Inhalational Anesthetics Disrupt Postsynaptic Density Protein-95, Drosophila Disc Large Tumor Suppressor, and Zonula Occludens-1 Domain Protein Interactions Critical to Action of Several Excitatory Receptor Channels Related to Anesthesia

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ABSTRACT

Background: The authors have shown previously that inhaled anesthetics disrupt the interaction between the second postsynaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1 (PDZ) domain of postsynaptic density protein-95 (PSD-95) and the C-terminus of N-methyl-D-aspartate receptor subunits NR2A and NR2B. The study data indicate that PDZ domains may serve as a molecular target for inhaled anesthetics. However, the underlying molecular mechanisms remain to be illustrated.

Methods: Glutathione S-transferase pull-down assay, coimmunoprecipitation, and yeast two-hybrid analysis were used to assess PDZ domain–mediated protein–protein interactions in different conditions. Nuclear magnetic resonance spectroscopy was used to investigate isoflurane-induced chemical shift changes in the PDZ1–3 domains of PSD-95. A surface plasmon resonance–based BiACore (Sweden) assay was used to examine the ability of isoflurane to inhibit the PDZ domain–mediated protein–protein interactions in real time.

Results: Halothane and isoflurane dose-dependently inhibited PDZ domain–mediated interactions between PSD-95 and Shaker-type potassium channel Kv1.4 and between α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subunit GluA2 and its interacting proteins—glutamate receptor–interacting protein or protein interacting with c kinase 1. However, halothane and isoflurane had no effect on PDZ domain–mediated interactions between γ-aminobutyric acid type B receptor and its interacting proteins. The inhaled anesthetic isoflurane mostly affected the residues close to or in the peptide-binding groove of PSD-95 PDZ1 and PDZ2 (especially PDZ2), while barely affecting the peptide-binding groove of PSD-95 PDZ3.

Conclusion: These results suggest that inhaled anesthetics interfere with PDZ domain–mediated protein–protein interactions at several receptors important to neuronal excitation, anesthesia, and pain processing. (Anesthesiology 2015; 122:776-86)

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NESTHETICS have been used in surgical procedures for well over a hundred years, but the molecular mechanism that underlies inhalational anesthesia is still poorly understood. Ion channels and receptors at synapses of the central nervous system (CNS) have been highlighted as potential targets for inhaled anesthetics.1–5 In the CNS, these ion channels and receptors are linked to their downstream signaling pathways through postsynaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1 (PDZ) domain–mediated protein–protein interactions. The PDZ domain can recognize and bind to specific amino acid sequences, including those at the C-termini of receptors and ion channels.6–8 Our previous studies have shown that knockdown of PDZ domain–containing scaffolding protein postsynaptic density protein-95 (PSD-95) facilitates isoflurane anesthesia9 and inhibits chronic pain behaviors.10,11 PSD-95, a member of the membrane-associated guanylate kinase family of proteins that assemble protein complexes at synapses, is composed of three tandem PDZ domains (PDZ1–3) at the N-terminus and guanylate kinase and Src homology 3 domains.12–14 The

What We Already Know about This Topic

• Ion channels and ligated-gated receptors are putative targets of anesthetic agents. These targets are associated with their downstream signaling pathways via protein interaction domains of approximately 90 residues called postsynaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1 (PDZ) domains.

• Disruption of PDZ domain–mediated protein–protein interactions can impact anesthetic effects. The interaction of anesthetics with different PDZ domains, however, is not clear.

• Using sophisticated and state-of-the-art techniques, anesthetic interaction with PDZ-binding domains 1 to 3 was investigated.

What This Article Tells Us That Is New

• Halothane and isoflurane disrupted PDZ domain–mediated interactions between potassium channel Kv1.4 and GluA2 subunit of AMPA receptor with their respective binding partners. Neither agent affected binding of γ-aminobutyric acid type B receptors with their binding partners.

• Anesthetics affected binding to PDZ1 and PDZ2 but not to PDZ3 domains.

• Anesthetics interfere with PDZ domain–mediated protein–protein interactions of several receptors that are important to excitatory neuronal function.

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first two PDZ domains of PSD-95 can bind the NR2A and NR2B subunits of N-methyl-D-aspartate (NMDA) receptors, neuronal nitric oxide synthase, Shaker-type potassium channel Kv1.4, and Src family tyrosine kinase. Furthermore, we have demonstrated that PDZ domain–mediated interactions between PSD-95 and NMDA receptors or neuronal nitric oxide synthase are disrupted by clinically relevant concentrations of inhaled anesthetic halothane. By injecting cell-permeable peptide Tat-PSD-95 PDZ2 (comprising the second PDZ domain of PSD-95) intraperitoneally into mice, we also showed that disrupting PSD-95 PDZ domain–mediated protein–protein interactions reduces the threshold for halothane anesthesia and diminishes chronic inflammatory pain.

