ANESTHESIOLOGY

Neonatal Isoflurane Anesthesia or Disruption of Postsynaptic Density-95 Protein Interactions Change Dendritic Spine Densities and Cognitive Function in Juvenile Mice

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- PDZ2 domains of postsynaptic density-95 proteins bind to the NR2 subunit of the *N*-methyl-D-aspartate (NMDA) receptor and promote synaptogenesis
- PDZ2 domains are molecular targets for inhalational anesthetics

What This Article Tells Us That Is New

- Disruption of PDZ2 domain-mediated protein–protein interactions or a 4-h-long isoflurane anesthesia in 7-day-old mice induced comparable lasting deficits in synaptogenesis and cognitive function, and these effects were prevented by administration of the nitric oxide donor molsidomine
- These experimental observations suggest the involvement of a pathway involving the NMDA receptor, postsynaptic density protein-95, and nitric oxide signaling in isoflurane exposure-induced cognitive impairment in mice

ABSTRACT

Background: Experimental evidence shows postnatal exposure to anesthesia negatively affects brain development. The PDZ2 domain, mediating protein–protein interactions of the postsynaptic density-95 protein, serves as a molecular target for several inhaled anesthetics. The authors hypothesized that early postnatal disruption of postsynaptic density-95 PDZ2 domain interactions has persistent effects on dendritic spines and cognitive function.

Methods: One-week-old mice were exposed to 1.5% isoflurane for 4 h or injected with 8 mg/kg active postsynaptic density-95 wild-type PDZ2 peptide along with their respective controls. A subset of these mice also received 4 mg/kg of the nitric oxide donor molsidomine. Hippocampal spine density, long-term potentiation, novel object recognition memory, and fear learning and memory were evaluated in mice.

Results: Exposure of 7-day-old mice to isoflurane or postsynaptic density-95 wild-type PDZ2 peptide relative to controls causes: (1) a long-term decrease in mushroom spines at 7 weeks (mean ± SD [spines per micrometer]): control (0.8 ± 0.2) *versus* isoflurane (0.4 ± 0.2), $P < 0.0001$, and PDZ2MUT (0.7 ± 0.2) *versus* PDZ2WT (0.4 ± 0.2), $P < 0.001$; (2) deficits in object recognition at 6 weeks (mean ± SD [recognition index]): naïve (70 ± 8) *versus* isoflurane (55 ± 14), $P = 0.010$, and control (65 ± 13) *versus* isoflurane (55 ± 14), $P = 0.045$, and PDZ2MUT (64 ±11) *versus* PDZ2WT (53 ± 18), $P = 0.045$; and (3) deficits in fear learning at 7 weeks and memory at 8 weeks (mean ± SD [% freezing duration]): Learning, control (69 ± 12) *versus* isoflurane (52 ± 13), $P < 0.0001$, and PDZ2MUT (65 ± 14) *versus* PDZ2WT (55 ± 14) $P = 0.011$, and Memory, control (80 ± 17) *versus* isoflurane (56 ± 23), $P < 0.0001$ and PDZ2MUT (73 ± 18) *versus* PDZ2WT (44 ± 19) $P < 0.0001$. Impairment in long-term potentiation has fully recovered here at 7 weeks (mean ± SD [% baseline]): control (140 ± 3) *versus* isoflurane (137 ± 8), $P = 0.560$, and PDZ2MUT (136 ± 17) *versus* PDZ2WT (128 ± 11), $P = 0.512$. The isoflurane induced decrease in mushroom spines was preventable by introduction of a nitric oxide donor.

Conclusions: Early disruption of PDZ2 domain-mediated protein–protein interactions mimics isoflurane in decreasing mushroom spine density and causing learning and memory deficits in mice. Prevention of the decrease in mushroom spine density with a nitric oxide donor supports a role for neuronal nitric oxide synthase pathway in mediating this cellular change associated with cognitive impairment.

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More than 500 preclinical studies of anesthesia neurotoxicity have demonstrated overwhelming evidence that general anesthetic drugs cause widespread adverse neurologic effects *in vitro* and in immature animals, including nonhuman primates.[1–3] Recently a group of experts identified a central goal of continuing to pursue research efforts to better understand the biologic pathways underlying anesthesia neurotoxicity and to try to causally link structural changes with long-term cognitive abnormalities.[2] In line with this goal, we study a specific molecular

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pathway underlying anesthesia neurotoxicity and link its disruption to a loss of mature dendritic spines and long-term cognitive abnormalities in mice. Our laboratory previously showed that anesthetics can disrupt PDZ2 domain-mediated protein–protein interactions *in vitro* and *in vivo*.[4,5] Using clinically relevant concentrations of inhalational anesthetics we dose-dependently and specifically inhibited PDZ2 domain-mediated protein interactions between postsynaptic density-95 or postsynaptic density-93 and the *N*-methyl-D-aspartate (NMDA) receptor or neuronal nitric oxide synthase.[4] We are able to mimic this action of anesthesia with postsynaptic density-95 wild-type PDZ2 peptide which disrupts postsynaptic density-PDZ2–mediated protein interactions by binding to interaction partners. Specifically, we demonstrated disruption of protein–protein interactions between NMDA receptor NR2A/B subunits and postsynaptic density-95.[6] This disruption significantly reduced minimum alveolar concentration (MAC) and righting reflex EC50, indicating that this domain and protein are important for anesthetic action.

Given that (1) the PDZ2 domain is a molecular target for inhalational anesthetics,[4,5] (2) postsynaptic density-95 PDZ2 interacts with NMDA receptor and promotes synaptogenesis,[7,8] (3) multi-innervated spine formation is prevented by deletion of the postsynaptic density-95 PDZ2 domain,[7] (4) disruption of postsynaptic density-PDZ2 mediated protein interactions increases anesthetic sensitivity in adult mice,[6] and (5) neonatal disruption of postsynaptic density-PDZ2 mediated protein interactions leads to a decrease of long thin spines, impairs long-term potentiation, and impairs novel object recognition in weanling mice,[9] we hypothesize that early postnatal disruption of PDZ2 domain mediated protein–protein interactions can have persistent effects extending beyond onset of sexual maturation that include long-term loss of mushroom spines and impaired cognitive functioning.

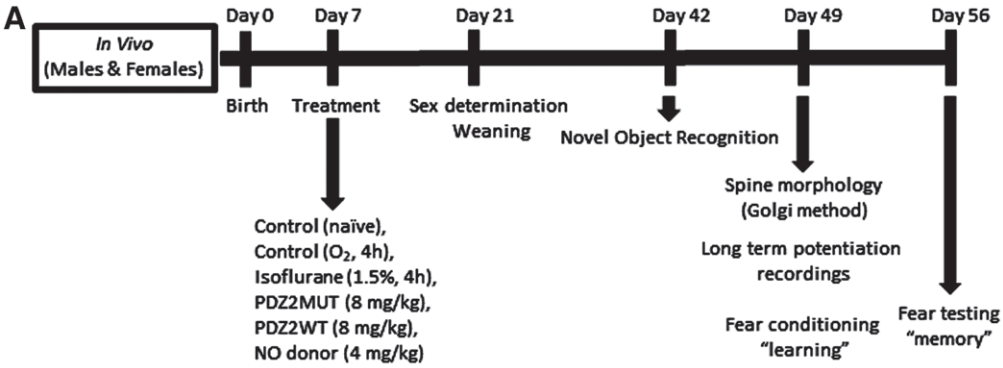
This study investigates the long-term effect of disrupting postsynaptic density-95 PDZ2 domain-mediated protein–protein interactions on mature dendritic spines, plasticity, and cognition. We examine, *in vivo*, the long-term outcome in mice of disrupting postsynaptic density-95 PDZ2 domain-mediated protein–protein interactions early in development on dendritic spine density, long-term potentiation, and hippocampal-dependent behaviors including novel object recognition memory (nonspatial) and contextual fear learning and memory. To specifically determine the involvement of the NMDA NR2-postsynaptic density-95 PDZ2–neuronal nitric oxide synthase pathway we introduced nitric oxide donor at the time of isoflurane anesthesia or postsynaptic density-95 wild-type PDZ2 peptide exposure to test whether spine loss is preventable.

