

Effect of Disrupting N-Methyl-D-aspartate Receptor–Postsynaptic Density Protein-95 Interactions on the Threshold for Halothane Anesthesia in Mice

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Background: The authors' previous studies have shown that clinically relevant concentrations of inhalational anesthetics dose-dependently and specifically inhibit the PSD-95, Dlg, and ZO-1 (PDZ) domain-mediated protein interactions between postsynaptic density protein 95 (PSD-95) and N-methyl-D-aspartate receptors, and that the knockdown of spinal PSD-95 by intrathecal injection of PSD-95 antisense oligodeoxynucleotide significantly reduces the minimum alveolar anesthetic concentration for isoflurane in rats.

Methods: The authors constructed a fusion peptide, Tat-PSD-95 PDZ2, comprising the second PDZ domain of PSD-95, which can specifically disrupt PSD-95 PDZ2-mediated protein interactions by binding to its interaction partner. By intraperitoneal injection of this fusion peptide into mice, the authors investigated the effect of disrupting the PSD-95 PDZ2-mediated protein interactions on the threshold for halothane anesthesia.

Results: Systemically injected fusion peptide Tat-PSD-95 PDZ2 was delivered into the central nervous system, disrupted the protein-protein interactions between N-methyl-D-aspartate receptor NR2 subunits and PSD-95, and significantly reduced the minimum alveolar anesthetic concentration and righting reflex EC₅₀ for halothane.

Conclusions: By disrupting PSD-95 PDZ2 domain-mediated protein interactions, intraperitoneal injection of cell-permeant fusion peptide Tat-PSD-95 PDZ2 dose-dependently reduces the threshold for halothane anesthesia. These results suggest that PDZ domain-mediated protein interactions at synapses in the central nervous system might play an important role in the molecular mechanisms of halothane anesthesia.

N-METHYL-D-ASPARTATE receptor (NMDAR) activation has been demonstrated to play an important role in the processing of spinal nociceptive information¹⁻⁴ and in the determination of the minimum alveolar anesthetic concentration (MAC) of inhalational anesthetics.⁵⁻¹¹ Postsynaptic density protein 95 (PSD-95), a scaffolding protein, has been identified to attach NMDARs to internal signaling molecules at neuronal synapses of the central nervous system (CNS).^{12,13} This function suggests that PSD-95 might be involved in physiologic and pathophysiologic actions triggered *via* the activation of NMDARs in the CNS. NMDAR-PSD-95 protein interactions are mediated by a PDZ domain (a term derived

from the names of the first three proteins identified to contain the domain: PSD-95, Dlg, and ZO-1). PSD-95 possesses three PDZ domains. The second PDZ domain of PSD-95 (PSD-95 PDZ2) interacts with the seven-amino acid, COOH-terminal domain containing a terminal tSXV motif (where S is serine, X is any amino acid, and V is valine) common to NR2 subunits of NMDARs.¹³ PSD-95 PDZ2 also interacts with the Shaker-type Kv1.4 potassium channel and this interaction regulates the clustering of PSD-95 with the Kv1.4 channel.¹⁴

Our previous studies have shown that clinically relevant concentrations of inhalational anesthetics dose-dependently and specifically inhibit the PDZ domain-mediated protein interaction between PSD-95 and NMDARs.¹⁵ These inhibitory effects are immediate, potent, and reversible and occur at a hydrophobic peptide-binding groove on the surface of the PSD-95 PDZ2 in a manner relevant to anesthetic action.¹⁵ These findings reveal the PDZ domain as a new molecular target for inhalational anesthetics. We have also found that PSD-95 knockdown significantly reduced MAC for isoflurane and attenuated the NMDA-induced increase in isoflurane MAC.¹⁶

To further define the role of PSD-95 PDZ2 domain-mediated protein interactions in the molecular mechanisms of inhalational anesthetics, we constructed a peptide comprising the PSD-95 PDZ2 and rendered it cell permeable by fusing it to the protein transduction domain (PTD) of the human immunodeficiency virus type 1 Tat protein to obtain the fusion peptide Tat-PSD-95 PDZ2. To investigate the effect of disrupting the PDZ domain-mediated protein interactions on the threshold for halothane anesthesia, we injected mice intraperitoneally with this fusion peptide and then measured their MAC and righting reflex EC₅₀ (RREC₅₀) for halothane.