Taken together, these results suggest that the PDZ domain at synapses of the CNS may be an important molecular target for inhaled anesthetics and that alteration of PDZ domain–mediated protein–protein interactions may contribute to the molecular mechanism of inhaled anesthesia. However, it is unclear whether and how inhaled anesthetics interact with different PDZ domains in the CNS.

In the current study, we used nuclear magnetic resonance (NMR) and other state-of-the-art techniques to examine in greater detail the nature of the anesthetic interaction with the PDZ protein–binding domains.

**Materials and Methods**

**Glutathione S-transferase Pull-down Assay**

Glutathione S-transferase (GST) and GST fusion proteins (GST-PSD-95 PDZ2 and GST-Kv1.4 C-terminus 100) were prepared in BL21 (Novagen, Germany) with glutathione sepharose 4B (Amersham Pharmacia Biotech Inc., USA) as an affinity resin for purification. 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 Laboratory Animals. Membrane-bound proteins were extracted from mouse cerebral cortex and solubilized. Mouse cerebral cortex was lysed in homogenizing buffer (containing 10 mM Tris-HCl [pH 8.0], 5 mM MgCl₂, 250 mM sucrose, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethanesulfonylfluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 20 µg/ml pepstatin A) with a rotor-stator Polytron homogenizer (Brinkmann Instruments, USA). The homogenized solution was centrifuged at 900 g for 15 min, and the supernatant was removed and centrifuged again at 37,000 g for 40 min. The precipitate was solubilized with resuspension buffer (containing 500 mM Tris-HCl, 1% [vol/vol] TritonX-100, and 1% [wt/vol] sodium deoxycholate) and centrifuged at 37,000 g for 20 min. The supernatant contained the membrane-bound proteins. For binding experiments, glutathione sepharose 4B (40 µl) was preincubated with 1 nmol of GST or GST fusion protein for 30 min and then the membrane-bound proteins (400 µg) were mixed with the GST fusion protein at room temperature for 1 h in the presence of different concentrations of halothane delivered in an incubation chamber (described under Yeast Two-hybrid Analysis). After the resin was washed five times with washing buffer (500 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethanesulfonylfluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 20 µg/ml pepstatin A in phosphate-buffered saline), the bound proteins were eluted in sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer, separated by electrophoresis, and detected by immunoblotting with anti-PSD-95 (Chemicon, USA), anti-Kv1.4 (Chemicon), and anti-GST (Santa Cruz Biotechnology Inc., USA) antibodies.

**Coimmunoprecipitation Assay**

The membrane-bound proteins were prepared as described above. The affinity-purified rabbit anti-Kv1.4 antibody (4 µg) was incubated with 100 µl of protein A–sepharose slurry for 1 h. The solubilized membrane proteins (400 µg) were then added to the sepharose beads, and the mixture was incubated with different concentrations of isoflurane for 2 h at room temperature. The mixture was washed once with 1% Triton X-100 in immunoprecipitation buffer (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, and 0.1 mM phenylmethylsulfonyl fluoride plus 20 U/ml Trasylol), twice with 1% Triton X-100 in immunoprecipitation buffer plus 300 mM NaCl, and three times with immunoprecipitation buffer. The bound proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and detected by immunoblotting with anti-PSD-95 and anti-Kv1.4 antibodies. As a positive control (input), 40 µg of the solubilized membrane proteins were loaded onto the gel. The specificity of the anti-Kv1.4 antibody was verified by preincubation with Kv1.4 fusion peptide.

**Yeast Two-hybrid Analysis**

The two partners in each interaction were subcloned into EcoRI and BamHI sites of the yeast vectors pGADT7 and pGBKAT7 (Clontech Laboratories Inc., USA), respectively. The yeast reporter strain AH109 (Clontech Laboratories Inc.) was transformed with the pGBT9 and pGAD424 plasmids. Protein–protein interactions were evaluated as described in our previous study by growth of yeast on selective agar plates sealed in incubator chambers containing different concentrations of isoflurane, which was introduced into the chambers by its conventional calibrated anesthesia vaporizer (OHIO Medical Co.,...
protein was extended with His Tag for facilitating the Ni. The N-terminus of the Escherichia coli PDZ3 (302 to 393) was incorporated into the pET28 vector. The concentration of 5 to 6.8 mg/ml or 125 to 170 μM. The isoflurane concentration was calibrated by 19F NMR with an external reference of LNSCSNRRVYKKSPIESDV and NR2B (FNGSSNG-) of the last 20 amino acids from the C-terminal of NR2A. The baseline sensorgram was obtained by injecting PSD-95 PDZ2 and NR2B C-terminal peptide was analyzed on a BIAcore 2000 system (BIAcore). PSD-95 PDZ2 (20 μM) was injected with different concentrations of isoflurane over the NR2B peptide–coupled surface at a flow rate of 5 μl/min. Anesthetic concentrations were determined by head-space sampling from an aliquot of the anesthetic-containing solution. Sensorgrams were recorded and normalized by subtracting the baseline resonance unit value. The baseline sensorgram was obtained by injecting PSD-95 PDZ2 over a nonprotein, blocked surface. Between successive measurements, the surface was regenerated with 50 mM phosphoric acid (3 min contact time). The analysis of kinetic parameters was performed with BIAevaluation version 3.0 software according to the manufacturer's instructions.