Materials and Methods

This study was carried out with approval from the Animal Care and Use Committee at Johns Hopkins University (Baltimore, Maryland) and was consistent with the National Institutes of Health (Bethesda, Maryland) Guide for the Care and Use of Laboratory Animals. No surgery was performed, and all efforts were made to minimize animal suffering and reduce the number of animals used. C57BL6 wild-type male and female mice were used in our study. At 1 week of age, animals from each litter were randomly assigned to control and treatment groups (fig. 1). Mice were maintained under standard lab housing with 12-h light/dark cycle. Water and food were available *ad libitum* until mice were transported to the laboratory approximately 1 h before the experiments.

Anesthesia, Peptide, and Moldsidomine Injections

One-week-old control and experimental mice were placed in a clear plastic cone and body temperature maintained by a heating blanket set to 35°C. Heart rate (HR) and oxyhemoglobin saturation (Spo_2) were continuously monitored using pulse-oximetry (PhysioSuite, Kent Scientific, USA). Arterial blood was collected by terminal cardiac puncture of the left ventricle from sentinels and pH (pHa) determined using a blood gas machine (ABL800 FLEX Series Radiometer, Radiometer, USA). At the end of the 4-h exposure to isoflurane (1.5% in 98.5% O_2), our data (mean ± SD) suggested mice were adequately oxygenated (Spo_2, 93 ± 6, n = 5) and were within acceptable ranges for HR (611 ± 84, n = 5) and pHa (pH, 7.26 ± 0.05, n = 2). Naïve control animals were left with the dams. Gas control animals were exposed to oxygen only. Anesthesia was initiated with 2.4% isoflurane in oxygen for 2 min and tapered down to 1.5% within 15 min. Exposure to 1.5% isoflurane was continued for 3 h 45 min (total 4 h). At the end of the exposure, animals were maintained in oxygen on the heating blanket for 10 min then returned to dams. The purified fusion peptides, active Tat-postsynaptic density-95 wild-type PDZ2 (referred to as wild-type PDZ2 peptide and PDZ2WT in manuscript) or inactive Tat-postsynaptic density-95 mutant PDZ2 (referred to as mutant PDZ2 peptide and PDZ2MUT) at 8 mg/kg were injected into naive mice intraperitoneally in 150 μl of phosphate buffer saline, as previously described.[6] Immediately after peptide injection, pups were returned to their home cage and remained with dams until weaning. Purification of fusion peptides was performed by Creative BioMart (USA) and verified by Coomassie blue staining and Western blot analysis and then stored in 10% glycerol/phosphate-buffered saline at −80°C until use. The Tat-postsynaptic density-95 wild-type PDZ2 and mutant plasmids used to generate proteins containing an amino-terminal, in-frame, 11–amino acid, minimal transduction domain (residues 47 to 57 of human immunodeficiency virus Tat protein) termed Tat. Inactive control plasmid, mutated Tat-postsynaptic density-95 PDZ2, has three sites critical for interactions between NMDA receptors and postsynaptic density-95 mutated (K165T, L170R, and H182L).[4] The nitric oxide donor, Molsidomine [(N-[ethoxycarbonyl]-3- [4-morpholinosydnomine] (Sigma, USA), was injected at 4 mg/kg into mice intraperitoneally in 100 μl sterile saline.



B

Treatment	Novel Object Recognition		Spine Analysis		Long term potentiation		Fear behavior	
	Females	Males	Females	Males	Females	Males	Females	Males
Naïve control (pups left with dams)	5	8						
Carrier gas control (100% O_2, 4h)	10	15	6	10	1	5	12	11
Isoflurane (1.5% in 98.5% O_2, 4h)	10	10	5	6	1	4	15	15
PDZ2MUT peptide (8 mg/kg)	11	9	4	10	2	2	10	13
PDZ2WT peptide (8 mg/kg)	10	9	8	8	-	4	12	12
Rescue: Carrier gas control (100% O_2, 4h) + NO donor (4 mg/kg)			6	6				
Rescue: Isoflurane (1.5% in 98.5% O_2, 4h) + NO donor (4 mg/kg)			6	7				
Rescue: PDZ2MUT peptide (8 mg/kg) + NO donor (4 mg/kg)			6	6				
Rescue: PDZ2WT peptide (8 mg/kg) + NO donor (4 mg/kg)			6	6				

Fig. 1. Schematic representation of experimental timeline and treatments *in vivo*. (*A*) The general experimental timeline *in vivo*. Mice were treated postnatally at day 7, weaned and sexed at day 21, followed by testing of individual cohorts on day 42 (novel object recognition), day 49 (spine analysis, long-term potentiation, and fear conditioning), and day 56 (fear memory testing). Peptides and nitric oxide (NO) donor were administered by intraperitoneal injection. (*B*) Table indicating animal cohorts used in experiments.

Golgi Staining, Microscopy, and Spine Reconstruction

Seven-week-old mice were deeply anesthetized and perfused transcardially with a brief flush of 0.01 M phosphate-buffered saline (pH 7.4) followed by 50 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After the perfusion, the brains were removed and hippocampi were grossly dissected and stained using FD Rapid GolgiStain Kit, (FD NeuroTechnologies, Inc., USA) as per vendor's instructions. Briefly, tissue was immersed in AB impregnation solution at room temperature in the dark for 2 weeks. Impregnation solution was replaced after the first overnight on the next day. Tissue was transferred to solution C for 72 h. Hippocampi were embedded in tissue freezing medium and stored at −80°C. Sixty-micrometer sections were cut on a cryostat at −20°C, mounted onto gelatin-coated slides, and air dried overnight. Slides were rinsed in Milli-Q water (Millipore Sigma, Germany), developed in the working solution DE for 10 min, rinsed, dehydrated in ethanol, cleared in xylene, and mounted with Permount (Fisher Scientific, USA).

Two different imaging fields per mouse, each containing at least three unique dendritic segments (six segments total per mouse) which contain dorsal hippocampus, were imaged. The six segments were averaged per mouse to contribute one datapoint per mouse. Dentate granule cells were identified by their location within the dentate gyrus and their distinct morphology. Spines along secondary and tertiary dendrites of these neurons were selected for analysis (fig. 2).[9] Z-stacks of Golgi stained dendrites (optical section thickness = 0.3 μm; *i.e.,* 50 to 100 images per stack) were taken at ×630 magnification on a Leica SPE confocal microscope (Leica, Germany). Spine analysis was performed as described by Risher *et al.*[10] using the freely available RECONSTRUCT software (Free Software Foundation, USA).[11] Briefly, objective classification of dendritic spines was done from the Golgi-Cox–stained tissue described above. This approach uses the distinct geometric characteristics of spines as the basis for their categorization. Spine head width and neck length measurements were obtained. Spines are classified in the algorithm according to the following measurements: Mushroom (width > 0.6 μm), Thin (width ≤ 0.6 μm; length < 1μm), Stubby (length:width ratio < 1). Individuals performing imaging and reconstruction were naïve to the presumed outcome (so essentially blinded).

