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Nitric oxide donor prevents neonatal isoflurane induced impairments in synaptic plasticity and memory

Michele L. Schaefer, Ph.D., **Meina Wang, Ph.D.**, **Patric J. Perez, B.A.**, **Wescley Coca Peralta, M.D.**, **Jing Xu, M.D.**, and **Roger A. Johns, M.D., MHS, Ph.D.**

Department of Anesthesiology and Critical Care Medicine, Johns Hopkins School of Medicine, Baltimore, MD.

Abstract

Background: In humans, multiple early exposures to procedures requiring anesthesia is a significant risk factor for development of learning disabilities and disorders of attention and anxiety. 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 (PND) 7 mice were exposed to 1.5% isoflurane (ISO) for 4 hrs or injected with 8 mg/kg active PSD-95 PDZ2WT peptide. Apoptosis, hippocampal dendritic spine changes, synapse density, long term potentiation (LTP), and cognition functions were evaluated (n=4-18).

Results: Exposure of PND7 mice to ISO or PSD-95 PDZ2WT peptide causes a reduction in long thin spines (median, IQ: WT CON (0.54, 0.52 to 0.86) vs WT ISO (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 LTP (median, IQ: WT CON (123, 119 to 147) and WT ISO (101, 96 to 118), p=0.0.049 and PDZ2MUT (125, 119 to 131) and PDZ2WT (104, 97 to 107), p=0.029), and deficits in acute object recognition (median, IQ: WT CON (79, 72 to 88) vs WT ISO (63, 55 to 72), p = 0.044 and PDZ2MUT (81, 69 to 84) vs PDZ2WT (67, 57 to 77), p=0.039) at PND21 without inducing

Corresponding Author: Roger A. Johns, M.D., MHS, Ph.D., Johns Hopkins University School of Medicine Department of Anesthesiology and Critical Care Medicine, 720 Rutland Ave., Ross 361, Baltimore, MD 21205, 410-952-0504, rajohns@jhmi.edu.

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detectable differences in apoptosis or changes in synaptic density. Impairments in recognition memory and LTP were preventable by introduction of a nitric oxide (NO) donor.

Conclusion: 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 LTP deficits with a NO donor supports a role for the *N*-methyl-D aspartate (NMDA) receptor/PSD-95 PDZ2/neuronal nitric oxide synthase (nNOS) pathway in mediating these aspects of ISO induced cognitive impairment.

Summary Statement:

Neonatal PSD-95 PDZ2WT peptide exposure mimics isoflurane (~1 MAC) by altering spine morphology, neural plasticity, and object recognition memory without inducing detectable increases in apoptosis or changes in synaptic density.

Introduction

While 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 United States Food and Drug Administration has identified pediatric anesthetic neurotoxicity (PAN) as a potentially important public health problem [7]. Evidence from epidemiologic studies suggest 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 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].

While a number of ion channels and receptors at synapses have been highlighted as potential targets for anesthetics the molecular mechanisms that underlie PAN are still poorly understood [20-24]. Many of these ion channels and receptors are linked to their downstream signaling pathways through 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 NMDA receptor or nNOS (fig. 1) [25]. These inhibitory effects are immediate, potent, and reversible and 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 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 3) multi-innervated spine formation is prevented by deletion of the PSD-95 PDZ2 domain [28], we hypothesize that alteration of PDZ domain mediated protein-protein interactions contribute to the molecular mechanisms of PAN 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 NMDAR NR2-PSD-95 PDZ2-nNOS pathway we introduced NO donor at the time of ISO anesthesia or PSD-95 PDZ2 exposure to test if 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 (WT) and PSD-93 null mutant male and female mice were used in our study. For all experiments, mice were assessed in their sexually immature state (≤PND21). On PND7, animals from each litter were randomly assigned to control and treatment groups. 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

PND7 control and experimental mice were placed in a clear plastic cone and body temperature maintained by a heating blanket set to 35C. Vital signs and physiological monitoring were assessed using Physiosuite and blood gasses 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% ISO in oxygen for 2 min and tapered down to 1.5% within 15 min. Exposure to 1.5% ISO was continued for 3 hours and 45 min (total 4 hours ISO). Control ‘CON’ animals were exposed to oxygen only. 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-PSD-95 PDZ2WT (referred to as PDZ2WT in manuscript) or inactive Tat-PSD-95 PDZ2MUT (referred to as PDZ2MUT in manuscript), at 8 mg/kg were injected into mice intraperitoneally (ip) in 150 μl of PBS and 10% glycerol, as previously described [27]. Purification of fusion peptides was performed by Creative BioMart (Shirley, NY) and

