ANESTHESIOLOGY

Nitric Oxide Donor Prevents Neonatal Isoflurane-induced Impairments in Synaptic Plasticity and Memory

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ABSTRACT

Background: In humans, multiple early exposures to procedures requiring anesthesia constitute a significant risk factor for development of learning disabilities and disorders of attention. In animal studies, newborns exposed to anesthetics develop long-term deficits in cognition. Previously, our laboratory showed that postsynaptic density (PSD)-95, discs large homolog, and zona occludens-1 (PDZ) domains may serve as a molecular target for inhaled anesthetics. This study investigated a role for PDZ interactions in spine development, plasticity, and memory as a potential mechanism for early anesthetic exposure-produced cognitive impairment.

Methods: Postnatal day 7 mice were exposed to 1.5% isoflurane for 4 h or injected with 8 mg/kg active PSD-95 PDZ2WT peptide. Apoptosis, hippocampal dendritic spine changes, synapse density, long-term potentiation, and cognition functions were evaluated (n = 4 to 18).

Results: Exposure of postnatal day 7 mice to isoflurane or PSD-95 PDZ2WT peptide causes a reduction in long thin spines (median, interquartile range [IQR]: wild type control [0.54, 0.52 to 0.86] *vs.* wild type isoflurane [0.31, 0.16 to 0.38], $P = 0.034$ and PDZ2MUT [0.86, 0.67 to 1.0] *vs.* PDZ2WT [0.55, 0.53 to 0.59], $P = 0.028$), impairment in long-term potentiation (median, IQR: wild type control [123, 119 to 147] and wild type isoflurane [101, 96 to 118], $P = 0.049$ and PDZ2MUT [125, 119 to 131] and PDZ2WT [104, 97 to 107], $P = 0.029$), and deficits in acute object recognition (median, IQR: wild type control [79, 72 to 88] *vs.* wild type isoflurane [63, 55 to 72], $P = 0.044$ and PDZ2MUT [81, 69 to 84] *vs.* PDZ2WT [67, 57 to 77], $P = 0.039$) at postnatal day 21 without inducing detectable differences in apoptosis or changes in synaptic density. Impairments in recognition memory and long-term potentiation were preventable by introduction of a NO donor.

Conclusions: Early disruption of PDZ domain–mediated protein–protein interactions alters spine morphology, synaptic function, and memory. These results support a role for PDZ interactions in early anesthetic exposure–produced cognitive impairment. Prevention of recognition memory and long-term potentiation deficits with a NO donor supports a role for the *N*-methyl-D-aspartate receptor/PSD-95/neuronal NO synthase pathway in mediating these aspects of isoflurane-induced cognitive impairment.

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Some general anesthetics have been shown to have adverse effects on neuronal development that affect neural function and cognitive behavior.
- Clinically relevant concentrations of inhalational anesthetics inhibit the postsynaptic density (PSD)-95, discs large homolog, and zona occludens-1 (PDZ) domain–mediated protein–protein interaction between PSD-95 or PSD-93 and *N*-methyl-D-aspartate receptors or neuronal NO synthase.

What This Article Tells Us That Is New

- Neonatal PSD-95 PDZ2WT peptide treatment mimics the effects of isoflurane (~1 minimum alveolar concentration) by altering dendritic spine morphology, neural plasticity, and memory without inducing detectable increases in apoptosis or changes in synaptic density.
- These results indicate that a single dose of isoflurane (~1 minimum alveolar concentration) or PSD-95 PDZ2WT peptide alters dendritic spine architecture and functions important for cognition in the developing brain. This impairment can be prevented by administration of the NO donor molsidomine.

Although pediatric anesthesia is considered safe in terms of mortality and gross morbidity, there is accumulating evidence that early exposure to anesthetic agents may interfere with brain development, cause neuronal death, and ultimately lead to permanent cognitive deficits.[1–6] Recently, the U.S. Food and Drug Administration has identified pediatric anesthetic neurotoxicity as a potentially important public health problem.[7] Evidence from epidemiologic studies suggests that humans are susceptible to long-term cognitive effects after anesthesia.[5,6,8–10] Multiple exposures before age 3 yr are associated with increased frequencies of learning disabilities and attention deficit/hyperactivity disorder.[11] Recent primate studies have revealed persistent abnormality in visual recognition memory[12] and emotional reactivity[13] after early repeated anesthesia exposure. Animal models have confirmed that early postnatal exposure to anesthetics results in long-lasting impairments in learning and memory.[3,14–19] Although a number of ion channels and receptors at synapses have been highlighted as potential targets for anesthetics, the molecular mechanisms that underlie pediatric anesthetic neurotoxicity are still poorly understood.[20–24] Many of these ion channels and receptors are linked to their downstream signaling pathways through postsynaptic density (PSD)-95, discs large homolog, and zona occludens-1 (PDZ) domain–mediated protein–protein interactions. Our laboratory previously showed that anesthetics can disrupt PDZ domain–mediated protein–protein interactions *in vitro* and *in vivo*.[25,26] Using clinically relevant concentrations of inhalational anesthetics, we dose-dependently and specifically inhibited PDZ domain–mediated protein interactions between PSD-95 or PSD-93 and the *N*-methyl-D-aspartate (NMDA) receptor or neuronal NO synthase (fig. 1).[25] These inhibitory effects are immediate, potent, and reversible and

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occur at a hydrophobic peptide-binding groove on the surface of the second PDZ domain of PSD-95. These findings reveal PSD-93 and PSD-95 proteins and specifically their PDZ domains as molecular targets for inhalational anesthetics. We are able to mimic this action of anesthesia with PSD-95 PDZ2WT peptide, which disrupts PSD-PDZ2–mediated protein interactions by binding to interaction partners (fig.1). Specifically, we demonstrated disruption of protein–protein interactions between NMDA receptor NR2 subunits and PSD-95.[27] This disruption significantly reduced minimum alveolar concentration (MAC) and righting reflex EC50 for halothane, indicating that this domain and protein are important for anesthetic action.

Given that (1) the PDZ domain is a molecular target for inhalational anesthetics,[25,26] (2) disruption of PSD–PDZ2–mediated protein interactions increases anesthetic sensitivity,[27] (3) PSD-95 PDZ2 interacts with NMDA receptor and promotes synaptogenesis,[28,29] and (4) multiinnervated spine formation is prevented by deletion of the PSD-95 PDZ2 domain,[28] we hypothesize that alteration of PDZ domain–mediated protein–protein interactions contributes to the molecular mechanisms of pediatric anesthetic neurotoxicity by uncoupling ion channels and receptors from their downstream signaling pathways. Here we investigate a role for PSD-95 PDZ2 domain–mediated protein–protein interactions in hippocampal development and plasticity as a potential mechanism for early anesthetic exposure–produced cognitive impairment. We examine, *in vivo*, the outcome of disrupting PSD-95 PDZ2 domain–mediated protein–protein interactions early in development on apoptosis, spinogenesis, synaptogenesis, long-term potentiation, and object recognition memory. To specifically determine the involvement of the NMDA receptor NR2–PSD-95 PDZ2–neuronal NO synthase pathway, we introduced NO donor at the time of isoflurane anesthesia or PSD-95 PDZ2 exposure to test whether impairments are preventable.

Materials and Methods

This study was carried out with approval from the Animal Care and Use Committee at Johns Hopkins University and was consistent with the National Institutes of Health 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 and PSD-93–null mutant male and female mice were used in our study. For all experiments, the mice were assessed in their sexually immature state (at or before postnatal day 21). On postnatal day 7, animals from each litter were randomly assigned to control and treatment groups. The mice were maintained under standard lab housing with a 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

Postnatal day 7 control and experimental mice were placed in a clear plastic cone, and body temperature was maintained by a heating blanket set to 35°C. Vital signs and physiologic monitoring were assessed using PhysioSuite (Kent Scientific, USA), and blood gasses were collected. Our data suggested that the mice were adequately oxygenated, and they were not overly acidotic (data not shown). Naïve animals were left with the dams. 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 and 45 min (total 4 h isoflurane). Control animals were exposed to oxygen only. At the end of the exposure, the animals were maintained in oxygen on the heating blanket for 10 min and then returned to dams. The purified fusion peptides, active Tat-PSD-95 PDZ2WT (referred to as PDZ2WT here) or inactive Tat-PSD-95 PDZ2MUT (referred to as PDZ2MUT here) at 8 mg/kg were injected into mice intraperitoneally in 150 μl of phosphate-buffered saline and 10% glycerol, as previously described.[27] 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-PSD-95 PDZ2WT and MUT 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-PSD-95 PDZ2, has three sites critical for interactions between NMDA receptors and PSD-95 mutated (K165T, L170R, and H182L).[25] The NO donor molsidomine (*N*-[ethoxycarbonyl]-3-[4-morpholinosydnomine]; Sigma, USA) was injected at 4 mg/kg into mice intraperitoneally in 100 μl of sterile saline as previously described. Control animals were injected intraperitoneally with the vehicle (saline).