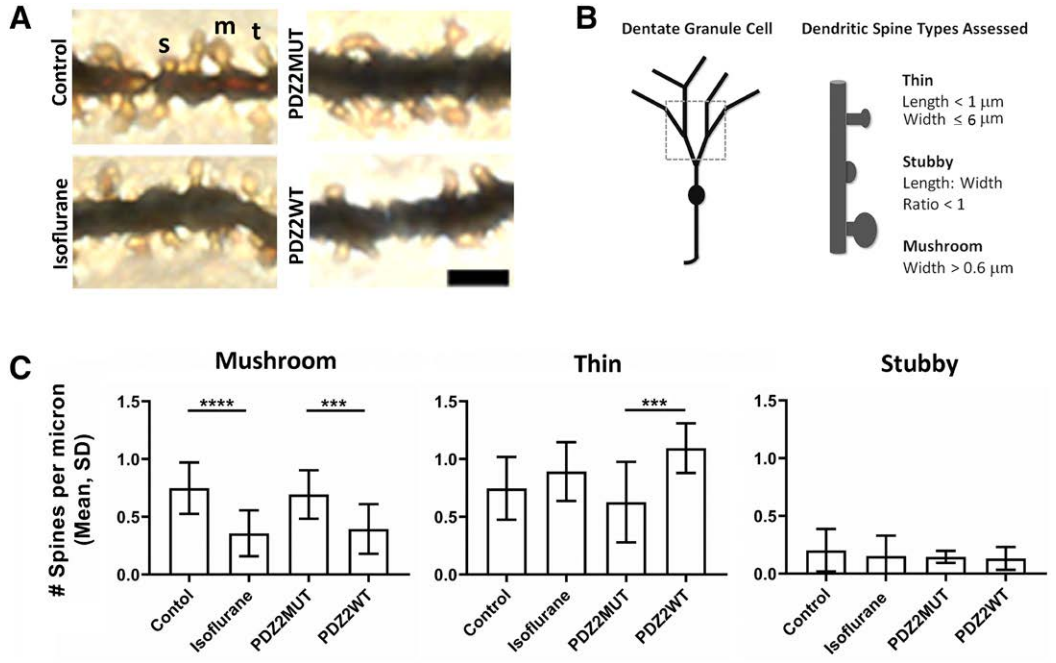


Fig. 2. Neonatal exposure to isoflurane or wild-type PDZ2 peptide decreases hippocampal mushroom spine density in mice. (*A*) Representative images showing dendritic segments and spines; s = stubby, m = mushroom, t = thin; scale bar, 2.5 µm. (*B*) Schematics showing dendrite branches and spine types sampled. (*C*) Density of spines among exposed groups assessed at 7 weeks. Mice in cohort 1 were exposed to oxygen (control, 100% O_2) or isoflurane (1.5% ISO in 98.5% O_2) and cohort 2 were injected with PDZ2MUT (8 mg/kg inactive mutant peptide) or PDZ2WT (8 mg/kg active wild-type peptide). Control (n = 16), Isoflurane (n = 11), PDZ2MUT (n = 14), and PDZ2WT (n = 16). n = number of animals. (*C*, *left*) Mushroom: control *versus* isoflurane, $P < 0.0001$; PDZ2MUT *versus* PDZ2WT, $P < 0.001$. (*C*, *middle*) Thin: control *versus* isoflurane, $P = 0.184$ and PDZ2MUT *versus* PDZ2WT, $P < 0.001$. (*C*, *right*) Stubby: control *versus* isoflurane, $P = 0.257$ and PDZ2MUT *versus* PDZ2WT, $P = 0.452$. Data were analyzed with 1-way ANOVA followed by Holm-Sidak's tests. *$P < 0.050$, **$P < 0.010$, ***$P < 0.001$, ****$P < 0.0001$.

Electrophysiology

Slice Preparation. Six weeks after exposure mice, were euthanized and coronal brain slices containing central part of hippocampus (300 µm thick) were made from Leica VT 1200S vibrotome (Leica, Germany) in ice-cold artificial cerebral spinal fluid containing (in mM): 128 NaCl, 3 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 1 $MgSO_4$, 10 glucose, and 2 $CaCl_2$ and saturated with 95% O_2 and 5% CO_2. The slices were incubated for at least 1 h at room temperature (22 to 24°C) in the interface-type holding chamber filled with artificial cerebral spinal fluid. Then a slice was transferred to the recording chamber, where artificial cerebral spinal fluid was perfused at a rate of 1.5 to 2.0 ml/min at room temperature.

Extracellular Field-Potential Recordings. Synaptic responses were recorded using a MultiClamp 700B amplifier (Molecular Devices, USA), and the signal was digitized with Digidita 1440A (Molecular Devices), analyzed with pClamp10 (Molecular Devices), and stored on a personal computer. Extracellular recordings of field excitatory postsynaptic potentials were made from the stratum radiatum of the hippocampal CA1 area. Evoked responses were elicited with 0.1 millisecond constant-current pulses through a concentric electrode in the Schaffer collateral pathway every 30 s at an intensity sufficient to elicit 40 to 50% maximal excitatory postsynaptic potentials. After establishing a stable baseline for 20 min, long-term potentiation was induced by applying three trains of 100Hz × 1 s high-frequency stimulus 20 s apart at the baseline stimulus intensity. Measurements of the field excitatory postsynaptic potentials slopes were made during the rising phase (5% to 50% of the peak) and the values normalized to the mean values recorded in 20 min baseline. The mean of normalized field excitatory postsynaptic potentials slopes 55 to 60 min after high frequency stimulation was used for comparison between groups. Experimenter was not blinded to group assignment. Each animal contributed one data point to obtain the mean for each group.

Novel Object Recognition

The novel object recognition assay was based upon the original Ennaceur *et al.*[12] procedure and was used to assess nonspatial hippocampal memory.[13–15] It consisted of a training familiarization phase followed by a testing phase. Training phase began

at 10 to 11 AM. Animals within a cohort were run randomly. During training, mice were allowed to freely explore within an opaque box (40 cm W × 40 cm L × 34 cm H) containing two identical objects for 10 min. Data were recorded with video camera and time spent with each object was recorded using ANYmaze software (Stoelting, USA). Object investigation time was determined by the amount of time the mouse spent in the zone immediately surrounding the object. Only mice that investigated the objects for at least 10 s (criterion) were taken forward to the testing phase (n = 5 animals were not taken forward as they did not meet criterion). After 2 h, object recognition was tested, using the same procedure as in training except that a novel object was substituted for one of the familiar training objects and mice were allowed to explore for 5 min. Mice inherently prefer to explore novel objects; thus, a preference for the novel object indicates intact memory for the familiar object. Individuals performing behavior experiments were blinded to group assignments.