**Statistical Analysis**

Data from the yeast two-hybrid experiments were expressed as means ± SD and statistically analyzed with SigmaStat version 3.1 software (Systat Software Inc., USA) by using one-way ANOVA followed by Student–Newman–Keul method. The nature of hypothesis testing is two tailed. The sample sizes were selected based on our previous study. Statistical significance was set at P value less than 0.05.

**Results**

**Effects of Halothane and Isoflurane on PDZ Domain–mediated Interactions between NMDA Receptor–Interacting Protein PSD-95 and Kv1.4**

Our previous studies showed that PDZ domain–mediated protein–protein interactions between PSD-95 and NMDA receptors or neuronal nitric oxide synthase are disrupted by clinically relevant concentrations of inhaled anesthetics. In this study, we used an yeast two-hybrid approach to investigate whether halothane and isoflurane also disrupt PDZ domain–mediated interactions between PSD-95 and...
potassium channel Kv1.4. In the absence of anesthetic, the second PDZ domain of PSD-95 (PSD-95 PDZ2) interacted with the C-terminal tail of Kv1.4 (Kv1.4 CT100), as evidenced by the growth of the yeast cells harboring both pGADT7-PSD-95 PDZ2 and pGBK7-Kv1.4 CT100 fusion protein plasmids in synthetic dropout agar plates lacking adenine, leucine, tryptophan, and histidine (fig. 1). However, the yeast cell growth was slowed by halothane (fig. 1, A and B) and isoflurane (fig. 1, C–E) in a dose-dependent manner, indicating that these anesthetics dose-dependently inhibit PDZ domain–mediated protein–protein interactions between PSD-95 and Kv1.4. Compared with isoflurane, halothane disrupted the interactions with a stronger potency. A high but still clinically relevant concentration of halothane (0.95 mM) completely blocked the yeast cell growth (fig. 1, A and B). We verified that halothane itself was not cytotoxic to yeast cells by growing the same strain on low-stringency media (synthetic dropout agar plates that lack leucine and tryptophan and are selective for the yeast cells cotransformed with pGADT7 and pGBK7) in the presence of the same concentrations of halothane (fig. 1A); halothane did not inhibit the growth of the control yeast cells.

Next, we used GST pull-down and coimmunoprecipitation assays to further confirm the yeast two-hybrid results. GST-PSD-95 PDZ2 and GST-Kv1.4 CT100, but not GST alone, precipitated Kv1.4 and PSD-95 (fig. 2A), in the absence of halothane. However, halothane dose-dependently inhibited the binding of Kv1.4 to GST-PSD-95 PDZ2 and the binding of PSD-95 to GST-Kv1.4 CT100 (fig. 2A). To determine whether inhaled anesthetics disrupt PDZ domain–mediated protein–protein interactions in a physiological setting, we used a coimmunoprecipitation assay to detect in vivo binding of PSD-95 to Kv1.4. We found that immunoprecipitation of Kv1.4 by its specific antibody resulted in coprecipitation of PSD-95 under normal conditions but that isoflurane dose-dependently inhibited the coprecipitation of PSD-95 (fig. 2B). These results suggest that inhaled anesthetics may disrupt the physiological complex of PSD-95 and Kv1.4 in the CNS.

Effects of Halothane and Isoflurane on PDZ Domain–mediated Interactions between α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptor and AMPA Receptor–interacting Proteins, Protein Interacting with c Kinase 1, or Glutamate Receptor-interacting Protein

To test whether inhaled anesthetics also disrupt AMPA receptor–involved PDZ domain–mediated protein–protein interactions, we investigated the effects of halothane and isoflurane on interactions between the AMPA receptor GluA2 subunit and glutamate receptor–interacting protein or protein interacting with c kinase 1 in an yeast two-hybrid system. Similar to our previous studies and the results described above for PSD-95, halothane and isoflurane dose-dependently inhibited the growth of yeast cells harboring both pGADT7-GluR2 C-terminus and pGBK7-glutamate receptor–interacting protein PDZ4,5 or pGBK7-protein interacting with c kinase 1 PDZ fusion protein plasmids (fig. 3). As in our other studies, halothane was more potent that isoflurane at disrupting these PDZ domain–mediated protein–protein interactions (fig. 3).

