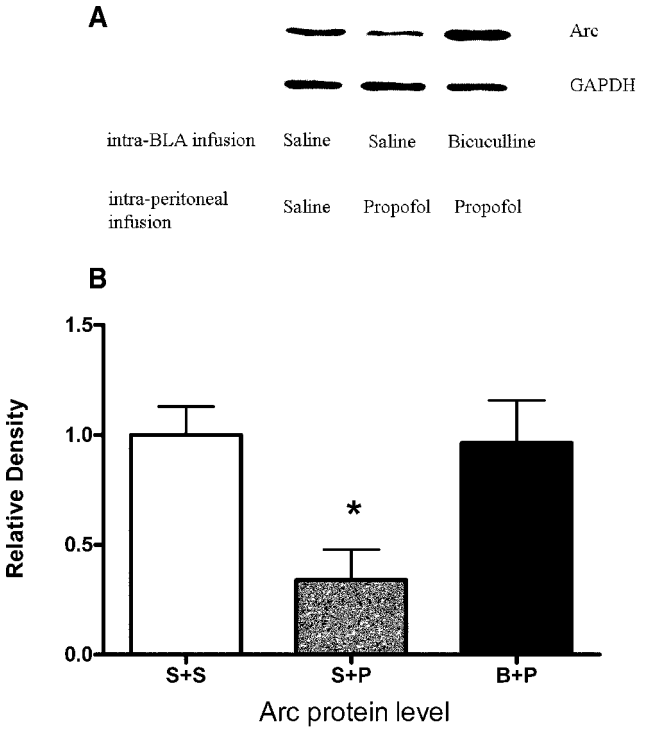

Fig. 4. Propofol (25 mg/kg) reduced activity-regulated cytoskeleton–associated protein (Arc) expression in the hippocampus, which could be reversed by 100 pmol bicuculline methiodide bilaterally injected into the basolateral amygdala (BLA). (*A*) Bands of Western blot of the Arc expression (55 kd). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a loading control. (*B*) Statistical analysis of relative density of Western blots between drug-treated group (S + P group or B + P group) and the control group (S + S group). S + S = rats received saline both intra-BLA and intraperitoneally; S + P = rats received saline intra-BLA and 25 mg/kg propofol intraperitoneally; B + P = rats received 100 pmol bicuculline methiodide intra-BLA and 25 mg/kg propofol intraperitoneally. n = 5 for each group. * $P < 0.05$ compared with control rats.

Discussion

There are two key findings of the current study. First, infusion of the GABAergic antagonist BMI into the BLA before training reversed amnesia induced by the systemic administration of propofol in a dose-dependent way. Second, the presumed $GABA_A$ block by BMI also reversed the effect of propofol on reducing Arc expression. These results indicate that propofol could impair memory consolidation in the hippocampus through a network interaction with the BLA.

Propofol is widely used in clinical anesthesia. It is believed to act on a broad variety of molecular targets. However, the potentiation of $GABA_A$ receptor is regarded as the most important effect for propofol to induce anesthesia.[25] It has been reported that direct effects of propofol on *N*-methyl-D-aspartate receptors occur only at supratherapeutic concentrations.[19,26] Nagashima *et al.*[26] found that propofol inhibits CA1 region long-term potentiation induction through modulation of $GABA_A$ receptors but not *via* inhibition of *N*-methyl-D-aspartate receptors.