Materials and Methods

Animal Preparation

Male C57Bl/6J mice (aged 8-10 weeks) were obtained from Jackson Laboratories (Bar Harbor, MA) and acclimated in our animal facility for a minimum of 1 week before use in experiments. The mice were housed under standard conditions with a 12-h light/dark cycle and allowed food and water *ad libitum*. All animal experiments were conducted with the approval of the Animal Care and Use Committee at Johns Hopkins University, Baltimore, Maryland, and were consistent with the National Institutes of Health *Guide for the Care and Use of*

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Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. The animal assignment was blinded to the observer for all of *in vivo* testing including MAC measurement, RREC₅₀ determination, and locomotor function test.

Construction and Purification of Tat Fusion Peptides

The complementary DNA (cDNA) encoding the PSD-95 PDZ2 was prepared in our laboratory as described previously.¹⁵ Here, we used subcloning to construct a Tat-PSD-95 PDZ2 plasmid by inserting PSD-95 PDZ2 cDNA into the pTAT-HA expression vector, which contains an amino-terminal, in-frame, 11-amino acid, minimal transduction domain (residues 47–57 of human immunodeficiency virus Tat protein) termed Tat.¹⁷ Two control plasmids were also constructed: mutated Tat-PSD-95 PDZ2, in which three sites critical for interactions between NMDARs and PSD-95 were mutated (K165T, L170R, and H182L), and PSD-95 PDZ2, which contained the same sequences as Tat-PSD-95 PDZ2 but without Tat PTD. To produce the fusion peptides, these plasmids were transformed into *Escherichia coli* BL21 cells, and protein expression was induced by 0.5 mM isopropylthiogalactoside at 37°C for 4 h. The fusion peptides were purified using Ni-NTA agarose (Qiagen, Valencia, CA) according to a standard 6× histidine-tagged protein purification protocol. The resulting fusion peptides were dialyzed twice against phosphate-buffered saline. The purified peptides were verified by Coomassie blue staining and Western blot analysis and then stored in 10% glycerol/phosphate-buffered saline at –80°C until use.

In Vivo Administration of Tat Fusion Peptides

The purified fusion peptides at the indicated amounts were injected intraperitoneally into mice in 300 μl phosphate-buffered saline and 10% glycerol. The mice were given Tat-PSD-95 PDZ2 or control peptide (mutated Tat-PSD-95 PDZ2 or PSD-95 PDZ2 without Tat) 4 h before MAC measurement and righting reflex testing. All of the animals were assigned randomly to experimental groups consisting of six to eight animals each. Western blot analysis was then used to verify the CNS delivery of these fusion peptides after intraperitoneal injection.

Western Blot Analysis

Cerebral cortex, hippocampus, and lumbar spinal cord were harvested 4 h after intraperitoneal injection of the fusion peptides. Total proteins from these tissues were extracted. In brief, the tissues were removed and homogenized in homogenization buffer¹⁸ (10 mM Tris-HCl, 5 mM MgCl₂, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 2 μM pepstatin A, and 320 mM sucrose, pH 7.4). The crude homogenates were centrifuged at 700g for 15 min at 4°C. The pellets were

rehomogenized and spun again at 700g, and the supernatants were combined and diluted in resuspension buffer¹⁸ (10 mM Tris-HCl, 5 mM MgCl₂, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 2 μM pepstatin A, and 250 mM sucrose, pH 7.4). Next, the protein extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrotransferred to nitrocellulose membranes, and then immunoblotted with monoclonal anti-His antibody (Sigma, St. Louis, MO) diluted (1:1,000) in blocking solution containing 3% nonfat dry milk and 0.1% Tween-20 in Tris-HCl-buffered saline for 1 h at room temperature. After extensive washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin (Bio-Rad Laboratories, Hercules, CA) at a dilution of 1:3,000 for another 1 h. Specific proteins were detected by enhanced chemiluminescence (Amersham, Piscataway, NJ). Tubulin served as a loading control, and cerebral cortex was used for its detection.