Fig. 1. Halothane and isoflurane dose-dependently disrupt postsynaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1 (PDZ) domain–mediated interactions between postsynaptic density protein-95 (PSD-95) and potassium channel Kv1.4 in an yeast two-hybrid system. (A and B) The effect of halothane on the growth of yeast cells harboring pGADT7-PSD-95 PDZ2 and pGBK7-Kv1.4 CT100 (n = 6). (A) Halothane dose-dependently inhibited the yeast growth. (B) Yeast growth is shown as a percent of control, relative to halothane concentration. *P < 0.05 and **P < 0.01 versus 0.00 mM halothane. (C–E) The effect of isoflurane on the growth of yeast cells harboring pGADT7-PSD-95 PDZ2 and pGBK7-Kv1.4 CT100 (n = 3). (C) Isoflurane dose-dependently inhibited the yeast growth. (D) Yeast growth is shown as a percent of control, relative to isoflurane concentration. *P < 0.05 versus 0.00 mM isoflurane. (E) Binned scatter plots of C. CT100 = C-terminal 100 amino acids.
Isoflurane Dose-dependently Disrupts PDZ Domain–mediated Interactions between PSD-95 and NR2B in a Yeast Two-hybrid System

We also used a yeast two-hybrid approach to determine the effect of isoflurane on the interaction of PSD-95 PDZ2 with NR2B C-terminus (C-terminal 20 amino acids). In the absence of isoflurane, PSD-95 PDZ2 interacted with the C-terminal tail of NMDA receptors NR2B subunit, as evidenced by the growth of the yeast cells harboring both pGADT7-PSD-95 PDZ2 and pGBK7-NR2B C-terminus fusion protein plasmids in synthetic dropout agar plates lacking adenine, leucine, tryptophan, and histidine (fig. 4A). However, the yeast cell growth was slowed by isoflurane (0.33, 0.66, and 0.99 mM) in a dose-dependent manner (fig. 4, A and B), indicating that isoflurane dose-dependently inhibits PDZ domain–mediated protein–protein interactions between PSD-95 and NR2B. To verify that isoflurane itself is not cytotoxic to yeast cells, we grew the same strain on low-stringency media (synthetic dropout agar plates that lack leucine and tryptophan and are selective for the yeast cells cotransformed with pGADT7 and pGBK7) in the presence of the same concentrations of isoflurane. It was shown that isoflurane did not inhibit the growth of the control yeast cells.

Isoflurane Dose-dependently Disrupts the Association between PSD-95 and NR2B in Real Time

We further used a surface plasmon resonance–based BIAcore assay to examine the ability of isoflurane to inhibit PSD-95

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**Fig. 2.** Halothane and isoflurane dose-dependently disrupt the association between postsynaptic density protein-95 (PSD-95) and potassium channel Kv1.4 in the forebrain. (A) The glutathione S-transferase (GST) pull-down assay showed that halothane dose-dependently inhibits the interaction between PSD-95 PDZ2 and Kv1.4 C-terminus. The bottom panels indicate equal amounts of loading of GST fusion proteins. (B) The coimmunoprecipitation assay showed that isoflurane dose-dependently disrupts the association between PSD-95/SAP90 and Kv1.4 in vivo. The specificity of the anti-Kv1.4 antibody was verified by preincubation with Kv1.4 fusion peptide (N). As a positive control (input), 40 μg of the solubilized membrane proteins were loaded onto the gel. IB = immunoblotting; IP = immunoprecipitation; PDZ = postsynaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1.

**Fig. 3.** Halothane and isoflurane dose-dependently disrupt postsynaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1 (PDZ) domain–mediated interactions between AMPA receptor subunit GluR2 and glutamate receptor–interacting protein (GRIP) or protein interacting with c kinase 1 (PICK1) in an yeast two-hybrid system. (A–C) The effect of halothane on the growth of yeast cells harboring pGADT7-GluR2 CT and pGBK7-GRIP PDZ2,4,5 (n = 3). (A) Halothane dose-dependently inhibited the yeast growth. (B) Yeast growth is shown as a percent of control, relative to halothane concentration. *P < 0.05 versus 0.00 mM halothane. (C) Binned scatter plots of A. (D–F) The effect of isoflurane on the growth of yeast cells harboring pGADT7-GluR2 CT and pGBK7-PICK1 PDZ (n = 3). (D) Isoflurane dose-dependently inhibited the yeast growth. (E) Yeast growth is shown as a percent of control, relative to isoflurane concentration. *P < 0.05 versus 0.00 mM isoflurane. (F) Binned scatter plots of D. CT = C-terminus.
PDZ domain–mediated protein–protein interactions in real
time. Binding of PSD-95 PDZ2 to NR2B C-terminus was
measured in real time as an increase in resonance units. In
the absence of isoflurane, 10 μM PSD-95 PDZ2 formed
a complex with immobilized NR2B C-terminal peptide as
indicated by a large increase in resonance unit level (fig. 5).
Interestingly, the inclusion of isoflurane in the mobile phase
inhibited the equilibrium binding in a dose-dependent man-
ner (fig. 5). These results suggest that isoflurane reduces the
number of sites available for the binding between PSD-
95-PDZ2 and NR2B C-terminus, thus preventing the
formation of the PDZ domain–mediated protein–protein
complex.