As shown in the current study, once the BLA GABAergic activity was inhibited by BMI, the memory-impairing effect of systemic infusion with propofol was blocked. This result strongly supported our hypothesis that enhancing GABAergic activity within the BLA is an important prerequisite for propofol to mediate amnesia. To date, the unitary mechanism of general anesthesia gives way to "multiple sites, multiple mechanisms." And, it has also been shown that anesthetic-induced amnesia is partly mediated by anesthetic actions in the BLA.[1,13,14] A powerful piece of evidence is that lesions of the BLA block propofol-induced amnesia for IA learning in rats.[1] Furthermore, the GABAergic system of the BLA is believed to play a critical role in synaptic plasticity and memory consolidation for both positive and negative emotional experiences. Infusion of bicuculline into the BLA, but not into the central amygdaloid nucleus, could increase fear conditioning in a contextual fear paradigm of the rat, which mimics the same behavioral effect of previous exposure to a restraint session.[27]

The effective dose of BMI to reverse amnesia of propofol was shown to be 100 pmol in the current study. Midazolam is also a GABA agonist, and previous studies have reported that BMI administered into the amygdala both before and after training blocked midazolam-induced amnesia. Interestingly, Dickinson-Anson *et al.*[28,29] found a much lower dose of bicuculline administered after training (2 pmol) than before training (56 pmol) to reverse the amnesia effect of midazolam. In addition, an even earlier study reported that intraamygdala infusion of the GABAergic agonist muscimol was 100–500 times more potent when administered after training as opposed to before training.[29,30] On the basis of these findings, it was hypothesized that the sensitivity of the amygdaloid GABAergic system to endogenous and exogenous ligands was increased after IA training. Hence, it is possible that a much lower dose of BMI ($<$ 100 pmol) administered after training is enough to reverse the amnesia induced by propofol. However, a contrary result was observed by O'Gorman *et al.*[31] They found that the dose of propofol administered by intraperitoneal injection required to impair IA memory was double after training (150 mg/kg) compared with before training (75 mg/kg). We assumed that after IA training, the sensitivity of the amygdaloid GABAergic system to ligands was transiently increased, which might have recovered to the baseline or even decreased when the intraperitoneally injected drug spread to the BLA. In any case, memory regulation efficiency differences between pretraining and posttraining drug administration, as well as such differences between systemic drug administration and local (BLA) certainly require further research.

Memory formation is a complicated process. Drugs can enhance or reduce memory *via* influencing three phases of memory processing (encoding, consolidation, and retention). It is widely accepted that propofol can induce amnesia independent of its sedation effect, *i.e.*, propofol can impair not only the acquisition phase but also the consolidation phase.[32] In our study, both propofol and BMI were administered before training, so they might influence both memory encoding and consolidation. In future studies, we may use BMI immediately after IA training to specifically identify whether the memory consolidation impairment of propofol is mainly through enhancing the BLA GABAergic activity.

The mechanism by which propofol's regulation of BLA GABAergic activity induces amnesia is still obscure. The current study might answer this question to some extent. It was observed that propofol reduced Arc protein expression in the hippocampus. Furthermore, the largest dose of BMI that blocked propofol amnesia effect also reversed the inhibition effect of propofol on Arc expression in the hippocampus. To fully appreciate these findings, the following two points must be considered. First, it should be noted that the immediate-early genes are rapidly induced in the brain in response to synaptic activity. Among these, Arc is unique in that, after long-term potentiation produced stimulation, its mRNA is robustly induced and transported to dendrites.[22,23] Infusions of Arc antisense oligodeoxynucleotides into the dorsal hippocampus impaired performance of an IA task.[22] More and more studies are continuously supporting the idea that Arc plays a key role in memory consolidation. Second, rapidly growing data have suggested that the BLA is not a storage site for many of the emotionally modulated memories[8] but that it can facilitate synaptic plasticity in other brain structures engaged in memory processing *via* efferent connections. Among these regions, the hippocampus is the most important site. It was proved that stimulation of the BLA, but not the central nucleus of the amygdala, facilitates long-term potentiation of perforant path inputs to the dentate gyrus.[33] Therefore, it seems that propofol impairs memory formation in the hippocampus *via* a network interaction with the amygdala.