Fear Behavior Testing

Contextual Fear Conditioning. Six weeks after anesthetic exposure, the 7-week-old animals were acclimated to the behavior testing room for 60 min. The conditioning trial protocol was set up to allow testing of both cued and contextual fear behavior, although only contextual fear testing was performed. Conditioning began at 1 to 2 PM. Animals within a cohort were run randomly. The conditioning trial consisted of a 3-min exploration period in conditioning chambers (Coulbourn Instruments, USA) followed by three conditioned stimulus–unconditioned stimulus pairings separated by 1.0 min each: unconditioned stimulus, 0.5 mA foot shock intensity, 1 s duration; conditioned stimulus, 90 db white noise tone, 30 s duration. The unconditioned stimulus was delivered during the last second of the conditioned stimulus presentation. The average percent freezing duration was calculated for the first 2.5 min (pre-shock) and the last 2.0 min (post-shock) of the conditioning trial.

Contextual Fear Testing. At 1 week after the conditioning, the 8-week-old mice were acclimated to the behavior room for 60 min. Testing began at 1 to 2 PM. Animals within a cohort were run randomly. Mice were placed into the chambers for 7 min and percent of freezing duration was captured by FreezeScan camera software (Clever Sys., USA). The average percent freezing duration was calculated for the first 2.5 min of the testing trial. Four mice were removed from the study because of equipment failure during conditioning or testing. Individuals performing behavior experiments were blinded to group assignments.

Statistical Analysis

Data were analyzed using *t* tests or ANOVA with Holm-Sidak multiple comparison correction in GraphPad Prism 8.3.1 (Graphpad Inc., USA). All data input used in one-way ANOVA had a normal distribution (*i.e.*, passed D'Agostino-Pearson omnibus normality test). Some data needed transformation before passing normality test, which was accomplished in GraphPad Prism by taking the square root of the raw data. Residuals were assessed with homoscedasticity and QQ plots. Male and female data were combined and analyzed in one-way ANOVA followed by Holm-Sidak's multiple comparison test (one family and two comparisons for all tests except novel object recognition index with one family and four comparisons). Data are expressed as mean ± SD, and statistical significance was set at $P < 0.050$. In all experiments each animal contributed one data point.

Sample Size and Effect Size Calculations. This study was not designed to study sex effects, and experimental results from male and female mice were combined.

Spine Analysis. Using means and SD of mushroom spine density from mixed sex control and isoflurane groups we determined the sample size needed for 90% power and $\alpha = 0.05$ is N = 7. Thus power is > 90% with female and male data combined (N = 11 to 16; Cohen's $d = 2.0$, effect size $r = 0.71$).

Novel Object Recognition Tests

Using means and SD of recognition index from mixed sex naïve control and isoflurane groups we determined the sample size needed for 90% power and $\alpha = 0.05$ is N = 6. Thus power is > 90% with female and male data combined (N = 13 to 25; Cohen's $d = 1.32$, effect size $r = 0.55$).

Electrophysiology. Using means and SD of normalized field excitatory postsynaptic potentials slope at 55 to 60 min of mixed sex control and isoflurane groups from our previous work on weanling mice (originally reported as median, IQ range) we determined the sample size needed for 90% power and $\alpha = 0.05$ is N = 3.[9] Thus power is > 90% with female and male data combined (N = 4 to 6; Cohen's $d = 1.96$, effect size $r = 0.70$).

Fear Behavior

Fear Learning. Using % freezing duration post-shock means and SD from mixed sex control and isoflurane groups we determined the sample size needed for 90% power and $\alpha = 0.05$ is N = 10. Thus power is > 90% with female and male data combined (N = 23 to 30; Cohen's $d = 1.36$, effect-size $r = 0.56$).

Fear Memory. Using % freezing duration means and SD from mixed sex control and isoflurane groups we determined the sample size needed for 90% power and $\alpha = 0.05$ is N = 10. Thus power is > 90% with female and male data combined (N = 23 to 30; Cohen's $d = 1.19$, effect-size $r = 0.51$).

Results

Neonatal Exposure to Isoflurane or Wild-type PDZ2 Peptide Leads to a Decrease in Mushroom Spines in 7-week-old Mice

To determine whether inhaled anesthetics interfere with spinogenesis and have long-lasting effects by disrupting

synaptic PDZ2 interactions in the developing hippocampus, we investigated the impact of isoflurane and disrupting postsynaptic density-95 PDZ2 domain-mediated protein–protein interactions on dendritic spines in 7-week-old mice 6 weeks after exposure. Neonatal mouse pups were exposed at 1 week to isoflurane or oxygen for 4 h (fig. 1, A and B). A separate cohort of animals were exposed to mutant PDZ2 or wild-type PDZ2 peptides. Animals were harvested 6 weeks later at 7 weeks of age for rapid golgi staining to visualize hippocampal dendritic spines within the superior blade of the dentate gyrus. Mushroom, thin, and stubby spines were quantified along the dendritic segments distal to the first and second branch points (fig. 2, A–C). Data were analyzed using one-way ANOVA. Isoflurane or wild-type PDZ2 peptide had a significant effect on number of mushroom type (width > 0.6 μm) protrusions present at 7 weeks of age (fig. 2C: Mean number of spines per micrometer ± SD, Left plot: control [0.8 ± 0.2] *versus* isoflurane [0.4 ± 0.2], $P < 0.0001$; PDZ2MUT [0.7 ± 0.2] *versus* PDZ2WT [0.4 ± 0.2], $P < 0.001$). PDZ2WT peptide had a significant effect on number of thin type (length < 1 μm; width ≤ 0.6 μm) protrusions present at 7 weeks of age (fig. 2C: Mean number of spines per micrometer ± SD, Middle plot: control [0.8 ± 0.3] *versus* isoflurane [0.9 ± 0.3], $P = 0.184$; PDZ2MUT [0.6 ± 0.4] *versus* PDZ2WT [1.0 ± 0.2], $P < 0.001$). Neither isoflurane nor PDZ2WT peptide had a significant effect on number of stubby type (length: width ratio < 1) protrusions present at 7 weeks of age (fig. 2C: Mean number of spines per micrometer ± SD, Right plot: control [0.2 ± 0.2] *versus* ISO [0.2 ± 0.2], $P = 0.257$; PDZ2MUT [0.2 ± 0.1] *versus* PDZ2WT [0.1 ± 0.1], $P = 0.452$.)

Neonatal Exposure to Isoflurane or Wild-type PDZ2 Peptide followed by 6 Weeks Recovery Does Not Result in Impaired Long-term Potentiation

Previously, we showed that early exposure at 1 week to isoflurane or disruption of postsynaptic density-95 PDZ2 domain-mediated protein–protein interactions impaired CA1 long-term potentiation in hippocampal slices prepared from mice at 3 weeks (2 weeks after exposure). Here we assess electrophysiologic effects after an even longer recovery period after isoflurane or wild-type PDZ2 peptide exposure at 1 week in hippocampal slices prepared from 7-week-old mice. Six weeks after exposure, robust CA1 long-term potentiation can be induced in all mice (fig. 3A). No significant differences were observed between controls and experimental groups (fig. 3B; mean normalized field excitatory postsynaptic potentials slopes 55 to 60 min after high frequency stimulation [% baseline] ± SD: control [140 ± 3] *versus* isoflurane [137 ± 8], $P = 0.560$; PDZ2MUT [136 ± 17] *versus* PDZ2WT [128 ± 11], $P = 0.512$).