Western Blotting

C57Bl6 wild type mice were euthanized by cervical dislocation, and the brains were harvested. Hippocampi were grossly dissected from the mouse brain under a dissecting microscope. Total proteins from these tissues were extracted. The tissues were homogenized in homogenization buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 2 μM pepstatin A, and 320 mM sucrose [pH 7.4]). The crude

Portions of the present work have been presented previously under the title "Neonatal Disruption of PSD-95 PDZ Domain-mediated Protein–Protein Interactions Alters Dendritic Spine Morphology, Long-term Potentiation, and Causes Deficits in Learning and Memory in Mice" at the following meetings: Association of University Anesthesiologists Annual Meeting, April 26, 2018, in Chicago, Illinois; Society of Critical Care Anesthesiologists Annual Meeting, April 27, 2018, in Chicago, Illinois; and the International Anesthesia Research Society Annual Meeting, April 29, 2018, in Chicago, Illinois.

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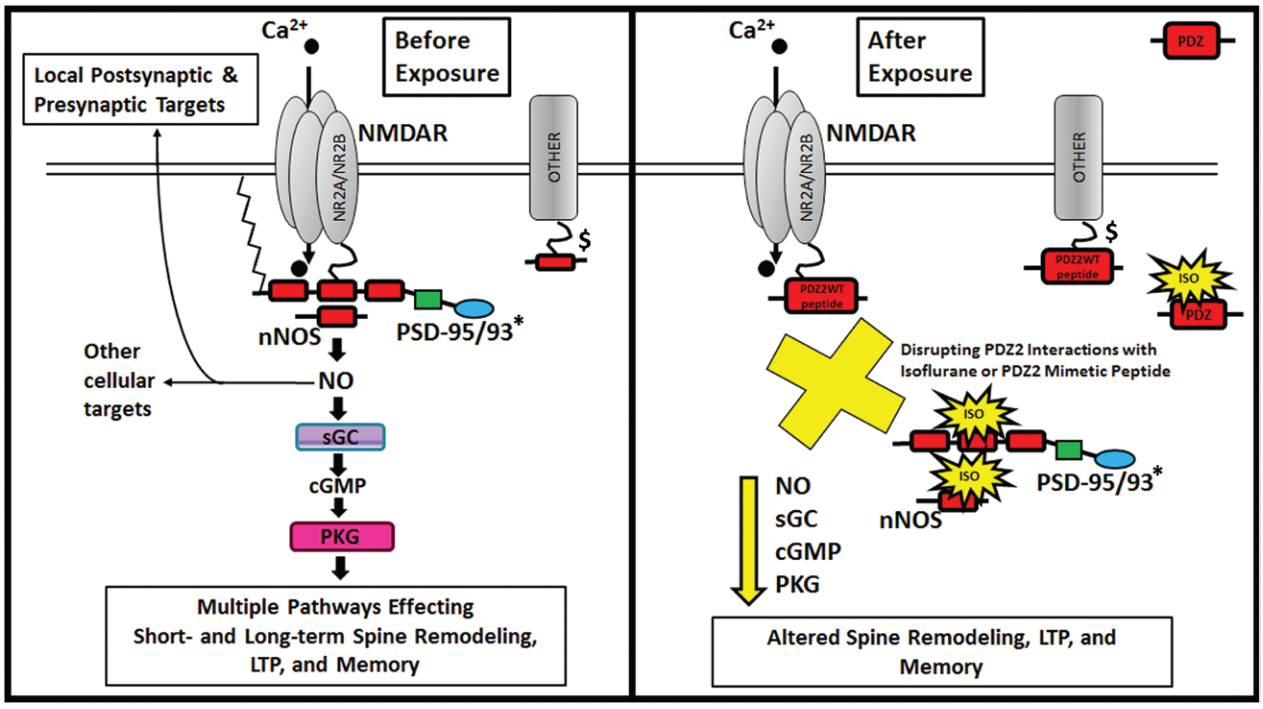

Fig. 1. Diagram illustrating dissociation of *N*-methyl-D-aspartate (NMDA) receptor–PSD95/93–neuronal NO synthase interaction by isoflurane or PDZ2WT (active) peptide (for simplicity, not all PSD-95 family members are represented). (*Left*) Before exposure, the NMDA receptor is linked to downstream molecules such as neuronal NO synthase through PSD-95. Through its first and second PDZ domain, PSD-95 forms a ternary complex by binding to both the tSXV (where S is serine, X is any amino acid, and V is valine) motif of NMDA receptor NR2 subunit and to the PDZ domain in neuronal NO synthase.[63] Disrupting NMDA receptor–PSD-95/93–neuronal NO synthase complexes can reduce the efficiency by which calcium ions activate the signaling molecule neuronal NO synthase. (*Right*) After exposure, this disruption is achieved by exposure to inhalational anesthetics[25,26] or the intracellular introduction of PDZ2WT peptide[64]; this is expected to bind to NMDA receptor NR2.[65] $Inhalational anesthetics (and presumably PDZ2WT peptide) can also inhibit interactions between PSD-95 PDZ2 domain and Shaker-type potassium channel Kv1.4 as well as other excitatory receptor channels related to anesthesia and other proteins not shown here for simplicity.[26] cGMP, cyclic guanosine monophosphate; ISO, isoflurane; LTP, long-term potentiation; NMDAR, NMDA receptor; nNOS, neuronal NO synthase; NO, nitric oxide; PDZ, PSD-95, discs large homolog, and zona occludens-1; PKG, protein kinase G; sGC, soluble guanylyl cyclase.

homogenates were centrifuged at 700*g* for 15 min at 4°C. Then the supernatants were combined and diluted in resuspension buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, 2mM 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 and electrotransferred to nitrocellulose membranes. The membranes were blocked in 0.1% Tween 20 in Tris-HCl–buffered saline containing 5% nonfat milk for 1 h at room temperature and then immunoblotted with primary antibodies (anti-caspase 3: 1:1,000 and poly-[adenosine diphosphate-ribose] polymerase 1:1,000 from Cell Signaling Technology [USA]; anti-β-actin: 1:100,000 from Sigma–Aldrich [USA]) in Tween 20 in Tris-HCl–buffered saline buffer containing 5% nonfat milk overnight at 4°C. After being washed extensively in Tween 20 in Tris-HCl–buffered saline, the membranes were incubated for 1 h with horseradish peroxidase–conjugated anti-rabbit or anti-mouse immunoglobulin (Bio-Rad, USA) at a dilution of 1:5,000. Proteins were detected by enhanced chemiluminescence (Amersham, USA). β-Actin served as a loading control.

Golgi Staining, Microscopy, and Spine Reconstruction

Postnatal day 21 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 the hippocampi were grossly dissected and stained using the FD Rapid GolgiStain kit (FD NeuroTechnologies, USA) as per the 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. The slides were rinsed in Milli-Q (Millipore, USA) water, developed in the working solution DE for 10 min, rinsed, dehydrated in ethanol, cleared in xylene, and mounted with Permount (Fisher, USA). Only samples that had optimal impregnation (contiguous staining across dendrites and spines) were taken forward for imaging (n = 2 excluded animals).

Two different imaging fields per mouse, each containing at least three unique dendritic segments (six segments total per mouse) that contain dorsal hippocampus, were imaged. The six segments were averaged per mouse to contribute one data point per mouse. Dentate granule cells were identified by their location within the dentate gyrus and their distinct morphology. Spines along the secondary and tertiary dendrites of these neurons were selected for analysis. *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. Spine analysis was performed as described by Risher *et al.*[30] using the freely available RECONSTRUCT software.[31]

Electron Microscopy

Postnatal day 21 mice were deeply anesthetized and perfused transcardially with a brief flush of 35C Mammalian Ringer solution (EMS 11763-10) with 5 U/ml of heparin (Sagent Pharmaceuticals, USA; NDC 25021-400-10) followed by 50 ml of 2.5% paraformaldehyde (freshly prepared from electron microscopy grade prill), 2% glutaraldehyde, 0.1 M sodium cacodylate, and 3 mM $MgCl_2$ (pH 7.2) at 1,314 milliosmols at a rate of 1 ml/min. After the perfusion, the mouse heads were stored at 4°C for 2 h. After incubation in the cold, the brains were removed, and the hippocampi were grossly dissected using a brain block and dissecting microscope. Tissue was immersed in fixative overnight and further dissected in the cold room the next morning to isolate the hippocampus (2 mm × 2 mm) and add notches for orientation. The following steps were kept cold (4°C) until the 70% ethanol step and then run at room temperature. Samples were rinsed in 100 mM cacodylate, 3.5% sucrose, 3 mM $MgCl_2$ (pH 7.2) at 324 milliosmols for 45 min. After buffer rinses, samples were microwave-fixed twice in 2% osmium tetroxide reduced with 1.6% potassium ferrocyanide, in the same buffer without sucrose. Sample temperatures did not exceed 9°C. After microwave processing, the samples were rocked in osmium on ice for 2 h in the dark. Tissue was then rinsed in 100 mM maleate buffer (pH 6.2) and then *en bloc* stained for 1 h with filtered 2% uranyl acetate in maleate buffer (pH 6.2). After *en bloc* staining, the samples were dehydrated through a graded series of ethanol to 100%, transferred through propylene oxide, embedded in Eponate 12 (Pella), and cured at 60°C for 2 days. Sections were cut on a Riechert Ultracut E microtome with a Diatome Diamond knife (45 degree).