Effects of Halothane and Isoflurane on PDZ Domain–
mediated Interactions between the γ-Aminobutyric
Acid Type B Receptor and γ-Aminobutyric Acid Type B
Receptor–interacting Proteins PAPIN and Mupp1

Different PDZ domain–mediated protein–protein interac-
tions exist in the CNS. To define whether inhaled anesthet-
ics disrupt PDZ domain–mediated interactions between the γ-aminobutyric acid type B (GABA B) receptor subunit
2 (GABAB2R) and GABA B receptor–interacting proteins
PAPIN and Mupp1, we investigated the effects of halothane
and isoflurane on these interactions in an yeast two-hybrid
system. We used the PDZ interaction between PSD-95

![Fig. 4. Isoflurane dose-dependently disrupts postsynaptic
density protein-95, Drosophila disc large tumor suppressor,
and zonula occludens-1 (PDZ) domain–mediated interactions
between postsynaptic density protein-95 (PSD-95) (PSD-95)
and NR2B in an yeast two-hybrid system. (A) The effect of isoflurane on
the growth of yeast cells harboring pGADT7-PSD-95 PDZ2
and pGBKTK7-NR2B C-terminus. Note that isoflurane dose-
dependently inhibited the yeast growth. (B) Yeast growth is
shown as a percent of control, relative to isoflurane concen-
tration (n = 6). *P < 0.05 and **P < 0.01 versus 0.00 mM iso-
flurane.](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/933643/)

![Fig. 5. Isoflurane dose-dependently disrupts the association
between postsynaptic density protein-95 (PSD-95) and NR2B
in real time. Surface plasmon resonance analysis revealed
that isoflurane dose-dependently reduces the real-time bind-
ing of PSD-95 PDZ2 to NR2B C-terminal peptide, as illus-
trated by superimposed sensorgrams. PDZ = postsynaptic
density protein-95, Drosophila disc large tumor suppressor,
and zonula occludens-1; RU = resonance units.](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/933643/)

![Fig. 8A shows a representative 15N-1H Heteronuclear Single
Quantum Coherence (HSQC) NMR spectrum of PDZ1–3,
in which 230 peaks are assigned to individual residues based
on previous publications.26,27 Those residues without reliable
assignments or with weak intensity are labeled in lower case.
To reveal an underlying cause of isoflurane perturbation on
PSD-95 PDZ domain–mediated protein–protein interac-
tions, we studied the interaction of isoflurane to the three
PDZ domains of PSD-95 (PDZ1, PDZ2, and PDZ3) using
NMR chemical shift as a probe. For each residue, its 15N and
1H chemical shifts can be affected by changes in the local
chemical environment, such as ligand-binding or the binding-
induced alteration in protein conformations. Upon titrating
different concentrations of isoflurane into a PDZ1–3 sample,
a number of residues showed chemical shift changes in an
isoflurane-concentration–dependent manner in the 15N-1H
HSQC spectra (fig. 8B). The fitting of chemical shift changes
as a function of isoflurane concentrations provided a disas-
sociation constant (Kd) of approximately 3 mM, suggesting a
low affinity binding of isoflurane to PDZ1–3. The combined
chemical shift changes of 15N and 1H quantified the anesthetic
perturbation. We found that isoflurane affected all three PDZ
domains, but PDZ2 had the most affected residues, whereas

PDZ2 and NR2B as a positive control. Although halothane
and isoflurane dose-dependently inhibited the growth of
yeast cells harboring both pGADT7-PSD-95 PDZ2 and
pGBKTK7-NR2B C-terminus fusion protein plasmids (figs. 6
and 7), treatment with the same concentrations of the two
inhaled anesthetics had no effect on the growth of yeast
cells harboring pGADT7-GABAB2R and pGBKTK7-PAPIN
PDZ1 or pGBKTK7-Mupp1 PDZ13 fusion protein plasmids
(figs. 6 and 7). Likewise, the yeast cells grew normally in the
absence of the inhaled anesthetics (figs. 6 and 7).

Isoflurane Dose-dependently Induces Chemical Shift
Changes in the Three PDZ Domains of PSD-95

Figure 8A shows a representative 15N-1H Heteronuclear Single
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chemical shift changes of 15N and 1H quantified the anesthetic
perturbation. We found that isoflurane affected all three PDZ
domains, but PDZ2 had the most affected residues, whereas
PDZ3 had the least (fig. 8C). The affected residues in PDZ1 and PDZ2 were mostly in, or close to, their peptide-binding groove, whereas very few residues close to the binding groove in PDZ3 were affected (fig. 8C). These findings are valuable for understanding how anesthetics perturb PDZ domain–mediated protein–protein interactions.