Neonatal Exposure to Isoflurane or Wild-type PDZ2 Peptide Impairs Object Recognition Memory in 6-week-old Mice

To determine whether the disruption of synaptic PDZ2 interactions contributes to cognitive impairment after early anesthetic exposure and has long-lasting effects, we investigated the impact of isoflurane and disrupting postsynaptic density-95 PDZ2 domain-mediated protein–protein interactions on non-spatial memory by assessing hippocampal dependent object recognition in 6-week-old mice 5 weeks after exposure. All control mice (naïve, control, and PDZ2MUT) were able to discriminate between novel and familiar objects revealed by significantly increased amounts of time investigating the novel object over the familiar object (fig. 4A; mean [seconds] ± SD for novel *versus* familiar: naïve [20 ± 8 *vs.* 9 ± 4], $P < 0.001$; control [17 ± 10 *vs.* 9 ± 4], $P < 0.001$; PDZ2MUT [18 ± 7 *vs.* 11 ± 5] $P = 0.002$). In contrast, experimental mice (isoflurane and PDZ2WT) showed no significant increase in investigation time between the novel and familiar objects (fig. 4A: mean [seconds] ± SD for novel *versus* familiar: isoflurane [14 ± 8 *versus* 10 ± 4], $P = 0.234$; PDZ2WT [13 ± 8 *vs.* 11 ± 8], $P = 0.382$). All groups spent at least 50% of their object interaction time with the novel object as can be seen in the recognition index (% time investigating novel object over time investigating novel object plus familiar object × 100) plot (fig. 4B). Data were analyzed using one-way ANOVA, which indicated that experimental mice performed less well than their respective controls (fig. 4B; mean recognition index ± SD: naïve [70 ± 8] *versus* isoflurane [55 ± 14], $P = 0.010$; control [65 ± 13] *versus* isoflurane [56 ± 14], $P = 0.045$; PDZ2MUT [64 ± 11] *versus* PDZ2WT [53 ± 18], $P = 0.045$). There was no difference between naïve and control animals ($P = 0.235$).

Neonatal Exposure to Isoflurane or Wild-type PDZ2 Peptide Leads to Impairment in Contextual Fear Behavior in 7- and 8-week-old Mice

Contextual fear learning is a form of Pavlovian conditioning elicited by pairing a neutral conditioned stimulus (for example, sound or context) with an aversive unconditioned stimulus. Acquisition of a context–unconditioned stimulus association usually requires both the hippocampus and amygdala.[16,17] We tested whether early exposure to isoflurane or disruption of postsynaptic density-95 PDZ2 domain-mediated protein–protein interactions impairs the ability of mice to learn and remotely retrieve information about the stored association (memory). Contextual fear learning was performed in 7-week-old mice (fig. 5A). All mice learned the association between the mild foot shock and context as indicated by $P < 0.0001$ for each group in the training session comparing percent freezing duration between preshock and postshock. To assess whether isoflurane or wild-type PDZ2 peptide had an impact on fear

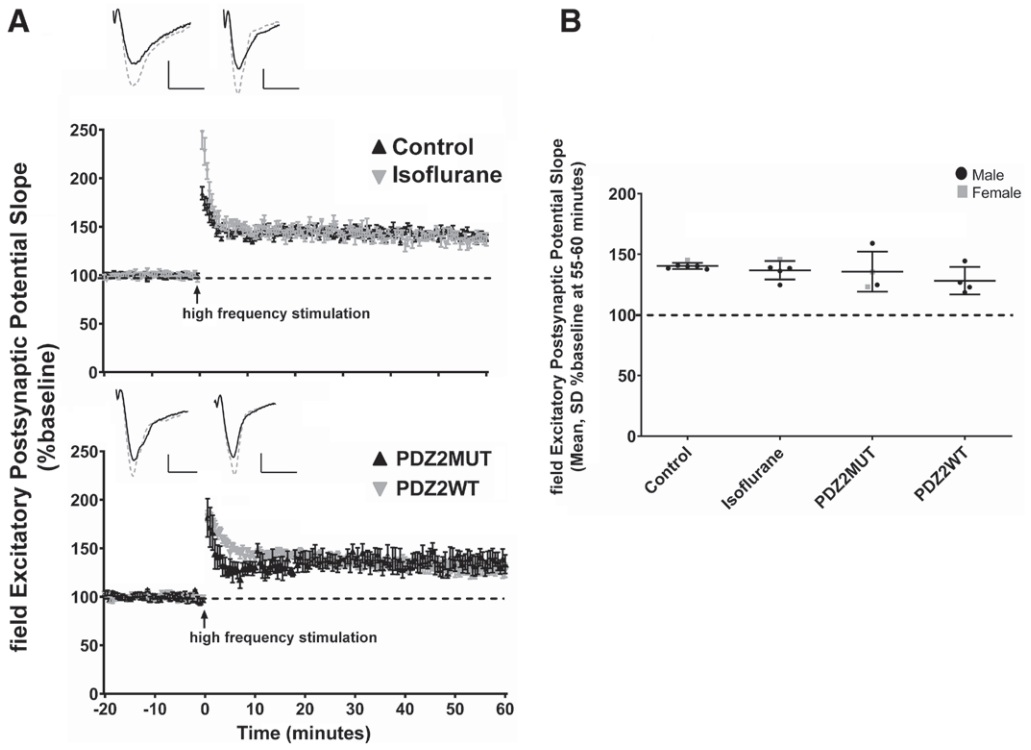


Fig. 3. Neonatal exposure to isoflurane or wild-type PDZ2 peptide followed by 6 weeks recovery period does not result in impaired hippocampal CA1 long-term potentiation in 7-week-old mice. At 1 week, mice in cohort 1 were exposed to oxygen (control, 100% O_2) or isoflurane (1.5% ISO in 98.5% O_2) and cohort 2 were injected with PDZ2MUT (8 mg/kg inactive mutant peptide) or PDZ2WT (8 mg/kg active wild-type peptide). (*A*) High-frequency stimulation induced robust long-term potentiation in all groups. (*Top*) Control (n = 6) and isoflurane (n = 5). (*Bottom*) PDZ2MUT (n = 4) and PDZ2WT (n = 4). Example traces are shown in upper left quadrants of field excitatory postsynaptic potentials plots. Control and isoflurane- (*top*) and PDZ2MUT- and PDZ2WT- (*bottom*) treated groups at baseline before high frequency stimulation (solid line trace) and the average of 55 to 60 min after high frequency stimulation (dashed line trace). (*B*) Mean of normalized field excitatory postsynaptic potentials 55 to 60 min after high frequency stimulation showed no significant differences between control (n = 6) *versus* isoflurane (n = 5), $P = 0.560$ and PDZ2MUT (n = 4) *versus* PDZ2WT (n = 4), $P = 0.512$ treated groups. n = number of animals. Data from individual animals are plotted and coded by gender (gray = female and black = male). Data were analyzed with one-way ANOVA followed by Holm-Sidak's tests. Values were considered significant at $^*P < 0.050$ or less. Scale bar, 10 ms, 0.25 mV.

learning relative to controls, percent freezing duration was compared across groups. One-way ANOVA was used to compare percent freezing duration between groups at the end of the training session (*i.e.*, postshock; fig. 5A). Mice exposed to isoflurane or wild-type PDZ2 peptide froze for less time at the end of the training session, suggesting that they learned less well than their respective controls (fig. 5A; Right panel, Mean percent freezing duration ± SD: control [69 ± 12] *vs.* isoflurane [52 ± 13], $P < 0.0001$; PDZ2MUT [65 ± 14] *vs.* PDZ2WT [55 ± 14] $P = 0.011$).