verified by Coomassie blue staining and Western blot analysis and then stored in 10% glycerol/phosphate-buffered saline at −80C 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 47-57 of human immunodeficiency virus Tat protein) termed Tat. Inactive control plasmid, mutated Tat-PSD-95 PDZ2, has three sites critical for interactions between NMDARs and PSD-95 mutated (K165T, L170R and H182L)[25]. The NO donor Molsidomine [(N-[ethoxycarbonyl]-3- [4-morpholinosydnomine] (Sigma, St. Louis, MO) was injected at 4 mg/kg into mice ip in 100 μl sterile saline as previously described. Control animals were injected ip with the vehicle (saline).

Western Blotting

C57Bl6 WT mice were sacrificed 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 MgCl2, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 2 μM pepstatin A, and 320 mM sucrose [pH 7.4]). The crude homogenates were centrifuged at 700g for 15 min at 4 °C. Then the supernatants were combined and diluted in resuspension buffer (10 mM Tris-HCl, 5 mM MgCl2, 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 (TBST) containing 5% nonfat milk for 1 h at room temperature and then immunoblotted with primary antibodies (anti-caspase 3: 1:1000 and poly-(adenosine diphosphate-ribose) polymerase (PARP) 1: 1000 were from Cell Signaling Technology, Beverly, MA; anti-β-actin: 1:100,000, Sigma-Aldrich, St. Louis, MO) in TBST buffer containing 5% nonfat milk overnight at 4 °C. After being washed extensively in TBST, the membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (Bio-Rad Laboratories, Hercules, CA) at a dilution of 1:5000. Proteins were detected by enhanced chemiluminescence (Amersham, Piscataway, NJ). β-Actin served as a loading control.

Golgi Staining, Microscopy, and Spine Reconstruction

PND21 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., Columbia, MD) as per vendors 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 hours. Hippocampi were embedded in tissue freezing medium (TFM) and stored at −80C. 60 μm sections were cut on a cryostat at −20C, mounted onto gelatin coated slides, and air dried overnight. Slides were rinsed in Milli-Q water, developed in the working solution DE for 10 min, rinsed, dehydrated in EtOH, cleared in xylene, and mounted with Permount®. 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 (6 segments total per mouse), which contain dorsal hippocampus were imaged. The 6 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 (see fig. 3). Z-stacks of Golgi stained dendrites (optical section thickness = 0.3 μm, i.e. 50-100 images per stack) were taken at 630x magnification on a Leica SPE confocal microscope. Spine analysis was performed as described in Risher et al (2014)[30] using the freely available RECONSTRUCT software[31].

Electron Microscopy

PND21 mice were deeply anesthetized and perfused transcardially with a brief flush of 35C Mammalian Ringer Solution (EMS 11763-10) with 5U/ml of Heparin (Sagent Pharmaceuticals NDC 25021-400-10) followed by 50 mL of 2.5% paraformaldehyde (freshly prepared from EM grade prill), 2% glutaraldehyde, 0.1M sodium cacodylate, 3mM MgCl2, (pH 7.2) at 1314 mOsmols at a rate of 1-ml/min. After the perfusion, the mouse heads were stored at 4C for 2 hours. After incubation in the cold, brains were removed and 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 (2mm × 2mm) and add notches for orientation. The following steps were kept cold (4 C) until the 70% ethanol step, then run at room temp. Samples were rinsed in 100 mM cacodylate 3.5% sucrose 3mM MgCl2, pH 7.2 at 324 mOsmols for 45 min. Following 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. Following microwave processing samples were rocked in osmium on ice for 2 hours in the dark. Tissue was then rinsed in 100 mM maleate buffer with pH 6.2, then en-bloc stained for 1 hour with filtered 2% uranyl acetate in maleate buffer, pH 6.2. Following en-bloc staining 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 two days. Sections were cut on a Riechert Ultracut E microtome with a Diatome Diamond knife (45 degree). 60nm 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 captured with an XR80- 8 megapixel CCD by AMT. Ten images (each image 6250 nm × 7500 nm) were averaged per animal. Each animal contributed one data point to obtain median number of PSD's for each group.