NMR Spectral Changes in the PDZ1−3 Domains of PSD-95 Produced by Binding Peptides NR2A/2B and/or Isoflurane

To further determine whether and how isoflurane perturbs peptide binding to PDZ1−3, we characterized NMR spectral changes of the PDZ1−3 domains of PSD-95 produced by binding of peptides NR2A-c20 or NR2B-c20 in the absence and presence of isoflurane. We found that NR2A-c20 and NR2B-c20 disturbed the motion of PSD-95 PDZ1−3. Upon peptide binding, dozens of NMR peaks in the 1H-15N HSQC NMR spectra of PDZ1−3 exhibit considerable intensity changes and some of them even disappeared from the spectra (Supplemental Digital Content 1, figs. 1 and 2, http://links.lww.com/ALN/B134; fig. 9, A and B).

The NMR peak intensity changes are often associated with changes in protein motions. The reduced intensity or disappearance of peaks signified that the peptide binding probably drove the residues into intermediate time-scale motions.28–32 Significant chemical shift changes along with peptide binding were also observed in the 1H-15N HSQC NMR spectra of PDZ1−3 (Supplemental Digital Content 1, figs. 1 and 2, http://links.lww.com/ALN/B134; fig. 9, C and D). The combined 1H and 15N chemical shift changes were mapped onto the structures of PDZ1−3 (fig. 9, E–G).

In the presence of peptides NR2A-c20 or NR2B-c20, isoflurane disturbed more extended regions of PSD-95 PDZ1−3 than in the absence of the peptides as evidenced by chemical shift changes in PDZ-peptide complexes upon adding isoflurane of approximately 3 mM (Supplemental Digital Content 1, figs. 3−6, http://links.lww.com/ALN/B134).

Fig. 6. Halothane has no effect on postsynaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1 (PDZ) domain–mediated protein–protein interactions between γ-aminobutyric acid type B (GABA_{B}) receptor subunit 2 and PAPIN or Mupp1 in an yeast two-hybrid system. (A) No concentration of halothane tested inhibited the growth of yeast cells harboring pGADT7-GABAB2 and pGBK7-PAPIN PDZ1 or pGBK7-Mupp1 PDZ13. Consistent with our previous study,15 halothane dose-dependently inhibited the growth of yeast cells harboring pGADT7-postsynaptic density protein-95 (PSD-95) PDZ2 and pGBK7-NR2B C-terminus, which were used as a positive control. (B) Yeast growth is shown as a percent of control, relative to halothane concentration (n = 6). *P < 0.05 and **P < 0.01 versus 0.00 mM halothane. GABAB2 = GABA_{B} receptor subunit 2.

Fig. 7. Isoflurane has no effect on postsynaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1 (PDZ) domain–mediated interactions between γ-aminobutyric acid type B (GABA_{B}) receptor subunit 2 and PAPIN or Mupp1 in an yeast two-hybrid system. (A) No concentration of isoflurane tested inhibited the growth of yeast cells harboring pGADT7-GABAB2 and pGBK7-PAPIN PDZ1 or pGBK7-Mupp1 PDZ13. Consistent with our previous study,15 isoflurane dose-dependently inhibited the growth of yeast cells harboring pGADT7-PSD-95 PDZ2 and pGBK7-NR2B C-terminus, which were used as a positive control. (B) Yeast growth is shown as a percent of control, relative to isoflurane concentration (n = 6). *P < 0.05 and **P < 0.01 versus 0.00 mM isoflurane. GABAB2 = GABA_{B} receptor subunit 2; PSD-95 = postsynaptic density protein-95.
Furthermore, the combined chemical shift showed that isoflurane introduced larger disturbance on the NR2A-c20 complex, but more extended disturbance on the NR2B-c20 complex (fig. 9, E–G). Several residues, including A106 and S116 of PDZ1 and S188, A201, and L241 of PDZ2, even disappeared in the spectrum of the NR2A-c20 complex. The disturbance of isoflurane on the PDZ-peptide complex could be beyond the binding groove (βB and αB). For the NR2A-c20 complex, the residues most perturbed by isoflurane were not within the binding groove, as was the case for the NR2B-c20 complex. Collectively, isoflurane interacts with residues outside of the

Fig. 8. Isoflurane concentration–dependent changes in chemical shift of the postsynaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1 (PDZ) domains of postsynaptic density protein-95. (A) A representative $^{15}$N-$^1$H Heteronuclear Single Quantum Coherence spectrum of the $^{15}$N-labeled PDZ1–3 of rat source postsynaptic density protein-95 (residues 61–393) showing 230 assigned residues. (B) Representative $^1$H chemical shift changes of residues in the PDZ1–3 as a function of isoflurane concentrations. (C) Structures of PDZ1–3 mapped with the combined chemical shifts $\Delta\delta_{ppm} = (\Delta\delta_{1H})^2 + (0.2 \times \Delta\delta_{15N})^2$ induced by isoflurane. Residues–experienced chemical shift changes are highlighted in red ($\Delta\delta \geq 0.03$ ppm), green ($\Delta\delta \sim 0.02–0.03$ ppm), and orange ($\Delta\delta \sim 0.01–0.02$ ppm). Isoflurane concentration was calibrated with the external reference of trifluoroacetic acid.