Contextual fear testing was performed in 8-week-old mice 1 week after conditioning and 7 weeks after exposure (fig. 5B). One-way ANOVA was used to compare test groups. Isoflurane- and wild-type PDZ2 peptide–exposed mice exhibited significantly reduced freezing behavior compared with controls (fig. 5B; Mean % freezing duration ± SD: control [80 ± 17] *vs.* isoflurane [56 ± 23], $P < 0.0001$; PDZ2MUT [73 ± 18] *vs.* PDZ2WT [44 ± 19], $P < 0.0001$).

Treatment with Nitric Oxide Donor Prevents Isoflurane or Wild-type PDZ2 Peptide–induced Decrease in Hippocampal Mushroom Spines in 7-week-old Mice

Treatment with nitric oxide donor prevents the decrease in mushroom spines caused by isoflurane or wild-type PDZ2 peptide. Representative images are shown in figure 6A. One-way ANOVA was used to compare groups for each spine type. With introduction of nitric oxide donor, neither isoflurane nor wild-type PDZ2 peptide has a significant effect on number of mushroom type protrusions present at 7 weeks of age relative to controls (fig. 6B; Mean number of spines per micrometer ± SD, Left panel, mushroom: control

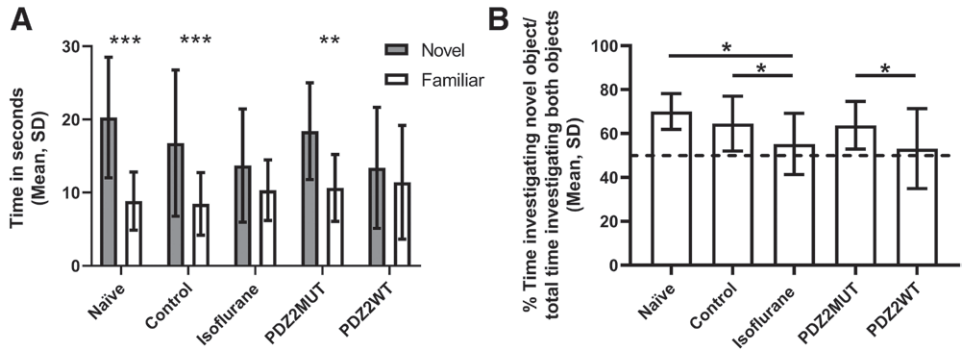


Fig. 4. Neonatal exposure to isoflurane or wild-type PDZ2 peptide impairs recognition memory at 7 weeks. Plots showing percent of time animals spent investigating novel or familiar objects among experimental groups. At 1 week, mice in cohort 1 were exposed to oxygen (control, 100% O_2) or isoflurane (1.5% ISO in 98.5% O_2) and cohort 2 were injected with PDZ2MUT (8 mg/kg inactive mutant peptide) or PDZ2WT (8 mg/kg active wild-type peptide). Data are plotted as mean ± SD. Left plot showing investigation time comparing novel *versus* familiar objects, naïve (n = 13), $P < 0.001$; control (n = 25), $P < 0.001$; isoflurane (n = 20), $P = 0.234$; PDZ2MUT (n = 20), $P = 0.002$; PDZ2WT (n = 19), $P = 0.382$. n = number of animals. Data were analyzed with *t* tests followed by Holm-Sidak multiple comparison correction. Right plot showing recognition index as % time investigating novel object/total time investigating both objects × 100, naïve *versus* control, $P = 0.235$; naïve *versus* isoflurane, $P = 0.010$; control *versus* isoflurane, $P = 0.045$; PDZ2MUT *versus* PDZ2WT, $P = 0.045$. Data were analyzed with 1-way ANOVA followed by Holm-Sidak multiple comparison correction. *$P < 0.050$, **$P < 0.010$, ***$P < 0.001$, ****$P < 0.0001$.

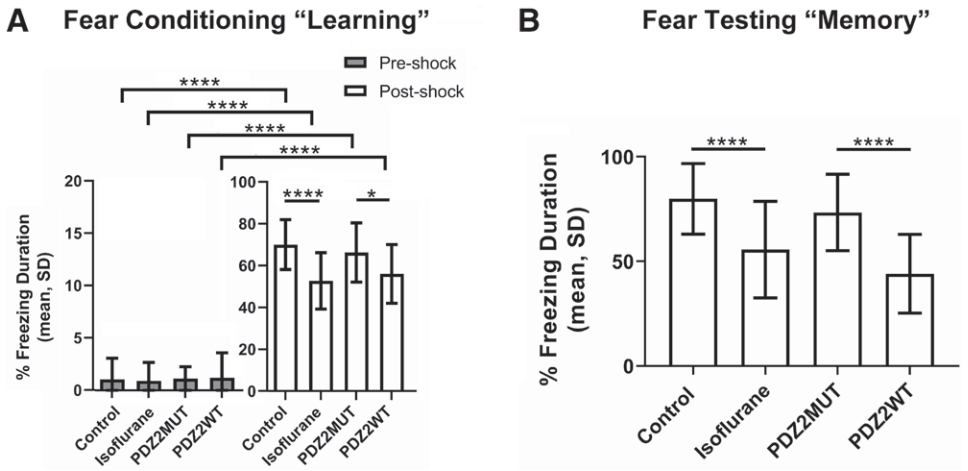


Fig. 5. The effect of neonatal exposure to isoflurane or wild-type PDZ2 peptide on fear learning and memory. At 1 week, mice in cohort 1 were exposed to O_2 (control, 100% O_2) or isoflurane (1.5% ISO in 98.5% O_2) and cohort 2 were injected with PDZ2MUT (8 mg/kg inactive mutant peptide) or PDZ2WT (8 mg/kg active wild-type peptide). Data are plotted showing % freezing duration mean ± SD. (*A*) Fear conditioning "learning" at 7 weeks: Pre- and postshock comparisons within each group were analyzed with *t* tests followed by Holm-Sidak multiple comparison correction and all $P < 0.0001$. (*A*, *left*) Control (n =23) *versus* Isoflurane (n = 30), $P < 0.0001$; PDZ2MUT (n = 23) *versus* PDZ2WT (n = 24), $P < 0.011$. (*B*) Fear testing "memory" at 8 weeks: Control (n = 23) *versus* Isoflurane (n = 30), $P < 0.0001$; PDZ2MUT (n = 23) *versus* PDZ2WT (n = 24), $P < 0.0001$. n = number of animals. Data were analyzed with one-way ANOVA followed by Holm-Sidak multiple comparison correction. *$P < 0.050$, **$P < 0.010$, ***$P < 0.001$, ****$P < 0.0001$.