In Vivo Binding Assay: Coimmunoprecipitation

Five micrograms of the affinity-purified rabbit NR2A/2B antibody (Chemicon, Temecula, CA) was incubated with 100 μl protein A-Sepharose slurry for 1 h, and the complex was spun down at 2,000 rpm for 4 min. The solubilized membrane fraction (500 μg) from the aforementioned different groups of treated mice then was added to the Sepharose beads, and the mixture was incubated for approximately 2–3 h at 4°C. The mixture was washed once with 1% Triton X-100 in immunoprecipitation buffer¹⁹ (containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 5 mM EGTA, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 50 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, and 20 U/ml Trasylol), twice with 1% Triton X-100 in immunoprecipitation buffer plus 300 mM NaCl, and three times with immunoprecipitation buffer. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by NR2A/2B or PSD-95 antibody (Upstate, Lake Placid, NY). As a positive control (input), 50 μg of the solubilized membrane fraction was loaded onto the gel. The NR2A/2B antibody was preincubated with excess NR2 peptide (100 μg/ml) to verify its specificity.

Measurement of Halothane Mac

Measurement of halothane MAC value was conducted as previously described, with minor modification.^{20–22} Mice were placed in individual hard plastic chambers 3 h after the injection of the fusion peptides. Each chamber was fitted with a rubber stopper at one end through which the mouse's tail and a rectal temperature probe protruded. Groups of four mice were given halothane in oxygen (4 l/min total gas flow). A gas sample was continuously drawn, and the anesthetic concentration was measured with an agent analyzer (Ohmeda 5250 RGM;

Louisville, CO). A rectal temperature probe was inserted during light general anesthesia, and temperature was kept at approximately 36°–38°C with heat lamps throughout the experiment. Mice initially breathed approximately 1.5% halothane for 60 min. Next, a 15-cm hemostatic forceps was applied to the tail for 1 min, and the mice were observed for movement in response to the stimulation. In each case, the tail was stimulated proximal to the previous test site. Only the middle third of the tail was used for tail clamping. The concentration of the anesthetic agent at which the mouse exhibited motor activity (gross movements of the head, extremities, and/or body) was considered one that permitted a positive motor response. The anesthetic concentration was increased (or decreased) in steps of 0.1% until the positive response disappeared (or *vice versa*), with 10 min for equilibration allowed after each change of anesthetic concentration. MAC is defined as the concentration midway between the highest concentration that permitted movement in response to the stimulus and the lowest concentration that prevented movement.

Determination of Halothane $RREC_{50}$

After the measurement of MAC, the halothane concentration was halved for 10 min, and the animal was turned on its back to test the righting reflex, defined as a return onto all four paws within 1 min.^{20–22} The halothane concentration was reduced by 0.1% for 10 min if the animal did not right itself, and the righting reflex was subsequently retested. $RREC_{50}$ was calculated for each mouse as the mean value of the anesthetic concentrations that just permitted and just prevented the righting reflex.

Tests for Locomotor Function

The effects of Tat fusion peptides on locomotor function were examined 4 h after intraperitoneal injection. The following tests were performed as described previously.²³ (1) Placing reflex: The mouse was held with hind limbs slightly lower than forelimbs, and the dorsal surface of the hind paws was brought into contact with the edge of a table. The experimenter recorded whether the mouse placed its hind paws on the table surface reflexively. (2) Grasping reflex: The mouse was placed on a wire grid, and the experimenter recorded whether the hind paws grasped the wire on contact. Scores for these reflexes were based on counts of each normal reflex exhibited in six trials.

Statistical Analysis

Data are expressed as mean \pm SEM and statistically analyzed with one-way analysis of variance followed by the Student-Newman-Keuls method. Statistical significance was set at $P < 0.05$. Statistical analysis was conducted using SigmaStat 2.0 software (SPSS, Inc., Chicago, IL).