Fig. 9. Changes in postsynaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1 (PDZ1–3) of postsynaptic density protein-95 (PSD-95) produced by binding of peptides NR2A-c20 or NR2B-c20 in the absence or presence of isoflurane. (A and B) The structure of PDZ1–3 of PSD-95 showing residues with diminished (left) and enhanced (right) nuclear magnetic resonance peak intensities after binding to NR2A-c20 (A) or NR2B-c20 (B). Color codes: blue = changed only by NR2A-c20 binding; red = changed only by NR2B-c20 binding; green = changed in both cases of binding. The same color codes are applied to all figures here. (C) The combined $^1$H and $^{15}$N chemical shift changes in PDZ1–3 of PSD-95 upon binding to NR2A-c20 (blue) or NR2B-c20 (red) binding. (D) The combined $^1$H and $^{15}$N chemical shift changes induced by isoflurane in PDZ1–3 of PSD-95 bound with NR2A-c20 (red) or NR2B-c20 (green). (E–G) The structure of PDZ1–3 of PSD-95 showing isoflurane-disturbed residues ($\Delta\delta \geq 0.04$ ppm) upon binding of NR2A-c20 (E), NR2B-c20 (F), or no peptide present (G). Concentrations used are 100 μM for PDZ1–3 of PSD-95, 310 μM for peptides, and 3 mM for isoflurane.
peptide-binding region once either one of the peptides bound to PDZ1–3.

Discussion

Cumulative evidence suggests that inhaled anesthetics act on multiple targets in the CNS.\(^3\)–\(^9\) It has been hypothesized that to achieve their anesthetic effect, they primarily modulate membrane-associated proteins that are involved in synaptic transmission.\(^3\) Within synapses, ion channels regulate ionic flow across the cellular membrane, thereby influencing the presynaptic release of neurotransmitters and altering postsynaptic excitability. Several ion channels have been reported to contribute to the physiological actions of anesthetics.\(^3\) Some, including nicotinic acetylcholine, serotonin type 3, GABA type A, glycine, NMDA, and AMPA receptors, are sensitive to inhaled anesthetics at clinically effective concentrations.\(^3\)–\(^6\) The alteration of potassium channel function has also been reported to be involved in the central effects of inhaled anesthetics.\(^9\)–\(^10\) In the CNS, PDZ domain–mediated protein–protein interactions have been identified in different signaling complexes at synapses and are critical to synaptic organization and for the activity of several excitatory receptors.\(^11\)–\(^14\) Our previous studies have shown that clinically relevant concentrations of inhaled anesthetics dose-dependently inhibit PDZ domain–mediated interactions between PSD-95 and NMDA receptors or neuronal nitric oxide synthase but have no effect on the non-PDZ domain–mediated interaction between the guanylate kinase domain of PSD-95 and Src homology 3 domain of SAP102.\(^15\) We have also shown that disrupting PDZ domain–mediated protein–protein interactions by systematic injection of the cell-permeable peptide Tat-PSD-95 PDZ2 reduces the threshold for halothane anesthesia.\(^16\) In the current study, we show that halothane and isoflurane inhibit PDZ domain–mediated interactions between PSD-95 and Shaker-type potassium channel Kv1.4 and between AMPA receptor subunit GluR2 and glutamate receptor–interacting protein or protein interacting with c kinase 1 and that isoflurane inhibits PDZ domain–mediated interactions between PSD-95 and NMDA receptors. A previous study\(^46\) has shown that mutations of the homologous Shaker channel in flies causes anesthetic resistance. The discrepancy may be due to the difference of properties of potassium channels between Drosophila and mammalian species. For instance, it has been reported that the potassium channel in Drosophila differs kinetically from mammalian species by exhibiting a faster inactivation time course.\(^47\) We further demonstrate that isoflurane mostly affects the residues close to or in the peptide-binding groove of PDZ1 and PDZ2 of PSD-95, while barely affecting the peptide-binding groove of PDZ3. Among three PDZ domains of PSD-95, PDZ2 domain has the most residues affected by isoflurane. Given that our current studies are investigating PDZ domain–mediated protein–protein interactions in in vitro preparations, our experiments are only suggestive of conditions in synapses, and actions in neuronal milieu could be different. Yet our combined studies of plasmon resonance, yeast two-hybrid, GST pull-down, and coimmunoprecipitation as well as our previous in vivo minimum alveolar anesthetic concentration estimates are all consistent with a functional effect of anesthetic disruption of PDZ domain–mediated protein–protein interactions being important to the anesthetic state.