[1.5 ± 0.4] *vs.* isoflurane [1.2 ± 0.4], $P = 0.100$; PDZ2MUT [1.3 ± 0.3] *vs.* PDZ2WT [1.3 ± 0.3], $P = 0.710$). With introduction of nitric oxide donor, a difference in number of thin type protrusions was detected between isoflurane and control groups but not between mutant PDZ2 and wild-type PDZ2 peptide groups [fig. 6B; number of spines per micrometer ± SD, Middle panel, thin: control [0.2 ± 0.1] *vs.* isoflurane [0.3 ± 0.2], $P = 0.033$; PDZ2MUT [0.3 ± 0.1] *vs.* PDZ2WT [0.3 ± 0.1], $P = 0.540$). No differences were observed in the number of stubby spines after

introduction of nitric oxide donor (fig. 6B; Mean number of spines per micrometer ± SD, Right panel, stubby: control [0.1 ± 0.1] *vs.* isoflurane [0.1 ± 0.1], $P = 0.794$; PDZ2MUT [0.3 ± 0.1] *vs.* PDZ2WT [0.3 ± 0.1], $P = 0.891$).

Discussion

To better understand the mechanisms underlying anesthesia neurotoxicity and to try to link structural changes with long-term cognitive abnormalities, we focused on one specific molecular target of anesthesia. Our laboratory previously reported that inhalational anesthetics, including halothane, isoflurane, and sevoflurane, can disrupt PDZ2 domain-mediated protein–protein interactions *in vitro* and *in vivo*[4,5] and specifically inhibit the PDZ2 domain-mediated protein interaction between postsynaptic density-95 or postsynaptic density-93 and the NMDA receptor NR2 subunits or neuronal nitric oxide synthase.[4,6] Recently, we determined the effects of this disruption, *in vivo,* in the context of isoflurane exposure by specifically mimicking this one action of anesthesia with wild-type PDZ2 peptide.[9] Wild-type PDZ2 peptide disrupts postsynaptic density-PDZ2-mediated protein interactions by binding to interaction partner NMDA receptor NR2.[9] We found isoflurane and disruption of postsynaptic density-95 PDZ2 domain-mediated protein–protein interactions at 1 week alters spine morphology, impairs CA1 long-term potentiation, and impairs memory in weanling aged mice (*i.e.*, 3 weeks old). In addition, further support was presented specifically linking disruption of PSD93/95 and PDZ2 domain-mediated protein–protein interactions with isoflurane's effect using PSD93 knockout mice and double hit studies combining peptide and isoflurane together.[9] We showed PSD93-null mice exposed only to oxygen had impaired expression of LTP and loss of long thin spines similar to isoflurane-exposed wild-type mice (and were not worsened by exposure to isoflurane) and neither PSD93 null mice nor wild-type PDZ2 peptide–injected mice showed significant changes in object recognition memory after exposure to isoflurane suggesting the effect is in the same pathway. Introduction of nitric oxide donor at the time of exposure prevented impairment in long-term potentiation and recognition memory further implicating the involvement of the NMDAR NR2-postsynaptic density-95 PDZ2-neuronal nitric oxide synthase signaling

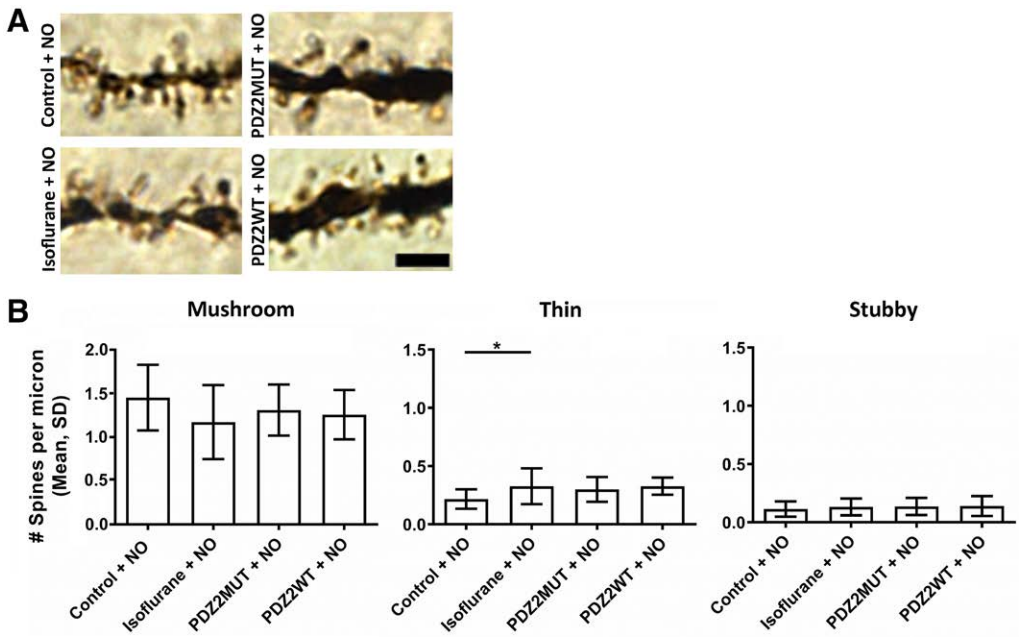


Fig. 6. Treatment with nitric oxide (NO) donor prevents the decrease in mushroom spine density in 7-week-old mice induced by neonatal exposure to isoflurane or wild-type PDZ2 peptide. Density of mushroom spines among exposed groups assessed at 7 weeks. At 1 week, mice in cohort 1 were exposed to oxygen (control, 100% O_2) or isoflurane (1.5% ISO in 98.5% O_2) and cohort 2 were injected with PDZ2MUT (8 mg/kg inactive mutant peptide) or PDZ2WT (8 mg/kg active wild-type peptide). At the end of the exposure period (4 h after onset of exposure) all animals received 4 mg/kg of nitric oxide donor molsidomine (intraperitoneal). Data are plotted as mean ± SD. Control (n = 12), Isoflurane (n = 13), PDZ2MUT (n = 12), and PDZ2WT (n = 12). n = number of animals. (*C*, *left*) Mushroom: control *versus* isoflurane, $P = 0.100$; PDZ2MUT *versus* PDZ2WT, $P = 0.710$. (*C*, *middle*) Thin: control *versus* isoflurane, $P = 0.033$ and PDZ2MUT *versus* PDZ2WT, $P = 0.540$. (*C*, *right*) Stubby: control *versus* isoflurane, $P = 0.794$ and PDZ2MUT *versus* PDZ2WT, $P = 0.891$. Data were analyzed with one-way ANOVA followed by Holm-Sidak's tests. A $P < 0.050$ was considered significant.

pathway in early anesthetic exposure-produced cognitive impairment. In the current study we sought to determine what changes occur following our exposure paradigm in mice allowed to recover for longer periods of time.

Anesthesia exposure, neuronal nitric oxide synthase activity, and modulation of membrane-associated guanylate kinase levels have all been associated with spine morphologic and density changes.[7,18,19] Dendritic spines are critical for learning and memory functions.[20] We asked whether neonatal isoflurane exposure could lead to a persistent change in mature spines of hippocampal dentate granule cells. Mushroom spines typically represent long-lasting, stable synaptic connections.[21] Mushroom spines were measured on secondary and tertiary dendrites which receive input from medial entorhinal cortex in the middle molecular layer of the hippocampus. Results of our spine analysis indicate a significant loss of mushroom spines in isoflurane or wild-type PDZ2 peptide exposed animals compared with controls measured 6 weeks after exposure. Others have also shown selective loss of spines from anesthesia such as the persistent decrease (up to 90 days in rat prefrontal cortex) in spine density of spines with head diameter between 0.3 to 0.4 μm[22] and hippocampal mushroom spines in mice at postnatal day 60.[23] The reduction in mushroom spine number suggests a substantial loss of functionally mature glutamatergic synapses and might involve a role in circuitry development and permanently altered neural connectivity. A decrease in mushroom spines suggest association of functional defects in glutamatergic transmission at the perforant path-dentate gyrus granule cell synapse that could reasonably account for reduced cognitive performance reported in individuals with early postnatal exposure to anesthesia.