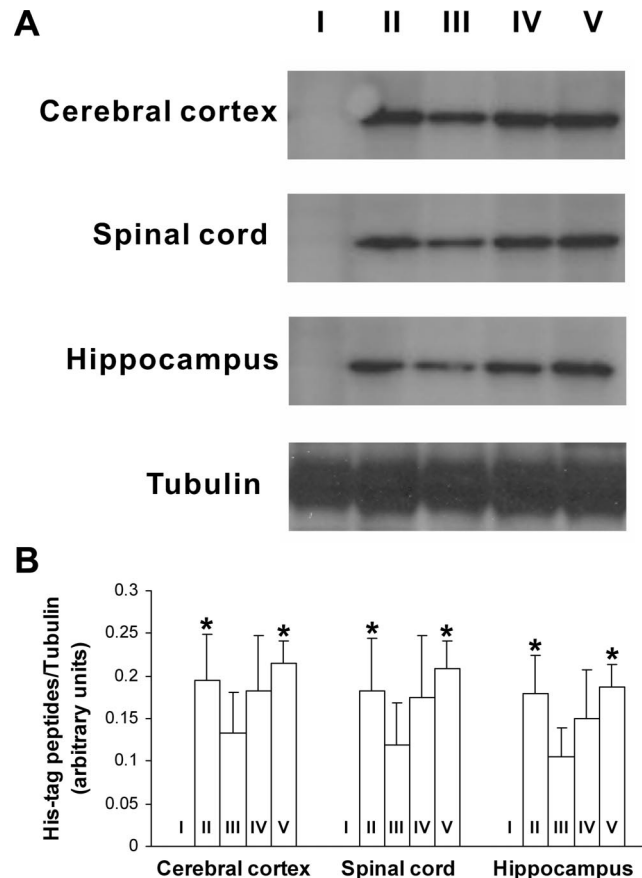


Fig. 1. Tat peptides were delivered into the central nervous system after intraperitoneal injection. I: PSD-95 PDZ2 (8 mg/kg); II: mutated Tat-PSD-95 PDZ2 (8 mg/kg); III: Tat-PSD-95 PDZ2 (2 mg/kg); IV: Tat-PSD-95 PDZ2 (4 mg/kg); V: Tat-PSD-95 PDZ2 (8 mg/kg). Tubulin served as a loading control. The data shown are representative of three independent experiments (A) and the results after statistical analysis (B). * $P < 0.05$ versus group III.

Results

CNS Delivery of Tat Peptides after Intraperitoneal Injection

Western blotting showed that after intraperitoneal injection, Tat-linked fusion peptides (Tat-PSD-95 PDZ2 and mutated Tat-PSD-95 PDZ2), but not PSD-95 PDZ2 without Tat, were delivered into cerebral cortex, hippocampus, and lumbar spinal cord of the mice (fig. 1). Moreover, Tat-PSD-95 PDZ2 was delivered into the spinal cord in a dose-dependent manner (fig. 1). No significant difference was observed in the protein transduction domain-mediated spinal delivery of Tat-PSD-95 PDZ2 and mutated Tat-PSD-95 PDZ2 (fig. 1).

Tat-PSD-95 PDZ2 Markedly Disrupted the Interactions between NMDAR NR2 Subunits and PSD-95

Coimmunoprecipitation assay was used to discover whether NMDAR-PSD-95 protein interactions were interrupted by Tat fusion peptides. We found that Tat-PSD-95 PDZ2 markedly disrupted the interactions between