Because the BLA does not project to the dentate gyrus, the BLA influence on long-term potentiation of perforant path inputs to the dentate gyrus must be indirect. It has been assumed to involve activation of noradrenergic and cholinergic cell groups projecting into the hippocampus.[8,34] Indeed, the β-adrenergic state of the BLA was also found to influence Arc expression in the hippocampus. This, in turn, affects memory consolidation of IA training.[22] Previous findings indicated that systemic administration of the GABAergic antagonist picrotoxin induced norepinephrine release in the amygdala.[35] Posttraining intra-BLA or systemic administration of a β-adrenergic antagonist blocks the memory-modulating effects of systemically administered GABAergic drugs.[36] A recent study showed that propranolol (a β-adrenergic antagonist) infused into the right BLA together with bicuculline blocked the bicuculline-induced contextual

fear conditioning extinction enhancement. On the other hand, norepinephrine infused into the right BLA enhanced extinction, and this effect was not blocked by coinfusions of muscimol (a GABAergic agonist).[37] Taken together with these findings, it seemed that GABAergic influences on memory consolidation act upstream from noradrenergic activation. Propofol might influence the BLA noradrenergic activity indirectly to modulate IA memory and Arc expression in the hippocampus. Therefore, further research is needed into the involvement of the noradrenergic state in the BLA during propofol-induced amnesia.

The last finding was that propofol was found to inhibit Arc protein expression but not reduce mRNA levels. These results were in accord with the work of McIntyre *et al.*,[22] who found that although both clenbuterol and lidocaine injected into the BLA significantly altered Arc protein levels in the dorsal hippocampus in trained rats, neither treatment significantly affected mRNA levels as compared with the vehicle treated hemisphere. Furthermore, Alkire's group has also observed that amnesic doses of sevoflurane could reduce hippocampal Arc protein expression of rats with IA training but not alter Arc mRNA level.[24,38] Therefore, it seems that the modulation of Arc expression and synaptic plasticity by propofol-stimulated BLA $GABA_A$ receptors occurs at a posttranscriptional level in the hippocampus.

To better appreciate the findings in the context of existing data, it should always be taken into consideration that bicuculline is not such a specific GABA blocker, as previously thought. A number of studies have questioned its selectivity in vertebrate inhibitory neurotransmission. Indeed, it also blocks mouse muscle $\alpha\beta\gamma\delta$, rat $\alpha_2\beta_4$ and $\alpha_4\beta_2$, and human α_7 neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes,[39] which may be relevant in the BLA, and thus contributes to the effect on modulating performance in amygdala-based learning tasks. Moreover, using whole cell patch clamp recording, Zhu *et al.*[40] found that activation of nicotinic acetylcholine receptors in principal neurons in the BLA lead to an action potential–dependent increase in the frequency of spontaneous GABAergic currents. They also found that such effect involved predominantly $\alpha_3\beta_4$-containing nicotinic acetylcholine receptors subunits.[40] Because the effect of bicuculline on $\alpha_3\beta_4$ subunits is still unclear, the actual *in vivo* effect of BMI on BLA is complicated and obscure.

Finally, one limitation in our study to be noted is that the cannula location for rats used in the Arc expression analysis experiments (Western blotting and real-time reverse transcription polymerase chain reaction) could not be microscopically detected, which might introduce slight inaccuracy. However, the potential for error was decreased by two factors. One, these rats were bilaterally implanted with cannula after those for IA memory testing, so the location accuracy was greatly improved after a large number of operations. Two, the location of the cannulae was analyzed by a group-blind examiner. Despite this limitation, the study indicates, to a certain extent, that BMI injected into the BLA reversed the inhibitory effect of propofol on Arc expression in the hippocampus.

In conclusion, the findings presented here support the hypothesis that the amnesic effect of propofol stems partly from its impairment of memory formation in the hippocampus *via* its enhancing effect on BLA $GABA_A$ receptor function. $GABA_A$ receptor inhibition by BMI infusions into the BLA blocked propofol-induced amnesia and restored propofol-induced suppression of Arc protein expression in the hippocampus. Arc mRNA was not affected, suggesting that the modulation of Arc protein level was posttranscriptional.

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