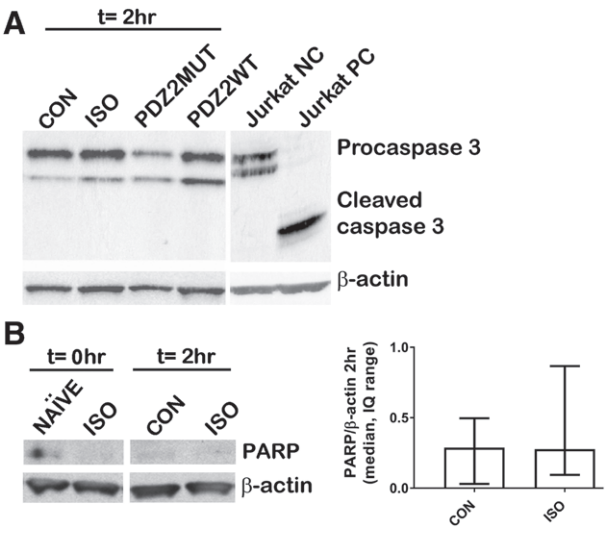

Fig. 2. Western blot assays did not show any significant differences in apoptosis in our exposure paradigm. Postnatal day 7 mice were exposed for 4 h and harvested 2 h after cessation of exposure. (*A*) Western blot analysis for total caspase-3 revealed the presence of procaspase 3 but did not reveal any detectable cleaved caspase-3 in mice exposed to control (CON, O_2), isoflurane (ISO, 1.5% isoflurane in O_2), PDZ2MUT (8 mg/kg inactive peptide), or PDZ2WT (8 mg/kg active peptide). Negative (NC) and positive (PC) Jurkat control proteins were run to demonstrate sensitivity of the caspase-3 antibody. (*B*) Western blot analysis for poly-(adenosine diphosphate-ribose) polymerase (PARP) did not reveal significant differences between control (O_2) and isoflurane (1.5% isoflurane in O_2). (*Left*) Representative blot. (*Right*) Densitometry data are plotted as median, interquartile (IQ) range. CON (n = 6) *versus* isoflurane (n = 6), $P = 0.818$. The data were analyzed using Mann–Whitney test. Sex was not determined.

Sixty-nanometer sections were picked up on Formvar-coated 1 × 2–mm copper slot grids and stained with methanolic uranyl acetate. Grids were viewed on a Phillips CM 120 TEM operating at 80 kV, and digital images were captured with an XR80-8 megapixel CCD by Advanced Microscopy Techniques, Corp. (USA). Ten images (each image 6,250 × 7,500 nm) were averaged per animal. Each animal contributed one data point to obtain median number of PSDs for each group.

Electrophysiology

Slice Preparation. Two weeks after exposure (postnatal days 21 to 35), mice were euthanized, and coronal brain slices containing central part of hippocampus (300-μm thick) were made from a Leica VT 1200S vibrotome in ice-cold artificial cerebrospinal fluid containing 128 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 10 mM glucose, and 2 mM $CaCl_2$ and saturated with 95% O_2 and 5% CO_2. The slices were incubated

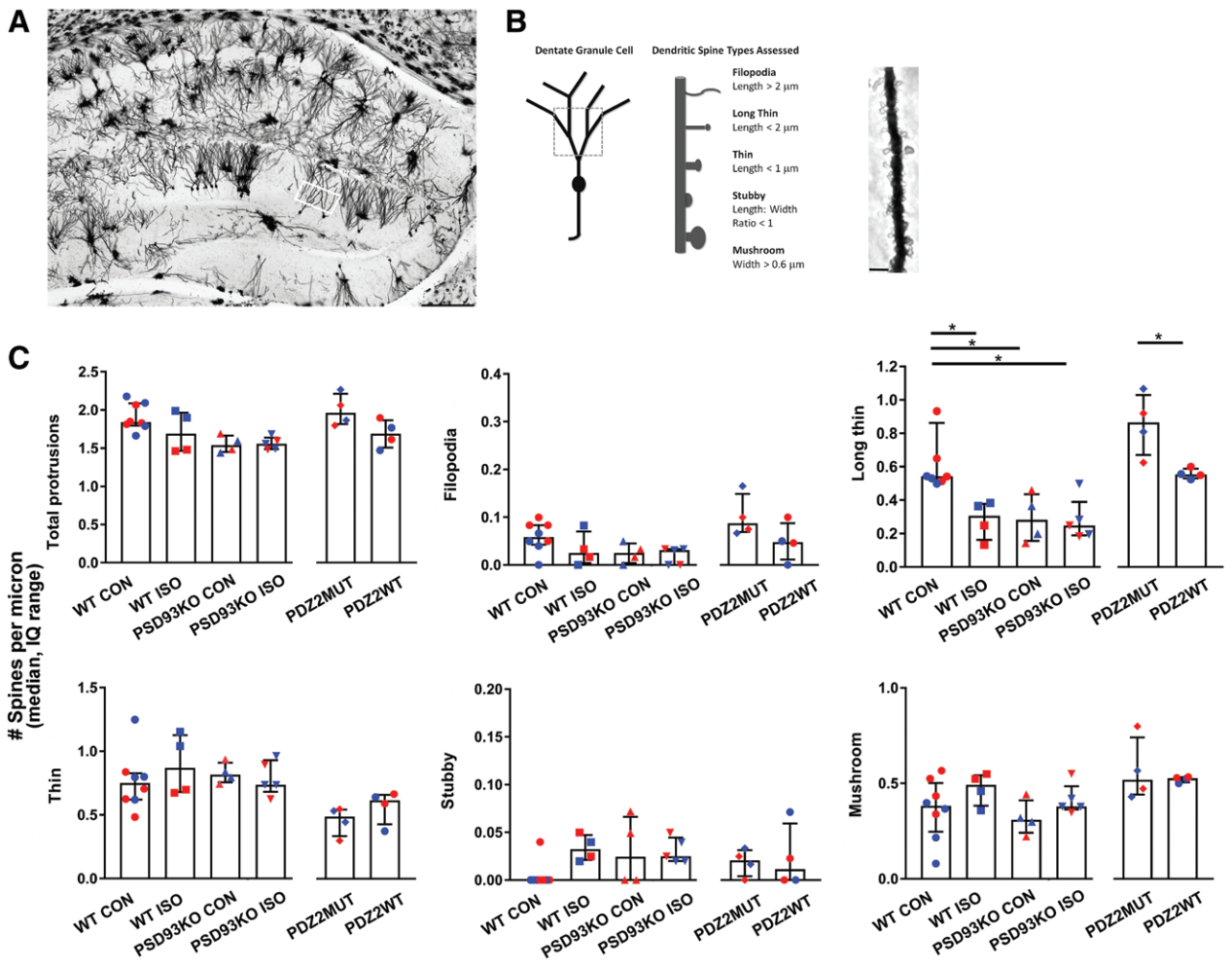

Fig. 3. Neonatal exposure to isoflurane (ISO) or PDZ2WT peptide alters hippocampal dendritic spine morphology and development. (*A*) Dorsal hippocampal region of a Golgi preparation illustrating dendrites from the superior blade of the dentate gyrus subregion of interest (*white box*); *scale bar* represents 250 µm. (*B*) Schematics showing dendrite branches and spine types sampled and a representative dendritic segment with spines; *scale bar* represents 2.5 µm. Spines were assessed on dendritic segments distal to the first and second branch points. (*C*) Distribution of dendritic spines according to morphological type in dentate gyrus among exposed groups assessed at postnatal day 21. Wild-type (WT) and PSD93KO knockout postnatal day 7 mice were exposed to O_2 (control [CON], 100% O_2) or isoflurane (1.5% isoflurane in O_2); WT postnatal day 7 mice were also injected with PDZ2MUT (8 mg/kg inactive peptide) or PDZ2WT (8 mg/kg active peptide; WT CON = 8, WT isoflurane = 4, PSD93KO CON = 4, PSD93KO isoflurane = 5, PDZ2MUT = 4, PDZ2WT = 4). Data from individual animals are plotted and color-coded by sex (*red* = female and *blue* = male). The data are plotted as median number of spines per micron with interquartile (IQ) range. The data were analyzed with Kruskal–Wallis followed by Dunn's multiple comparison correction and Mann–Whitney tests. *$P < 0.05$ was considered significant.

for at least 1 h at room temperature (22 to 24°C) in the interface-type holding chamber filled with artificial cerebrospinal fluid. Then a slice was transferred to the recording chamber, where artificial cerebrospinal 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, and the signal was digitized with Digidita 1440A, analyzed with pClamp10, 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-ms 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 100 Hz × 1 s high-frequency stimulus 20 s apart at the baseline stimulus intensity. Measurements of the field

excitatory postsynaptic potential slopes were made during the rising phase (5 to 50% of the peak), and the values were normalized to the mean values recorded in 20-min baseline. The median of normalized field excitatory postsynaptic potential slopes 55 to 60 min after high-frequency stimulus was used for comparison between groups. Each animal contributed one data point to obtain the median for each group.