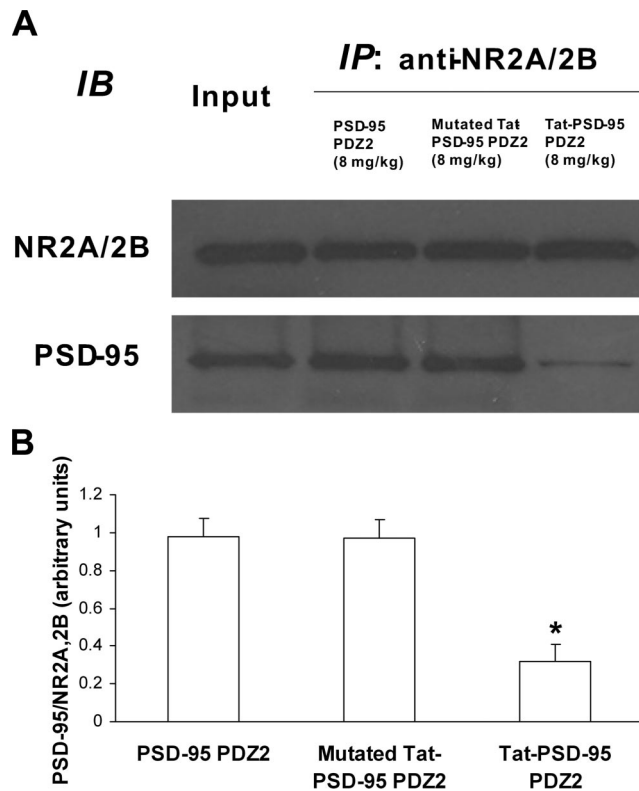


Fig. 2. Interaction of *N*-methyl-D-aspartate receptor NR2A/2B with PSD-95 in the spinal cord was disrupted by systemic administration with Tat-PSD-95 PDZ2. NR2A/2B antibody was used to immunoprecipitate NR2A/2B and its interacting proteins after intraperitoneal injection of Tat-PSD-95 PDZ2, mutated Tat-PSD-95 PDZ2, and PSD-95 PDZ2 without Tat. Note that Tat-PSD-95 PDZ2 (8 mg/kg) markedly blocked the interaction between NR2A/2B and PSD-95 and that mutated Tat-PSD-95 PDZ2 (8 mg/kg) had no effect on this interaction compared with the effect of PSD-95 PDZ2 (8 mg/kg). The amount of sample loaded for the input was 10% of that for the immunoprecipitation. The data shown are representative of three independent experiments (A) and the results after statistical analysis (B). **P* < 0.05 versus PSD-95 PDZ2 group. IB = immunoblotting; IP = immunoprecipitation.

NMDAR NR2 subunits and PSD-95 (fig. 2). However, mutated Tat-PSD-95 PDZ2 had no effect (fig. 2).

After mice were given intraperitoneal injection of Tat-PSD-95 PDZ2, mutated Tat-PSD-95 PDZ2, or PSD-95 PDZ2 without Tat, NR2A/2B antibody was used to immunoprecipitate NR2A/2B and its interacting proteins from spinal cord homogenates (fig. 2). We found that Tat-PSD-95 PDZ2 (8 mg/kg) markedly blocked the interaction between NR2A/2B and PSD-95 but that neither mutated Tat-PSD-95 PDZ2 (8 mg/kg) nor PSD-95 PDZ2 (8 mg/kg) had an effect on this interaction. The specificity of the NR2A/2B antibody was verified by preincubation with NR2 peptide, and no bands were detected in this condition (data not shown).

Effect of Tat Fusion Peptides on the Threshold for Halothane Anesthesia

After mice were given intraperitoneal injection of the fusion peptides, halothane MAC and RREC₅₀ were mea-

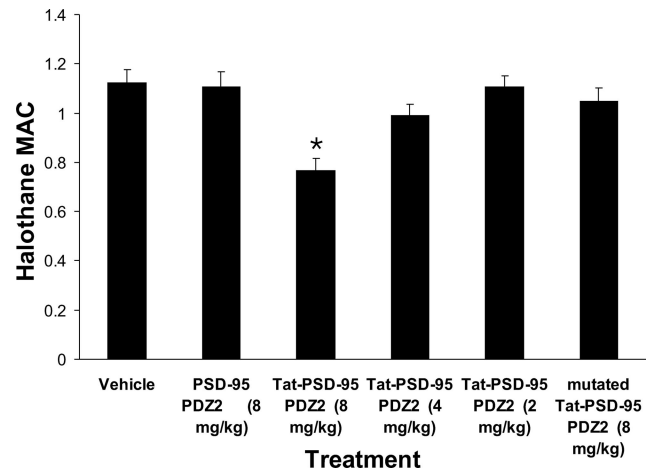


Fig. 3. Systemic pretreatment with Tat-PSD-95 PDZ2 significantly reduced halothane minimum alveolar concentration (MAC). Halothane MAC was measured after intraperitoneal injection of Tat-PSD-95 PDZ2 (n = 8), mutated Tat-PSD-95 PDZ2 (n = 8), and PSD-95 PDZ2 without Tat (n = 6). Note that Tat-PSD-95 PDZ2 dose-dependently reduced halothane MAC. However, mutated Tat-PSD-95 PDZ2 and PSD-95 PDZ2 had no effect. **P* < 0.05 versus vehicle group.

sured. We found that Tat-PSD-95 PDZ2 dose-dependently reduced halothane MAC and RREC₅₀ (figs. 3 and 4). However, mutated Tat-PSD-95 PDZ2 and PSD-95 PDZ2 without Tat had no effect (figs. 3 and 4). As a control, we observed that these peptides had no effect on locomotor function of unanesthetized mice (table 1). The mice showed normal grooming behavior, normal levels of activity, and no significant change in either blood pressure or heart rate after intraperitoneal injection of these peptides.