To further explore whether the disruption of synaptic PDZ2 interactions could contribute to persistent learning and memory deficits through altered synaptic function after early anesthetic exposure, we investigated CA1 long-term potentiation in sexually mature mice. Long-term potentiation is widely considered as a major cellular mechanism underlying learning and memory.[24] Hippocampal granule neurons that we show here to have decreased mushroom spine density after early exposure to isoflurane or wild-type PDZ2 peptide emit mossy fibers that synapse on pyramidal neurons of area CA3 of Ammon's horn, which synapse on pyramidal neurons of area CA1 of Ammon's horn. These connections are involved in long-term potentiation and long-term depression. Thus we were curious to determine whether the long-term decrease in dentate gyrus granule cell mushroom spines parallels suppressed CA1 long-term potentiation. Previous work demonstrated early exposure to a combination anesthetic induced a profound suppression of long-term potentiation in the hippocampus of adolescent rats.[25] In addition, we found suppression of CA1 long-term potentiation in the hippocampus of weanling mice exposed early to isoflurane and wild-type PDZ2 peptide as compared with controls.[9] Thus disrupting PDZ2 domain-mediated protein interactions mimicked the effect of isoflurane on CA1 long-term potentiation. These results suggested synaptic PDZ2 interactions may contribute to the mechanism underlying anesthesia induced impairment in synaptic function in mice and therefore could contribute to learning and memory deficits. Here, we found the suppression of CA1 long-term potentiation by either isoflurane or wild-type PDZ2 peptide had fully recovered by 7 weeks of age. Thus exposure to isoflurane or wild-type PDZ2 peptide causes long-lasting (2 weeks after exposure)[9]—but not permanent (6 weeks after exposure)—impairment of synaptic plasticity in area CA1 of the hippocampus. It should be mentioned that although traditionally, with respect to learning and memory, focus has been more on the CA3-CA1 area of the hippocampus recent proposals suggest a differential role of the dentate gyrus in aspects of memory, such as pattern separation.[26,27] Added to this view is the possibility that dentate gyrus-associated aspects of memory will be enhanced under more emotional or stressful conditions.[28] Thus, the persistent dendritic spine abnormalities we observe in isoflurane- and peptide-exposed mice suggest association of functional defects in glutamatergic transmission at the perforant path-dentate gyrus granule cell synapse that appear independent of CA1 hippocampal synaptic plasticity.

We explored whether the disruption of synaptic PDZ2 interactions contributes to persistent learning and memory deficits after early anesthetic exposure. We assessed cognitive performance in mice using two different behavior tests that have previously indicated impairment after early exposure to anesthesia and that involve different types of hippocampal dependent learning and memory including non-spatial recognition memory (novel object recognition)[9,29,30] and contextual fear learning and memory (fear conditioning).[31,32] Previously we found that weanling mice exposed early to isoflurane or injected with wild-type PDZ2 peptide exhibited reduced recognition memory performance as compared with controls.[9] Here, we asked whether this behavior impairment persists after an even longer recovery period. We found 6-week-old mice exposed early to isoflurane or injected with wild-type PDZ2 peptide also exhibited reduced recognition memory performance. These results indicate that intact postsynaptic density-PDZ2–mediated protein interactions are important for hippocampal-dependent recognition memory performance in weanling and sexually mature mice. Long-lasting impairments in fear conditioning, persisting into adulthood, have been observed after exposure of neonatal mice to sevoflurane.[31] Contextual fear tests evaluate hippocampus and amygdala dependent learning and memory functions.[33] During fear conditioning we found all mice learned the association between unconditioned stimulus and conditioned stimulus. Subsequent tests comparing experimental animals with their respective controls in this training phase indicated that isoflurane and wild-type PDZ2 peptide–exposed mice froze for a shorter duration of time suggesting they had impaired learning. Long-term memory was

assessed based on the freezing reaction of mice in response to the previously conditioned context (*i.e.*, the box). The freezing response to the same context in mice with neonatal exposure to isoflurane or wild-type PDZ2 peptide was reduced significantly compared with controls after a 1-week retention delay at 8 weeks of age. Thus, we found isoflurane or disruption of synaptic PDZ2 interactions can impair fear learning and causes persistent memory deficits as evidenced by decreased freezing response at 8 weeks of age (1 week after fear conditioning and 7 weeks after exposure). Because CA1 long-term potentiation recovered by 7 weeks, the isoflurane-induced impairment in fear learning and memory is likely independent of CA1 hippocampal synaptic plasticity but leaves unanswered a possible role for dentate gyrus, amygdala, or other.

We have demonstrated that PDZ2 domain-mediated interactions between postsynaptic density-95 or postsynaptic density-93 and NMDA receptors or neuronal nitric oxide synthase are disrupted by clinically relevant concentrations of anesthetics.[4] We hypothesized that introduction of certain downstream signaling components during the time of isoflurane or wild-type PDZ2 peptide exposure may prevent the decrease in mushroom spines. Indeed, treatment with the nitric oxide donor Molsidomine prevents the decrease in mushroom spine density caused by isoflurane or wild-type PDZ2 peptide suggesting the effect of nitric oxide is downstream of the disrupted PDZ2 interactions. These results argue in favor of a role for NMDAR NR2-postsynaptic density-95 PDZ2-neuronal nitric oxide synthase signaling in the isoflurane mediated decrease in mushroom spine density. A better understanding of how nitric oxide signaling affects mushroom spine density is an important area to pursue.

Potential limitations to our study are the high oxygen concentration used for carrier gas and not having run naïve mice in all experiments. There is the potential for increased production of reactive oxygen species and also for enhanced toxicity by combining an inhalational anesthetic with oxygen so our use of 98.5% oxygen as a carrier gas may be questioned. This is unlikely to explain our results, however, because anesthesia control mice received 100% oxygen for the same period of time and peptide injected mice were not exposed to the oxygen carrier gas or separated from their dams. There is the risk that maternal separation may have a confounding effect such as obscuring a real effect and could explain why we were unable to detect a significant difference in thin spines (but do see a trend in the expected direction) between oxygen controls and isoflurane exposed mice. Low animal number and data composed of a mixture of male and female data may be one of the reasons we did not detect significant changes in the hippocampal CA1 LTP. However, this is unlikely because every animal tested exhibited robust LTP at this timepoint.

In conclusion, our findings indicate that a single 4-h exposure of infant mice to 1.5% isoflurane or targeted disruption of postsynaptic density 95-PDZ2-mediated protein interactions (*i.e.*, a specific molecular target of isoflurane) with wild-type PDZ2 peptide results in a persistent decrease in mushroom spine density and impairments in hippocampus and amygdala dependent learning and memory (nonspatial and contextual) in sexually mature mice. The observed decrease in mushroom spine density can be prevented by introduction of a nitric oxide donor suggesting the involvement of NMDAR NR2-postsynaptic density-95 PDZ2-neuronal nitric oxide synthase signaling pathway in these processes.

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Competing Interests

The authors declare no competing interests.

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