Novel Object Recognition

The novel object recognition procedure was used to assess nonspatial hippocampal memory.[32–34] It consisted of a training familiarization phase followed by a testing phase. During training, the mice were allowed to freely explore within an opaque box (40 cm wide × 40 cm long × 34 cm high) containing two identical objects for 10 min. The data were recorded with a 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 = 6 animals were not taken forward because 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. The mice inherently prefer to explore novel objects; thus, a preference for the novel object indicates intact memory for the familiar object.

Statistical Analysis

Statistical analysis was carried out by unpaired two-tailed Student's *t*, Mann–Whitney, and Kruskal–Wallis tests followed by *post hoc* Dunn's tests with GraphPad Prism version 7.0 software (GraphPad Inc., USA). Data sets that failed D'Agostino and Pearson normality test were analyzed using nonparametric statistics. Two-tailed Student's *t* test was used to compare novel and known object investigation times in the novel object recognition assay. Two-tailed Mann–Whitney test was used to compare wild-type control *versus* wild-type isoflurane and PDZ2MUT *versus* PDZ2WT groups (PSD quantification, novel object recognition + NO donor, and long-term potentiation + NO donor), two-tailed Mann–Whitney test was used to compare wild-type control *versus* wild-type isoflurane groups (poly-[adenosine diphosphate-ribose] polymerase western blot) and PDZ2MUT *versus* PDZ2WT groups (spine analysis and long-term potentiation). Kruskal–Wallis test was used in analysis of spines and long-term potentiation (comparing wild-type control, wild-type isoflurane, PSD93KO control, and PSD93KO isoflurane, which included one family, four treatments, and six comparisons) and novel object recognition (comparing wild-type naïve, wild-type control, wild-type isoflurane, PSD93KO control, and PSD93KO isoflurane, which included one family, five treatments, and ten comparisons) and (PDZ2MUT, PDZ2WT, and PDZ2WT + isoflurane, which included one family, three treatments, and three comparisons). The data are expressed as means ± SD or median, interquartile range, respectively, and statistical significance was set at $P < 0.05$. Sample sizes were chosen based on previous experience or published literature. A sample size of N = 6 was chosen for poly-(adenosine diphosphate-ribose) polymerase western blot because an N = 3 was sufficient to detect significant differences after forebrain injury in rats.[35] A sample size of N = 3 to 6 was chosen for ultrastructure analysis because this number of animals was sufficient to detect significant differences in PSDs in the dentate gyrus after cerebral ischemia.[36] A sample size of N = 4 to 8 was chosen for spine analysis because an N = 4 was sufficient to show differences in hippocampal proportional densities of different types of spines after excitoxicity.[37] A sample size of N = 4 to 7 was chosen for long-term potentiation measurements because an N = 3 was sufficient to show differences in long-term potentiation amplitude induced by NMDA antagonist.[38] A sample size of N = 6 to 18 was chosen for novel object recognition because sample sizes of 10 were sufficient to show age-specific differences in rodents.[34,39] In all experiments each animal contributed one data point to obtain the median for each group.

Results

Effect of a Single Dose of Isoflurane or PDZ2WT Peptide on Apoptosis

Jurkat cell control protein obtained from Cell Signaling Technology (USA) was run to determine parameters for running caspase-3 Western blot. The negative control was generated by lysing in Chaps cell extract buffer, and positive control was treated with cytochrome C. Representative image shows expected uncleaved (procaspase 3) and cleaved (cleaved caspase 3) bands (fig. 2A). Four-hour treatment in mice including isoflurane or PDZ2WT peptide exposure did not yield any detectable cleaved caspase at 2 h after cessation of exposure (fig. 2A), nor were we able to detect cleaved caspase 3 at 0 or 20 h after cessation of exposure (data not shown). A representative image shows expected cleaved poly-(adenosine diphosphate-ribose) polymerase band (fig. 2B left). Four-hour isoflurane treatment in mice did not yield significantly different levels of poly-(adenosine diphosphate-ribose) polymerase compared with control at 2 h after cessation of exposure (fig. 2B right; median, interquartile range: control [0.29, 0.31 to 0.50] vs. isoflurane [0.28, 0.09 to 0.87], $P = 0.818$). The data were analyzed using Mann–Whitney test.

Isoflurane, PSD93 Deficiency, and PDZ2WT Peptide Alter Hippocampal Dendritic Spine Morphology

To determine whether inhaled anesthetics interfere with spinogenesis by disrupting synaptic PDZ interactions in the developing hippocampus, we investigated the impact of isoflurane, PSD-93 deficiency, and disrupting PSD-95 PDZ2 domain–mediated protein–protein interactions on dendritic spine morphology in postnatal day 21 mice 2 weeks after an exposure at postnatal day 7. Of note, the PSD-95 family (PSD-95, PSD-93, synapse-associated protein 102, and synapse-associated protein 97) of membrane-associated guanylate kinases share high sequence similarity, as well as similar domain structure: three PDZ domains followed by an SH3 and a GK domain.[40,41] PDZ domains of PSD93 are remarkably similar to the PDZ domains of PSD95 in sequence and structure.[42] Both PSD-93 and PSD-95 PDZ domain–mediated interactions with NMDA NR2 or neuronal NO synthase can be disrupted with anesthetics (fig. 1).[25] Because PSD-93 knockout (KO) mice show impaired long-term potentiation[43] similar to anesthesia-exposed wild type rodents,[3] we used them here as a representative global knockout for the PSD-95 family of membrane-associated guanylate kinases in assessments on spine morphology, long-term potentiation induction, and memory. Neonatal wild type and PSD93KO mouse pups (postnatal day 7) were exposed to isoflurane or O_2 for 4 h. A separate cohort of wild type animals were also exposed to PDZ2MUT or PDZ2WT peptides. The animals were harvested 2 weeks later (postnatal day 21) for rapid Golgi staining to visualize hippocampal dendritic spines (fig. 3). An example tiled image of a Golgi stained region of the hippocampus is shown in figure 3A. The *white box* indicates the subregion of interest within the superior blade of the dentate gyrus. The total number of protrusions and different spine types were assessed based on dendritic segments distal to the first and second branch points (fig. 3B). The data were analyzed using Kruskal–Wallis and Mann–Whitney tests.

A Kruskal–Wallis test indicated a main effect on the number of total dendritic protrusions across the following four groups (fig. 3C, top left, median, interquartile range: wild-type control [1.8, 1.8 to 2.1], wild-type isoflurane [1.7, 1.5 to 2.0], PSD93KO control [1.5, 1.5 to 1.7], PSD93KO isoflurane [1.6, 1.5 to 1.6], $P = 0.029$). However, no difference was detected after Dunn's multiple comparison tests (wild-type control *vs.* wild-type isoflurane, $P = 0.766$; wild-type control *vs.* PSD93KO control, $P = 0.329$; wild-type control *vs.* PSK93KO isoflurane, $P = 0.094$; wild-type isoflurane *vs.* PSD93KO control, $P > 0.999$; wild-type isoflurane *vs.* PSD93KO isoflurane, $P > 0.999$; PSD93KO control *vs.* PSD93KO isoflurane, $P > 0.999$). No significant effect on total protrusions was observed between PDZ2MUT (2.0, 1.8 to 2.2) *versus* PDZ2WT (1.7, 1.5 to 1.9), $P = 0.114$. Thus, exposure to isoflurane, PSD93 deficiency, or PDZ2WT peptide did not have a significant effect on total number of dendritic protrusions assessed at postnatal day postnatal day 21.

Isoflurane, PSD93 deficiency, or PDZ2WT peptide did not have significant effects on the number of filopodial type protrusions (length > 2 μm; fig. 3C, top middle, wild-type control [0.06, 0.04 to 0.08], wild-type isoflurane [0.02, 0.00 to 0.07], PSD93KO control [0.02, 0.00 to 0.05], PSD93KO isoflurane [0.03, 0.00 to 0.03], $P = 0.062$. PDZ2MUT [0.09, 0.07 to 0.15] *vs.* PDZ2WT [0.05, 0.01 to 0.09], $P = 0.200$).

A main effect of treatment was observed across the following groups on the number of long thin spines (less than 2 μm length and less than 0.6 μm width; fig. 3C, top right, wild-type control [0.54, 0.52 to 0.86], wild-type isoflurane [0.31, 0.16 to 0.38], PSD93KO control [0.28, 0.16 to 0.44], PSD93KO isoflurane [0.25, 0.19 to 0.39], $P = 0.003$). Isoflurane caused a reduction in long thin spines in wild-type mice, as did PSD93 deficiency (wild-type control *vs.* wild-type isoflurane, $P = 0.034$; wild-type control *vs.* PSD93KO control, $P = 0.042$; wild-type control *vs.* PSD93KO isoflurane, $P = 0.015$; wild-type isoflurane *vs.* PSD93KO control, $P > 0.999$; wild-type isoflurane *vs.* PSD93KO isoflurane, $P > 0.999$). Isoflurane did not further reduce the number of long thin spines in PSD93KO mice (PSD93KO control *vs.* PSD93KO isoflurane, $P > 0.999$). PDZ2WT peptide caused a reduction in long thin spines (PDZ2MUT [0.86, 0.67 to 1.0] *vs.* PDZ2WT [0.55, 0.53 to 0.59], $P = 0.028$).