In the MAC study, the value for halothane MAC in vehicle-treated group was 1.12 ± 0.05. In the groups treated with Tat-PSD-95 PDZ2 at doses of 2, 4, or 8

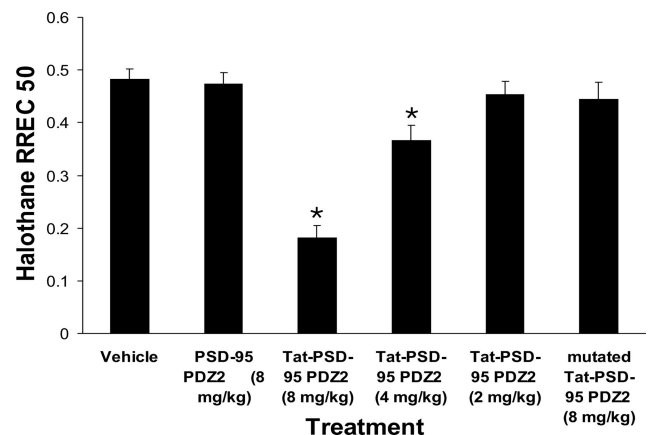


Fig. 4. Systemic pretreatment with Tat-PSD-95 PDZ2 significantly reduced halothane righting reflex EC₅₀ (RREC₅₀). Halothane RREC₅₀ was determined after minimum alveolar concentration measurement after intraperitoneal injection of Tat-PSD-95 PDZ2 (n = 8), mutated Tat-PSD-95 PDZ2 (n = 8), and PSD-95 PDZ2 without Tat (n = 6). Note that Tat-PSD-95 PDZ2 dose-dependently reduced halothane RREC₅₀. However, mutated Tat-PSD-95 PDZ2 and PSD-95 PDZ2 had no effect. **P* < 0.05 versus vehicle group.

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Table 1. Intraperitoneal Injection of Tat Peptides Had No Effect on Locomotor Function of Unanesthetized Mice

Agents	Placing	Grasping
Vehicle	6 (0)	6 (0)
PSD-95 PDZ2, 8 mg/kg	6 (0)	6 (0)
Tat-PSD-95 PDZ2, 8 mg/kg	6 (0)	6 (0)
Mutated Tat-PSD-95 PDZ2, 8 mg/kg	6 (0)	6 (0)

Data are mean (SEM). Six trials are taken for each testing. Score 1 for each normal response and score 0 when the animals fail.

PDZ = PSD-95, Dlg, and ZO-1; PSD-95 = postsynaptic density protein 95.

mg/kg, the halothane MAC values were 1.11 ± 0.05 , 0.99 ± 0.05 , or 0.77 ± 0.05 , respectively (fig. 3). One-way analysis of variance showed that halothane MAC was significantly altered after pretreatment with this peptide ($P < 0.05$; fig. 3). The highest dose (8 mg/kg) of Tat-PSD-95 PDZ2 significantly reduced the halothane MAC compared with the vehicle-treated group ($P < 0.05$). In contrast, intraperitoneal injection with the same dose of mutated Tat-PSD-95 PDZ2 (8 mg/kg) or PSD-95 PDZ2 without Tat (8 mg/kg) had no effect on the halothane MAC ($P > 0.05$; fig. 3).

In the RREC₅₀ study, the value for halothane RREC₅₀ in vehicle-treated group was 0.48 ± 0.02 . In the groups treated with Tat-PSD-95 PDZ2 at doses of 2, 4, or 8 mg/kg, the halothane RREC₅₀ values were 0.45 ± 0.03 , 0.37 ± 0.03 , or 0.18 ± 0.02 , respectively (fig. 4). One-way analysis of variance showed that halothane RREC₅₀ was significantly altered after pretreatment with this peptide ($P < 0.05$; fig. 4). The two higher doses (4 and 8 mg/kg) of Tat-PSD-95 PDZ2 significantly reduced the halothane RREC₅₀ compared with the vehicle-treated group ($P < 0.05$; fig. 4). In contrast, intraperitoneal injection of mutated Tat-PSD-95 PDZ2 (8 mg/kg) or PSD-95 PDZ2 without Tat (8 mg/kg) had no effect on the halothane RREC₅₀ ($P > 0.05$; fig. 4).