Electrophysiology

Slice preparation.—Two weeks after exposure (PND21-35) mice were euthanized and coronal brain slices containing central part of hippocampus (300μm thick) were made from Leica VT 1200S vibrotome in ice-cold ACSF 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 1hr at room temperature (22-24°C) in the interface-type holding chamber filled with ACSF. Then a slice was transferred to the recording chamber, where ACSF was perfused at a rate of 1.5-2.0mL/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 (fEPSPs) were made from the stratum radiatum of the hippocampal CA1 area. Evoked responses were elicited with 0.1msec constant-current pulses through a concentric electrode in the Schaffer collateral pathway every 30sec at an intensity sufficient to elicit 40-50% maximal EPSPs. After establishing a stable baseline for 20min, LTP was induced by applying three trains of 100Hz X 1sec high frequency stimulus (HFS) 20sec apart at the baseline stimulus intensity. Measurements of the fEPSP slopes were made during the rising phase (5-50% of the peak) and the values normalized to the mean values recorded in 20min baseline. The median of normalized fEPSP slopes 55-60min after HFS 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 (NOR) procedure was based upon the original Ennaceur et al (1988) procedure and was used to assess nonspatial hippocampal memory [32, 3334]. It consisted of a training ‘familiarization’ phase followed by a testing phase. 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). 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 sec (criterion) were taken forward to the testing phase (n=6 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.

Statistical Analysis

Statistical analysis was carried out by unpaired Student's t-test, two-tailed, Mann-Whitney, and Kruskal-Wallis followed by post-hoc Dunn’s tests with Graphpad Prism version 7.0 software (Graphpad Inc., La Jolla, CA). Datasets that failed D'Agostino & Pearson normality test were analyzed using non-parametric statistics. Student's t-test, two-tailed was used to compare novel and known object investigation times in the NOR assay. Mann-Whitney, two-tailed test was used to compare WT CON versus WT ISO and PDZ2MUT versus PDZ2WT groups (PSD quantification, NOR + NO donor, and LTP + NO donor), Mann-Whitney, two-tailed test was used to compare WT CON versus WT ISO groups (PARP WB) and PDZ2MUT versus PDZ2WT groups (spine analysis and LTP). Kruskal-Wallis test was used in analysis of spines and LTP (comparing WT CON, WT ISO, PSD93KO CON, PSD93KO ISO which included 1 family, 4 treatments, and 6 comparisons)

and NOR (comparing WT NAÏVE, WT CON, WT ISO, PSD93KO CON, PSD93KO ISO which included 1 family, 5 treatments and 10 comparisons) and (PDZ2MUT, PDZ2WT, PDZ2WT+ISO that include 1 family, 3 treatments, and 3 comparisons). Data are expressed as mean± standard deviation (SD) or median, interquartile range respectively, and statistical significance was set at $P < 0.05$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. Sample sizes were chosen based on previous experience and/or published literature. A sample size of N=6 was chosen for PARP WB because an N=3 was sufficient to detect significant differences following 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 DG 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 following excitoxicity[37]. A sample size of N=4 to 7 was chosen for LTP measurements because an N=3 was sufficient to show differences in LTP amplitude induced by NMDA antagonist [38]. A sample size of N=6 to 18 was chosen for NOR 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 ISO or PDZ2WT peptide on apoptosis

Jurkat cell control protein obtained from cell signaling technology 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). 4 h treatment in mice including ISO or PDZ2WT peptide exposure did not yield any detectable cleaved caspase at 2 h following cessation of exposure (fig. 2A). Neither were we able to detect cleaved caspase 3 at 0 h or 20 h following cessation of exposure (data not shown). Representative image shows expected cleaved PARP band (fig. 2B left). 4 h ISO treatment in mice did not yield significantly different levels of PARP compared to CON at 2 h following cessation of exposure (fig. 2B right, median, interquartile range: CON (0.29, 0.31 to 0.50) vs ISO (0.28, 0.09 to 0.87), p=0.818). Data were analyzed using Mann-Whitney test.