Isoflurane, PSD93 deficiency, or PDZ2WT peptide did not have a significant effect on number of thin spines (length of less than 1 μm; fig. 3C, bottom left, wild-type control [0.75, 0.62 to 0.83], wild-type isoflurane [0.87, 0.68 to 1.13], PSD93KO control [0.82, 0.76 to 0.91], PSD93KO isoflurane [0.74, 0.68 to 0.93], $P = 0.705$; PDZ2MUT [0.49, 0.34 to 0.54] *vs.* PDZ2WT [0.62, 0.43 to 0.66], $P = 0.200$).

Isoflurane, PSD93 deficiency, or PDZ2WT peptide did not have a significant effect on stubby spines (length:width ratio of less than 1; fig. 3C, bottom middle, wild-type control [0.00, 0.00 to 0.00], wild-type isoflurane [0.03, 0.02 to 0.05], PSD93KO control [0.02, 0.00 to 0.07], PSD93KO isoflurane [0.02, 0.02 to 0.04], $P = 0.051$; PDZ2MUT [0.02, 0.00 to 0.03] *vs.* PDZ2WT [0.01, 0.00 to 0.06], $P = 0.914$).

Isoflurane, PSD93 deficiency, or PDZ2WT peptide did not have a significant effect on number of mushroom-type protrusions (fig. 3C, bottom right, wild-type control [0.38, 0.25 to 0.50], wild-type isoflurane [0.49, 0.38 to 0.54], PSD93KO control [0.31, 0.24 to 0.41], PSD93KO isoflurane [0.38, 0.36 to 0.48], $P = 0.283$; PDZ2MUT [0.52, 0.44 to 0.74] *vs.* PDZ2WT [0.53, 0.51 to 0.53], $P > 0.999$).

Isoflurane and PDZ2WT Peptide Do Not Have an Effect on the Number of Postsynaptic Densities in the Hippocampus at Postnatal Day 21

To investigate the impact of neonatal exposure to isoflurane or disruption of PSD-95 PDZ2 domain–mediated

protein–protein interactions on synaptogenesis, we assessed the number of PSDs in the hippocampus in postnatal day 21 mice (2 weeks after exposure; fig. 4). Neonatal exposure to isoflurane or PDZ2WT peptide did not have a significant effect on number of PSDs as compared with controls (fig. 4C, median, interquartile range: wild-type control [12.8, 10.9 to 14.1] *vs.* wild-type isoflurane [12.9, 10.7 to 13.8], $P = 0.829$; PDZ2MUT [12.0, 8.5 to 18] *vs.* PDZ2WT [12.8, 10.5 to 15.7], $P = 0.743$). The data were analyzed using a Mann–Whitney test.

Isoflurane, PSD93 Deficiency, and PDZ2WT Peptide Impair Long-term Potentiation Induction in Hippocampal CA1 at Postnatal Day 21

To assess long-term electrophysiologic effects of early isoflurane exposure, PSD-93 deficiency, and disruption of PSD-95 PDZ2 domain–mediated protein–protein interactions, we examined synaptic function and long-term potentiation in hippocampal slices prepared at postnatal day 21 from mice treated at postnatal day 7. Two weeks after exposure, robust long-term potentiation can be induced in wild-type mice that received oxygen control exposure (fig. 5A, top, 5B; median, interquartile range: 123, 119 to 147) or inactive PDZ2MUT peptide (fig. 5A, bottom, 5B; 125, 119 to 131) 55 to 60 min after high-frequency stimulus. Expression of long-term potentiation was impaired in isoflurane (fig. 5A, top, 5B; wild-type isoflurane, 101, 96 to 118; PSD93KO isoflurane, 107, 97 to 117), PDZ2WT peptide (fig. 5A, bottom, 5B; 104, 97 to 107), and PSD93KO control (fig. 5A, middle, 5B; 102, 94 to 112) groups. A Kruskal–Wallis test indicated a significant effect of treatment (wild-type control, wild-type isoflurane, PSD93KO control, PSD93 isoflurane, $P = 0.009$). *Post hoc* Dunn's analysis showed significant differences between wild-type control *versus* wild-type isoflurane (fig. 5B; $P = 0.049$) and wild-type control *versus* PSD93KO isoflurane (fig. 5B; $P = 0.026$). No differences were observed in the following comparisons (wild-type control *vs.* PSD93KO control, $P = 0.056$; wild-type isoflurane *vs.* PSD93KO control, $P > 0.999$; wild-type isoflurane *vs.* PSD93KO isoflurane, $P > 0.999$; PSD93KO control *vs.* PSD93KO isoflurane, $P > 0.999$). PDZ2MUT *versus* PDZ2WT (fig. 5B; $P = 0.029$) groups differed during the last 5 min of recording after high-frequency stimulus.

Isoflurane and PDZ2WT Peptide Cause Subtle but Significant Decreases in Recognition Memory in the Novel Object Recognition Test

To determine whether the disruption of synaptic PDZ interactions contributes to cognitive impairment after early anesthetic exposure, we investigated the impact of isoflurane, PSD93 deficiency, and disrupting PSD-95 PDZ2 domain–mediated protein–protein interactions on memory by assessing hippocampal dependent object recognition in postnatal day 21 mice 2 weeks after exposure at postnatal day 7. In the majority of conditions, the mice were able to discriminate at some level between novel and known objects, revealed by increased investigation times of the novel object (fig. 6A; mean ± SD: wild-type naïve [25 ± 11 *vs.* 6 ± 4], $P < 0.0001$; wild-type control [34 ± 19 *vs.* 9 ± 6], $P < 0.0001$; wild-type isoflurane [29 ± 11 *vs.* 18 ± 12], $P = 0.005$; PSD93KOCON [52 ± 30 *vs.* 15 ± 7], $P = 0.001$; PDZ2MUT [34 ± 16 *vs.* 10 ± 6], $P < 0.0001$; PDZ2WT [19 ± 8 *vs.* 10 ± 6], $P = 0.001$; two-tailed *t* test novel *vs.* known). The double-hit animals were unable to significantly discriminate between novel and known objects (PSD93KO isoflurane [42 ± 20 *vs.* 29 ± 13], $P = 0.098$; PDZ2WT + isoflurane [21 ± 15 *vs.* 14 ± 7], $P = 0.227$). All groups spent more than 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. 6B). A Kruskal–Wallis test indicates a significant effect of exposure treatment (fig. 6B; median, interquartile range: wild-type naïve [85, 78 to 87], wild-type control [79, 72 to 88], wild-type isoflurane [63, 55 to 72], PSD93KO control [78, 65 to 87], PSD93KO isoflurane [57, 47 to 73], $P = 0.0006$). *Post hoc* Dunn's comparisons across groups show that isoflurane-exposed wild-type animals have a subtle but significant decrement in recognition memory as compared to controls (wild-type naïve *vs.* wild-type isoflurane, $P = 0.023$ and wild-type control *vs.* wild-type isoflurane, $P = 0.044$). PSD93 deficiency did not have a significant effect on recognition memory (wild-type naïve *vs.* PSD93KOCON, $P > 0.999$ and wild-type control *vs.* PSD93KOCON, $P > 0.999$). Isoflurane exposure did not cause detectable impairment in PSD93-deficient animals (PSD93KO control *vs.* PSD93KO isoflurane, $P = 0.177$). A Kruskal–Wallis test indicates a significant effect of peptide treatment on memory (PDZ2MUT [81, 69 to 84], PDZ2WT [67, 57 to 77], PDZ2WT + isoflurane [56, 51 to 64], $P = 0.001$). PDZ2WT-exposed animals have a subtle but significant decrement in recognition memory as compared with PDZ2MUT controls (PDZ2MUT *vs.* PDZ2WT, $P = 0.039$ and PDZ2MUT *vs.* PDZ2WT + isoflurane, $P = 0.001$). Isoflurane did not cause further significant decrement in recognition memory in PDZ2WT-exposed animals (PDZ2WT *vs.* PDZ2WT + isoflurane, $P = 0.386$).