Discussion

Results from our current studies indicate that intraperitoneally injected fusion peptide Tat-PSD-95 PDZ2 (1) can be delivered into the CNS, (2) dose-dependently disrupts the protein-protein interactions between NMDAR NR2 subunits and PSD-95, and (3) significantly reduces halothane MAC and RREC₅₀. These results suggest that PDZ domain-mediated protein interactions at synapses in the CNS might play an important role in the molecular mechanisms of halothane anesthesia.

Protein transduction domain-mediated *in vivo* delivery of biologically active peptides represents a novel and promising strategy to treat CNS diseases. Although the exact mechanism of transduction across the cellular membrane is currently unknown, the first step of the transduction seems to involve a charge-charge interaction of the basic PTD with acidic motifs on the cellular

membrane. It has been demonstrated that fusion peptides containing the PTD sequence derived from human immunodeficiency virus Tat protein can be transduced into the CNS after systemic administration.²⁴ In our current study, we found that after intraperitoneal injection, Tat-PSD-95 PDZ2 and mutated Tat-PSD-95 PDZ2 were detected in cerebral cortex, hippocampus, and lumbar spinal cord of mice, but PSD-95 PDZ2 lacking Tat was not seen in these tissues. These results support the conclusion that a wide variety of cargo, including peptides and full-length proteins, can be delivered into cells when linked to the PTD sequence.²⁵

The interactions between NMDAR NR2 subunits and PSD-95 are mediated by the second PDZ domain of PSD-95 protein.¹³ The Shaker-type potassium channel, Kv1.4, also binds to the PSD-95 PDZ2.¹⁴ Therefore, we hypothesized that competition with a peptide consisting of PSD-95 PDZ2 could disrupt this PDZ domain-mediated protein interaction. Our current results support this hypothesis. By *in vivo* binding assay, we show here that fusion peptide Tat-PSD-95 PDZ2 dose-dependently suppresses the NMDAR-PSD-95 protein interaction. However, mutation of three critical amino acids (K165T, L170R, and H182L) of the PDZ2 domain in the fusion peptide eliminated its ability to affect the interaction. The mutated Tat-PSD-95 PDZ2 and PSD-95 PDZ2 without Tat served as controls for Tat-PSD-95 PDZ2 in our studies.

Inhalational anesthetics have been in widespread use in modern surgical procedures, but their molecular mechanisms remain poorly understood. PDZ domain-mediated protein interactions play a central role in organizing signaling complexes around synaptic receptors for efficient signal transduction. Our previous studies have demonstrated that halothane dose-dependently and reversibly inhibits PSD-95 PDZ domain-mediated protein interactions and that the halothane binding site on PSD-95 PDZ2 completely overlaps with the binding pocket of PSD-95 for NMDAR NR2 subunits,¹⁵ suggesting a new concept that affecting PDZ domain-mediated protein interactions at synapses in the CNS might be one of molecular mechanisms by which the general anesthetic state is achieved. By knocking down PSD-95 expression in the spinal cord, we have shown that the deficiency of spinal PSD-95 reduced isoflurane MAC in rats.¹⁶ In the current study, we found that fusion peptide Tat-PSD-95 PDZ2, but not mutated Tat-PSD-95 PDZ2 or PSD-95 PDZ2, dose-dependently reduced halothane MAC and RREC₅₀ in mice by disrupting the PDZ domain-mediated protein interactions. These results provide *in vivo* evidence to support this concept. On the other hand, a key concern with inhalational anesthetics is the narrow relation between the therapeutic and toxic doses. This concern has negative impact on clinical administration of the inhalational anesthetics. Tat-PSD-95 PDZ2, a novel agent, markedly reduces the amount of inhalational anesthetics needed to induce anesthesia, thereby reducing

the dose-dependent toxic side effects of the inhalational anesthetics.

In conclusion, this study demonstrates that by disrupting PDZ domain-mediated protein interactions, intraperitoneal injection of cell-permeable fusion peptide Tat-PSD-95 PDZ2 dose-dependently reduces the threshold for halothane anesthesia. These results provide a novel insight into the molecular mechanisms that underlie the inhalational anesthetic state and a new target for development of anesthetics.

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