As binding partners of PDZ domains, NR2B-c20 and NR2A-c20 interact with the PDZ domains of PSD-95. Due to the long length of both peptides, their interactions with the PDZ domains extend beyond the peptide-binding groove, which is formed by αβ, ββ, and the carboxylate-binding loop of the PDZ domain. The extended region includes βA (E65, K162), βB-βC link (T83, I88, G89, G177, G179), βC (I100, S339), αA (K202, G345, L349), βD (G209, V215, V362), and βE (L367, r368). The “disappeared” and “shifted” residues partially overlapped with the residues forming the three peptide-binding grooves of PSD-95 PDZ1–3, which include R70, G74, L75, G76, F77, S78, I79, G81, T97, H130, L137, and G141 in PSD1; K165, G169, L170, G171, F172, S173, I174, G176, T192, H225, L232, and Y236 in PDZ2; R318, G322, L323, G324, F325, N326, I327, G329, S339, H372, L379, and G383 in PDZ3. This divergence from the peptide-binding groove could come from at least three different sources. First, the real structure could be different from the model used here, the simple combination of PDZ1, PDZ2, and PDZ3. Second, the peptides used in the model are shorter than what we used in our experiments, which should cause the extended disturbance on the PSD-95 PDZ1–3. Third, some “disappeared” and “shifted” residues, which were not assigned by the current H\(^1\)N\(^{-15}\)N HSQC NMR spectra, could be other disturbed residues in the groove. About half of the residues in the groove of PDZ1 were affected by the peptides binding, whereas three quarters of the residues of PDZ2 were affected. These results suggest that the binding of NR2A-c20 and NR2B-c20 to the three PDZ domains of PSD-95 is not equivalent, and PDZ2 may be the primary target of the peptides.

Our yeast two-hybrid analysis and surface plasmon resonance assay further demonstrate that the inhaled anesthetic isoflurane disrupts the PDZ domain–mediated interaction between PSD-95 and NMDA receptors. PDZ domain–mediated protein–protein interactions provide a framework for the assembly of multiprotein signaling complexes at synapses. These PDZ proteins coordinate and guide the flow of regulatory information and regulate receptor and ion channel activities. One of the best-understood PDZ domain proteins at synapses is PSD-95, a modular protein highly enriched in the postsynaptic density. In our yeast two-hybrid experiments, the growth of the yeast cells harboring both pGADT7-PSD-95 PDZ2 and pGBKKT7-NR2B C-terminus was slowed by isoflurane (0.33, 0.66, and 0.99 mM) in a dose-dependent manner. Our surface plasmon resonance assay showed that the inclusion of isoflurane in the mobile
phase inhibited the equilibrium binding of PSD-95 PDZ2 to NR2B C-terminus in real time. These results suggest that the inhaled anesthetic isoflurane can inhibit the binding of PSD-95 with NMDA receptors and thereby interrupt relevant downstream signaling. Taken together, our data indicate that the ability of anesthetics to disrupt neuronal signaling pathways through inhibition of PDZ domain interactions plays a significant role in anesthesia itself. Different experimental conditions certainly affect quantitative data for peptide or isoflurane binding. For example, a protein-crowding effect may exist in cells but not in NMR sample tubes; the isolated PDZ2 is not comparable to the intact PDZ1–3; plasmon resonance experiments need to have the peptide immobilized. All of these may render variances in quantification of binding affinities.

We also investigated the effects of halothane and isoflurane on PDZ domain–mediated protein–protein interactions in GABA receptor signaling. We found that inhaled anesthetics had no effect on PDZ domain–mediated interactions between the GABA<sub>B</sub> receptor and its interacting proteins PAPIN and Mupp1, suggesting that inhaled anesthetics have differential effects on different PDZ domain–mediated protein–protein interactions in the CNS.

In conclusion, PDZ domain–mediated protein–protein interactions in NMDA/AMPA receptor signaling and Kv1.4 channels, but not GABA receptor signaling, can be inhibited by inhaled anesthetics. Our data also indicate that PSD-95 PDZ1–3 domains can interact with isoflurane, of which PDZ2 is the most affected domain. Thus, inhaled anesthetics may affect synaptic transmission by binding to PDZ domains, which can be considered as a new molecular target for inhaled anesthetics. The common inhibition seen on pull-down, yeast two-hybrid, and NMR (all controlled experiments) as well as a functional change in minimum alveolar anesthetic concentration provide support for our hypothesis and for a functional role for the anesthetic–PDZ interaction in anesthesia.

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Competing Interests

The authors declare no competing interests.

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References

9. Tao YX, Johns RA: Effect of the deficiency of spinal PSD-95/ SAP90 on the minimum alveolar anesthetic concentration of isoflurane in rats. ANESTHESIOLOGY 2001; 94:1010–5
36. Franks NP, Lieb WR: Which molecular targets are most relevant to general anaesthesia? Toxicol Lett 1998; 100:101–1–8