Treatment with NO Donor Prevents the Negative Effects of Isoflurane and PDZ2WT Peptide on Hippocampal Long-term Potentiation

Treatment with NO donor prevents the impairment in long-term potentiation caused by isoflurane or PDZ2WT peptide as indicated by the renewed expression of long-term potentiation (fig. 7A, 7B; median, interquartile range: wild-type control + NO [129, 123 to 130], isoflurane +

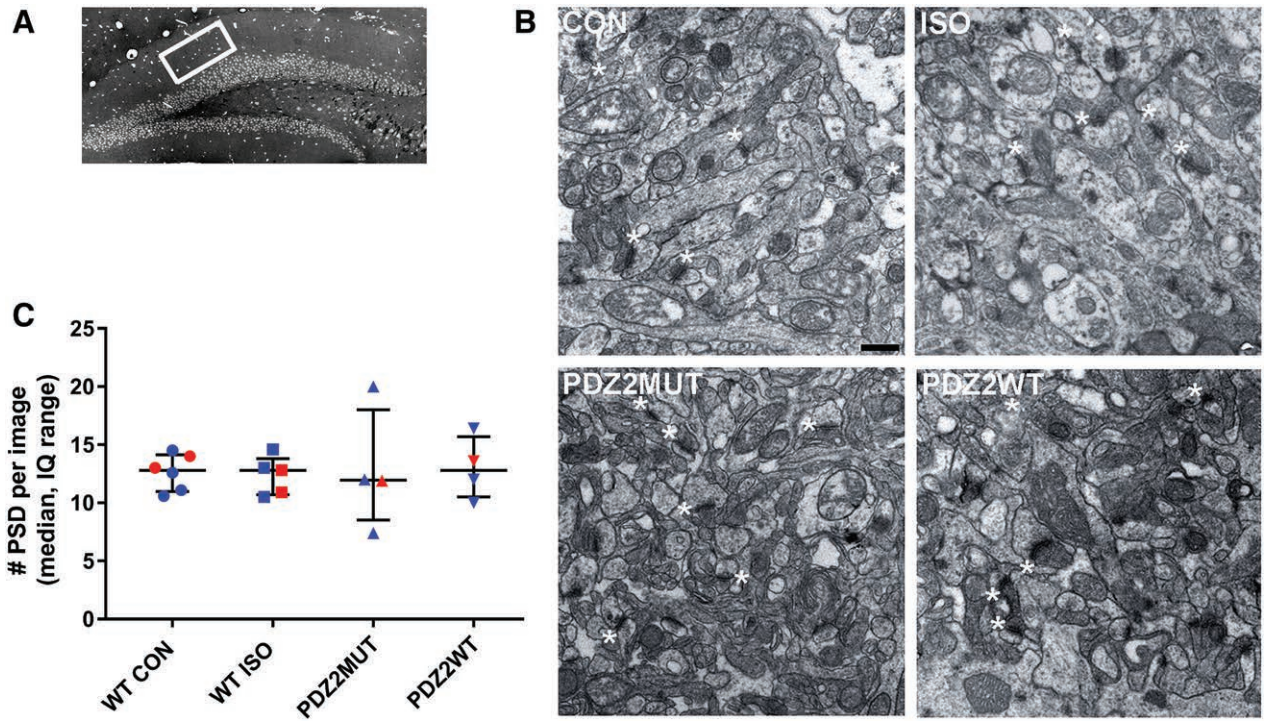

Fig. 4. Neonatal exposure to isoflurane (ISO) or PDZ2WT peptide did not have an acute impact on the number of hippocampal postsynaptic densities. (*A*) Dorsal hippocampal region of a semi-thin section illustrating dentate gyrus (DG) subregion of interest (*white box*). (*B*) Representative ultrastructure images from postnatal day 21 mice exposed at postnatal day 7 to O_2 (wild-type [WT] control [CON], 100% O_2), WT isoflurane (1.5% isoflurane in O_2), PDZ2MUT peptide (8 mg/kg inactive peptide), or PDZ2WT peptide (8 mg/kg active peptide); *scale bar* represents 500 nm. *Asterisks* showing some postsynaptic density (PSD) family members (not all are labeled). (*C*) Plots showing the median with interquartile (IQ) range number of PSDs. WT CON (n = 6) *versus* WT isoflurane (n = 5), $P = 0.829$; PDZ2MUT (n = 4) *versus* PDZ2WT (n = 4), $P = 0.742$. Data from individual animals are plotted and color-coded by sex (*red*, female; and *blue*, male). The data were analyzed with a Mann–Whitney test.

NO [136, 125 to 146], PDZ2MUT + NO [134, 128 to 142], and PDZ2WT + NO [139, 130 to 147]). There is no longer a significant difference between wild-type control and wild-type isoflurane when NO donor is added (fig. 7B; wild-type control + NO *vs.* wild-type isoflurane + NO, $P = 0.284$) or between PDZ2MUT and PDZ2WT (fig. 7B; PDZ2MUT + NO *vs.* PDZ2WT + NO, $P = 0.662$). The data were analyzed with a Mann–Whitney test.

Treatment with NO Donor Prevents Isoflurane- or PDZ2WT-induced Impairment in Acute Recognition Memory

Treatment with NO donor prevents the impairment in novel object recognition caused by isoflurane or PDZ2WT peptide as indicated by the increased discrimination in novel object recognition (fig. 8; median, interquartile range: wild-type control + NO [71, 61 to 82], wild-type isoflurane + NO [87, 73 to 93], PDZ2MUT + NO [84, 73 to 86], and PDZ22WT + NO [79, 73 to 85]). There is no longer a significant difference between wild-type control and wild-type isoflurane when NO donor is added (wild-type control + NO *vs.* isoflurane + NO, $P = 0.073$) or between PDZ2MUT and PDZ2WT (PDZ2MUT + NO *vs.* PDZ2WT + NO, $P = 0.779$). The data were analyzed with a Mann–Whitney test.

Discussion

To better understand the mechanisms mediating isoflurane-induced cognitive impairment, we focused on one specific molecular target of anesthesia. Previously, we demonstrated that inhalational anesthetics can disrupt PDZ domain–mediated protein–protein interactions *in vitro* and *in vivo*[25,26] and specifically inhibit the PDZ domain–mediated protein interaction between PSD-95 or PSD-93 and the NMDA receptor NR2 subunits or neuronal NO synthase (fig. 1).[25,27] Here, we determined the effects of this disruption *in vivo* in the context of isoflurane exposure by specifically mimicking this isolated action of anesthesia with the PDZ2WT peptide, which disrupts PSD-PDZ2–mediated protein interactions by binding to interaction

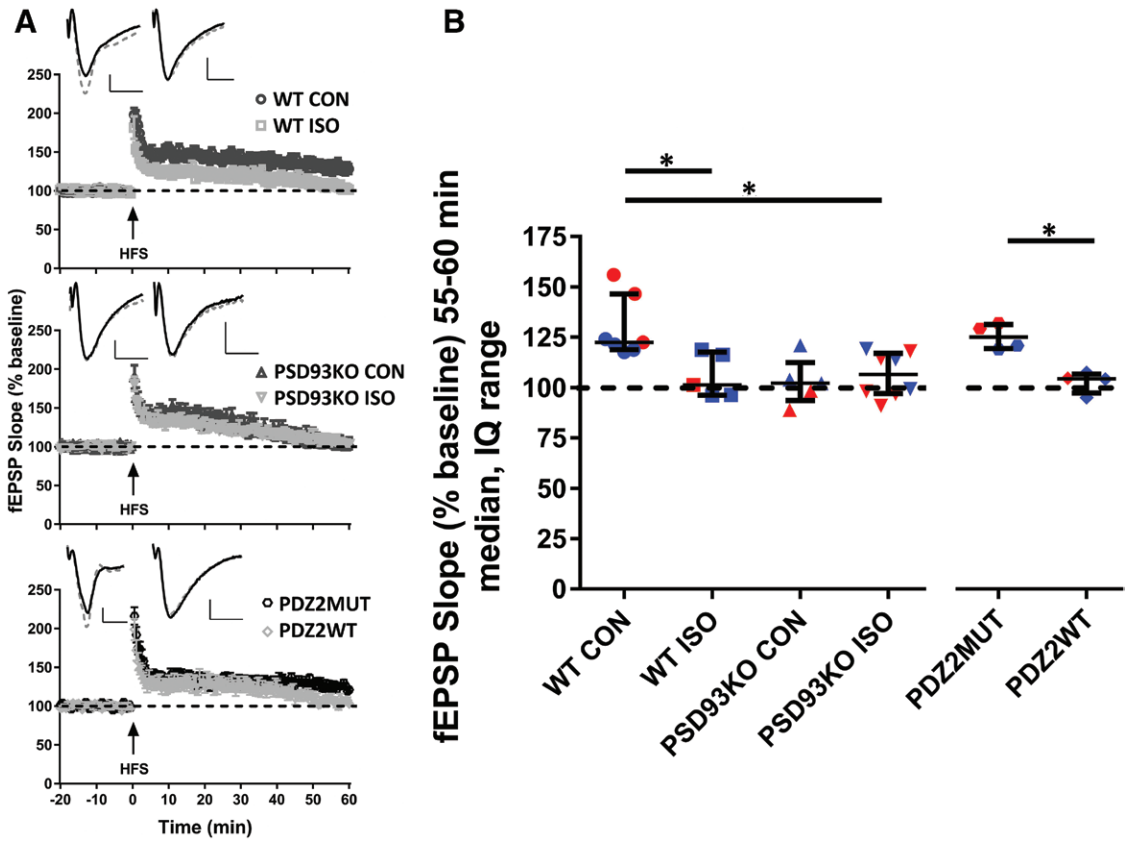

Fig. 5. Neonatal exposure to isoflurane (ISO) or PDZ2WT peptide impairs long-term potentiation in hippocampal CA1 at postnatal day 21. (*A*) High-frequency stimulation (HFS) induced robust long-term potentiation in wild-type control–treated (*top*; WT CON, 100% O_2) and inactive PDZ2MUT–treated (*bottom*) groups in hippocampal Schafer collateral to CA1 pathway. Isoflurane (*top*) and PDZ2WT (*bottom*) exposure as well as PSD93 deficiency (*middle*) impaired the expression of long-term potentiation. Example traces are shown in the upper left quadrants of field excitatory postsynaptic potential (fEPSP) plots. WT CON– and WT isoflurane–treated (*top*), PSD93KO CON– and PSD93KO isoflurane–treated (*middle*), and PDZ2MUT– and PDZ2WT–treated (*bottom*) groups at baseline before HFS (*solid line trace*) and the average of 55 to 60 min after HFS (*dashed line trace*). (*B*) The median of normalized field excitatory postsynaptic potential 55 to 60 min after HFS showed significant differences between WT CON– (n = 7) *versus* WT isoflurane–treated (n = 5, $P = 0.049$) and PDZ2MUT– (n = 4) *versus* PDZ2WT–treated (n = 4, $P = 0.028$) groups. Significant differences were not observed between WT CON *versus* PSD93KO CON (n = 5, $P = 0.056$) but were observed between WT CON *versus* PSD93KO isoflurane (n = 8, $P = 0.025$) groups. Data from individual animals are plotted and color-coded by sex (*red*, female; *blue*, male). The data were analyzed with Kruskal–Wallis followed by *post hoc* Dunn's test and Mann–Whitney test. Values were considered significant at $P < 0.05$ or less (*). The data were plotted as median and interquartile (IQ) range. *Scale bar* indicates 10 ms, 0.25 mV.

partners such as NMDA receptor NR2. In contrast to earlier work,[3] we found that isoflurane exposure did not induce a detectable increase in the level of apoptosis nor did PDZ2WT peptide (fig. 2). Our findings are consistent with recent findings of others showing that brief exposures to anesthesia did not induce apoptosis.[44–46] Further investigation into the sublethal effects of isoflurane, PSD-93 deficiency, and disruption of PSD-95 PDZ2 domain–mediated protein–protein interactions revealed changes in spine morphology, impairments in long-term potentiation induction, and impairments in memory. Introduction of NO donor at the time of exposure prevents impairment in long-term potentiation and recognition memory, further implicating the involvement of the NMDA receptor NR2–PSD-95 PDZ2–neuronal NO synthase signaling pathway in early anesthetic exposure-produced cognitive impairment.

Alterations in spine number and shape are associated with cognitive and developmental dysfunction in various neurologic disorders.[47] Anesthesia exposure, neuronal NO synthase activity, and modulation of membrane-associated guanylate kinase levels have all been associated with spine morphological and density changes.[28,48,49] The most striking result in our spine analysis is the significant loss of long thin spines (length less than 2 μm and width less than 0.6 μm) in isoflurane-exposed, PSD-93–null mutants, and PDZ2WT peptide–exposed animals compared with controls that persists 14 days after exposure. Others have also shown selective loss of spines from anesthesia such as the persistent decrease

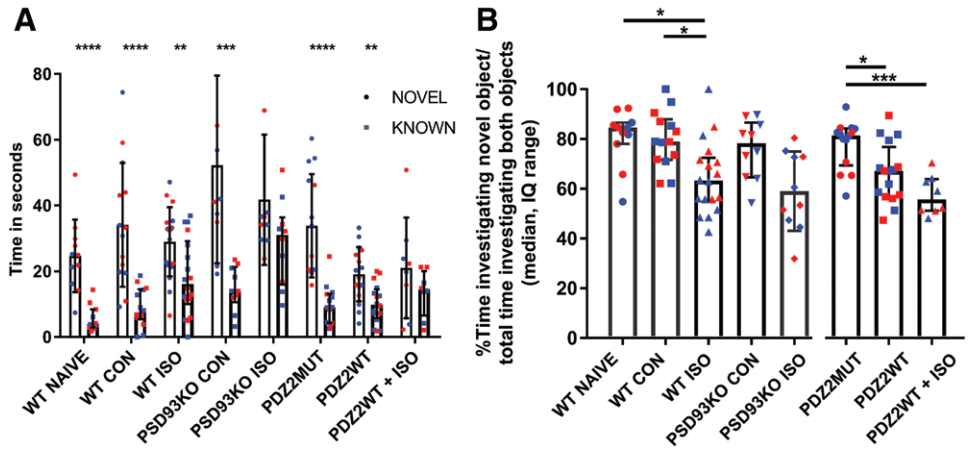

Fig. 6. Neonatal exposure to isoflurane (ISO) or PDZ2WT peptide causes a subtle but significant decrease in acute recognition memory. (*A*) Plots showing percent of time animals spent investigating novel or known objects among experimental groups (wild type [WT] naïve, n = 11, $P < 0.0001$; WT control [CON], n = 14, $P < 0.0001$; WT isoflurane, n = 18, $P = 0.005$; PSD93KO CON, n = 10, $P = 0.001$; PDZ2MUT, n = 14, $P < 0.0001$; PDZ2WT, n = 16, $P = 0.001$). The double hit animals were unable to significantly discriminate between novel and known objects (PSD93KO isoflurane, n = 10, $P = 0.098$; PDZ2WT + isoflurane, n = 8, $P = 0.227$). The data were plotted as mean and SD. The data were analyzed with two-tailed *t* tests, known *versus* novel. (*B*) Plots showing discrimination index as percent of time animals spent investigating novel object over the total time investigating novel and known objects multiplied by 100. Isoflurane-exposed WT animals have a subtle but significant decrement in recognition memory as compared with controls (WT naïve *vs.* WT isoflurane, $P = 0.022$; WT CON *vs.* WT isoflurane, $P = 0.043$; WT naïve *vs.* WT CON, $P > 0.999$). PSD93 deficiency did not have a significant effect on recognition memory (WT naïve *vs.* PSD93KO CON, $P > 0.999$; WT CON *vs.* PSD93KOCON, $P > 0.999$). Isoflurane-exposed PSD93KO animals were not significantly different from PSD93KO controls (PSD93KO CON *vs.* PSD93KO isoflurane, $P = 0.176$). Isoflurane-exposed PSD93KO animals differed from WT controls (WT naïve *vs.* PSD93KO isoflurane, $P = 0.011$; WT CON *vs.* PSD93KO isoflurane, $P = 0.022$). Active peptide exposed animals have a subtle but significant decrement in recognition memory as compared with inactive peptide controls (PDZ2MUT *vs.* PDZ2WT, $P = 0.038$; PDZ2MUT *vs.* PDZ2WT + isoflurane, $P < 0.001$). Isoflurane exposure did not further significantly impair peptide-exposed animals (PDZ2WT *vs.* PDZ2WT + isoflurane, $P = 0.385$). Data from individual animals are plotted and color-coded by sex (*red* = female and *blue* = male). The data were analyzed with Kruskal–Wallis followed by *post hoc* Dunn's test. The data were plotted as median and interquartile (IQ) range. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

(up to 90 days) in spine density of spines with head diameter between 0.3 and 0.4 μm.[50] Length shortening of dendritic protrusions after sevoflurane anesthesia[51] and reduction of long protrusions after isoflurane anesthesia[52,53] in culture have been reported. Platholi *et al.*[54] specifically showed that isoflurane reduces F-actin concentration in spines, suggesting a role in spine shrinkage and loss. Thus the loss of long thin spines that we observe in our study could be the result of length shortening after disruption of PDZ domain–mediated interactions that lead to F-actin depolymerization. Experiments are underway to determine whether the specific PDZ domain–mediated interaction involves NMDA receptor NR2–PSD-95 PDZ2–neuronal NO synthase or another pathway. The functional significance of this loss of long thin spines remains to be determined but might involve a role in circuitry development and permanently alter neural connectivity.

Anesthesia-induced changes in dendritic spine density have been shown to be accompanied by parallel changes in spine synapse number.[50,52] We did not observe any change in density of synapses after isoflurane or PDZ2WT peptide exposure. Our results demonstrate that exposure of immature mice to a sublethal dose of anesthesia or PDZ2WT peptide at the peak of synaptogenesis did not cause significant differences in hippocampal ultrastructural synaptic density 2 weeks later at postnatal day 21. Our findings are consistent with the lack of synaptic density change in the single 2-h anesthesia exposure observed in the study by Amrock *et al.*[55] and the lack of synaptic density loss in PSD-95–null mutants.[56] Follow-up studies are underway to determine whether synaptic density changes occur after our exposure paradigm in mice allowed to recover for longer periods (*i.e.*, after postnatal day 21).

To further explore whether the disruption of synaptic PDZ interactions could contribute to learning and memory deficits through altered synaptic function after early anesthetic exposure, we investigated long-term potentiation, a widely considered major cellular mechanism underlying learning and memory.[57] Previous work demonstrated early exposure to a combination anesthetic induced a profound suppression of long-term potentiation in the hippocampus of adolescent rats.[3] We found suppression of long-term potentiation in isoflurane-exposed wild-type animals. Similar to Carlisle *et al.*,[43] we found long-term

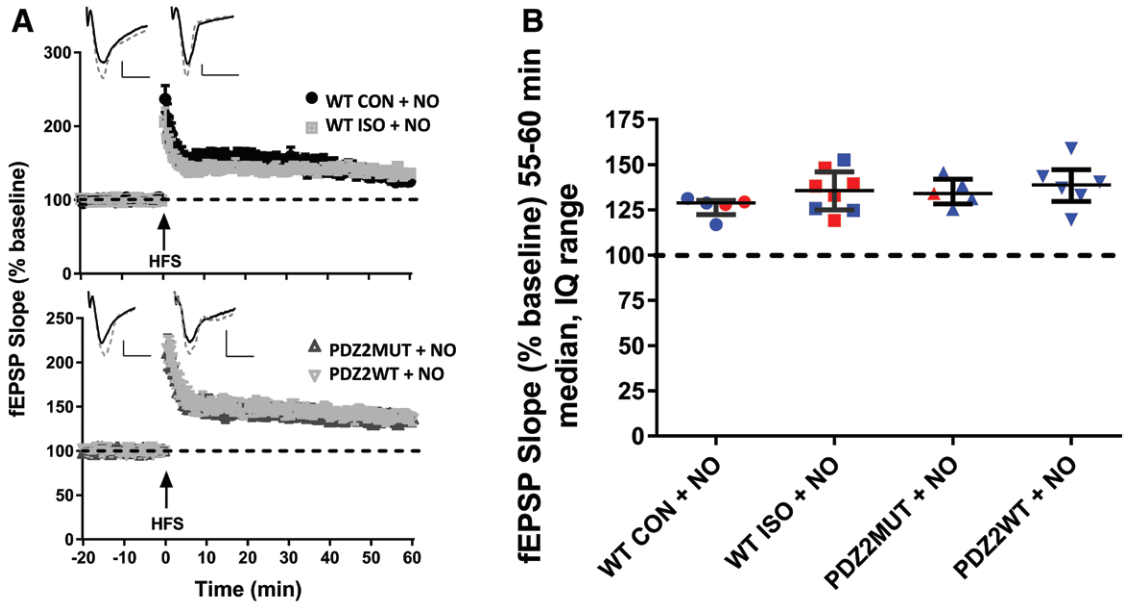

Fig. 7. Treatment with NO donor prevents the negative effect of isoflurane (ISO) and PDZ2WT peptide on hippocampal long-term potentiation. (*A*) Robust long-term potentiation was induced by high-frequency stimulation (HFS) in all groups. In the upper left quadrants, example traces of wild-type (WT) control (CON) + NO–treated and WT isoflurane + NO–treated (*top*), PDZ2MUT + NO–treated and PDZ2WT + NO–treated (*bottom*) groups before high-frequency stimulation (*solid line trace*) and 55 to 60 min after high-frequency stimulation (*dashed line trace*) are shown. (*B*) The median of normalized field excitatory postsynaptic potential (fEPSP) 55 to 60 min after high-frequency stimulation no longer shows a significant difference between WT CON and WT isoflurane when NO donor is added (WT CON + NO (n = 5) *versus* isoflurane + NO (n = 8), $P = 0.284$) or between PDZ2MUT and PDZ2WT (PDZ2MUT + NO (n = 5) *versus* PDZ2WT + NO (n = 6), $P = 0.662$). The data were analyzed with Mann–Whitney tests. Data from individual animals are plotted and color-coded by sex (*red*, female; *blue*, male). The data were plotted as median and interquartile (IQ) range. Values were considered significant at $^*P < 0.05$ or less. *Scale bar* indicates 10 ms, 0.25 mV.

potentiation to be impaired in PSD93 mutant mice. Like isoflurane-exposed animals, PDZ2WT peptide–exposed mice showed impaired long-term potentiation as compared with controls. Thus disrupting PDZ domain–mediated protein interactions mimicked the effect of isoflurane on long-term potentiation. These results suggest that synaptic PDZ interactions may contribute to the mechanism underlying anesthesia-induced impairment in synaptic function in mice and therefore could contribute to learning and memory deficits.

More than a decade ago, Jevtovic-Todorovic *et al.*[3] first reported persistent impairments in learning and memory after early exposure to anesthetics in rats.[3] Subsequent studies have linked early exposure to anesthesia to impairments on hippocampal-dependent recognition memory tests in rodents[44,58–60] and humans.[61] Thus, we hypothesized that if disruption of synaptic PDZ interactions contributes to impairments in learning and memory after early anesthetic exposure, we should see deficits in recognition memory performance. We found that wild-type mice exposed to isoflurane exhibited reduced recognition memory performance as compared with controls, as we expected. Injection of PDZ2WT peptide mimicked the effect of isoflurane, reducing recognition memory performance as compared with inactive peptide. These results indicate that intact PSD-PDZ2–mediated protein interactions are important for hippocampal-dependent recognition memory performance in weanling mice.

Several PDZ domain–mediated protein–protein interactions linking ion channels and receptors to their downstream signaling pathways have been shown to be disrupted by inhalational anesthetics. We have demonstrated that PDZ domain–mediated interactions between PSD-95 or PSD-93 and NMDA receptors or neuronal NO synthase,[25] PSD-95, and Shaker-type potassium channel Kv1.4[26] 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,[26] are disrupted by clinically relevant concentrations of anesthetics. We hypothesized that introduction of certain downstream signaling components during the time of isoflurane or PDZ2WT peptide exposure may prevent deficits in long-term potentiation and memory. Indeed, treatment with the NO donor molsidomine prevents impairment in long-term potentiation and recognition memory caused by isoflurane or PDZ2WT peptide, suggesting that the effect of NO is downstream of the disrupted PDZ interactions. These results support the involvement of

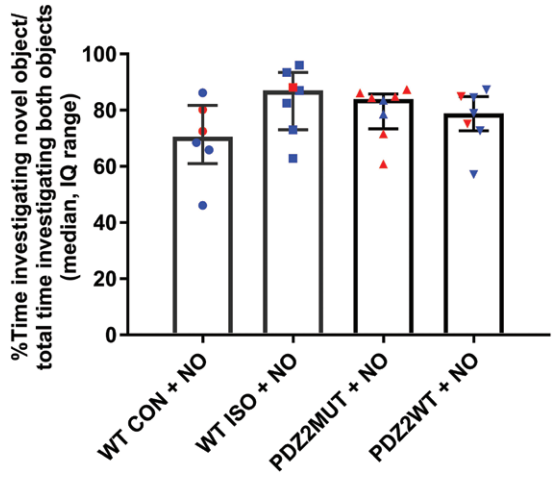

Fig. 8. Treatment with NO donor prevents isoflurane (ISO)– or PDZ2WT peptide–induced impairment in acute recognition memory. Plots show discrimination index as percentages of time animals spent investigating novel object over the total time investigating novel and known objects multiplied by 100. There is no longer a significant difference between wild-type (WT) control (CON) and WT isoflurane when NO donor is added (WT CON + NO [n = 6] *vs.* isoflurane + NO [n = 7], $P = 0.073$) or between PDZ2MUT and PDZ2WT (PDZ2MUT + NO [n = 8] *vs.* PDZ2WT + NO [n = 7], $P = 0.778$). Data from individual animals are plotted and color-coded by sex (*red*, female; *blue*, male). The data were plotted as median and interquartile (IQ) range. The data were analyzed with Mann–Whitney tests. Values were considered significant at $^*P < 0.05$ or less.

the NMDA receptor NR2–PSD-95 PDZ2–neuronal NO synthase signaling pathway in isoflurane-mediated impairment in long-term potentiation and recognition memory. A better understanding of how NO signaling affects changes in long-term potentiation and recognition memory in this developmental context is an important area to pursue.

This study was limited in several important respects. We did not phenotype animals on noncognitive behaviors, so other aspects of neurodevelopment are undefined here. *In vivo* evidence suggests that hyperoxia may be harmful to developing neurons,[62] and there is also the potential for enhanced toxicity by combining an inhalational anesthetic with 100% oxygen, so our use of 100% oxygen as a carrier gas may be questioned. This is unlikely to explain our results, however, because control mice received 100% oxygen for the same period of time, and peptide-injected mice were not exposed to the oxygen carrier gas or to anesthetic and showed parallel results. The number of animals in our spine and synapse analyses are small. To reduce the effect of a small sample size, we took multiple measurements per animal in an effort to lower variation (each animal provided one data point; six dendritic fields were averaged per animal in the spine analysis, and 10 images were averaged per animal in the electron microscopy analysis). Our ultrastructural resolution was not high enough to permit length and width assessments on PSDs.

In conclusion, our findings indicate that a single 4-h exposure of infant mice to isoflurane (~1 MAC) or targeted disruption of PSD-PDZ2–mediated protein interactions (*i.e.*, a specific molecular target of isoflurane) with PDZ2WT peptide results in spine morphological changes, impairments in long-term potentiation induction, and impairments in memory in mice without inducing apoptosis or changes in synaptic density. The observed impairments in long-term potentiation and object recognition memory can be prevented by introduction of an NO donor, suggesting the involvement of NMDA receptor NR2–PSD-95 PDZ2–neuronal NO synthase signaling pathway in these processes.

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Competing Interests

The authors declare no competing interests.

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