ISO, 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 ISO, PSD-93 deficiency, and disrupting PSD-95 PDZ2 domain-mediated protein-protein interactions on dendritic spine morphology in PND21 mice two weeks following an exposure at PND7. Of note, the PSD-95 family (PSD-95, PSD-93, SAP102, SAP97) of membrane associated guanylate kinases (MAGUKs) 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 nNOS can be disrupted with anesthetics (fig. 1) [25]. Since PSD-93 KO mice show impaired LTP [43] similar to anesthesia exposed WT rodents [3] we used them here as a representative global knockout for the PSD-95 family of MAGUKs in assessments on spine morphology, LTP induction, and memory. Neonatal WT and PSD93KO mouse pups (PND7) were exposed to ISO or O_2 for 4 hrs. A separate cohort of WT animals were also exposed to PDZ2MUT or PDZ2WT peptides. Animals were harvested two weeks later (PND21) 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 fig. 3A. The white box indicates the subregion of interest within the superior blade of the DG. 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). 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, IQ: WT CON (1.8, 1.8 to 2.1), WT ISO (1.7, 1.5 to 2.0), PSD93KO CON (1.5, 1.5 to 1.7), PSD93KO ISO (1.6, 1.5 to 1.6), p=0.029. However, no difference was detected following Dunn's multiple comparison tests (WT CON vs WT ISO, p=0.766; WT CON vs PSD93KO CON, p=0.329; WT CON vs PSK93KO ISO, p=0.094; WT ISO vs PSD93KO CON, p>0.999; WT ISO vs PSD93KO ISO, p>0.999; PSD93KO CON vs PSD93KO ISO, p>0.999). No significant effect on total protrusions was observed between PDZ2MUT (2.0, 1.8 to 2.2) vs PDZ2WT (1.7, 1.5 to 1.9), p=0.114. Thus, exposure to ISO, PSD93 deficiency, or PDZ2WT peptide did not have a significant effect on total number of dendritic protrusions assessed at PND21.

ISO, PSD93 deficiency, or PDZ2WT peptide did not have significant effects on the number of filopodial type protrusions (length > 2 μm) (fig. 3C top middle, WT CON (0.06, 0.04 to 0.08), WT ISO (0.02, 0.00 to 0.07), PSD93KO CON (0.02, 0.00 to 0.05),PSD93KO ISO (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 (< 2 μm length and < 0.6 μm width) (fig. 3C top right, WT CON (0.54, 0.52 to 0.86), WT ISO (0.31, 0.16 to 0.38), PSD93KO CON (0.28, 0.16 to 0.44), PSD93KO ISO (0.25, 0.19 to 0.39), p=0.003. ISO caused a reduction in long thin spines in WT mice as did PSD93 deficiency (WT CON vs WT ISO, p=0.034; WT CON vs PSD93KO CON, p=0.042; WT CON vs PSD93KO ISO, p=0.015; WT ISO vs PSD93KO CON, p>0.999; WT ISO vs PSD93KO ISO, p>0.999). ISO did not further reduce the number of long thin spines in PSD93 KO mice (PSD93KO CON vs PSD93KO ISO, 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.

ISO, PSD93 deficiency, or PDZ2WT peptide did not have a significant effect on number of thin spines (length< 1 μm) (fig. 3C bottom left, WT CON (0.75, 0.62 to 0.83), WT ISO (0.87, 0.68 to 1.13), PSD93KO CON (0.82, 0.76 to 0.91), PSD93KO ISO (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).

ISO, PSD93 deficiency, or PDZ2WT peptide did not have a significant effect on stubby spines (length:width ratio<1) (fig. 3C bottom middle, WT CON (0.00, 0.00 to 0.00), WT ISO (0.03, 0.02 to 0.05), PSD93KO CON (0.02, 0.00 to 0.07), PSD93KO ISO (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).

ISO, PSD93 deficiency, or PDZ2WT peptide did not have a significant effect on number of mushroom type protrusions (fig. 3C bottom right, WT CON (0.38, 0.25 to 0.50), WT ISO (0.49, 0.38 to 0.54),PSD93KO CON (0.31, 0.24 to 0.41), PSD93KO ISO (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).

ISO and PDZ2WT peptide do not have an effect on the number of postsynaptic densities in the hippocampus at PND21

To investigate the impact of neonatal exposure to ISO or disruption of PSD-95 PDZ2 domain-mediated protein-protein interactions on synaptogenesis, we assessed the number of PSDs in the hippocampus in PND21 mice (two weeks after exposure)(fig. 4). Neonatal exposure to ISO or PDZ2WT peptide did not have a significant effect on number of PSDs as compared to controls (fig. 4C, median, IQ: WT CON (12.8, 10.9 to 14.1) vs WT ISO (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). Data were analyzed using Mann-Whitney test.

ISO, PSD93 deficiency, and PDZ2WT peptide impair LTP induction in hippocampal CA1 at PND21

To assess long-term electrophysiological effects of early ISO exposure, PSD-93 deficiency, and disruption of PSD-95 PDZ2 domain-mediated protein-protein interactions, we examined synaptic function and LTP in hippocampal slices prepared at PND21 from mice treated at PND7. Two weeks after exposure, robust LTP can be induced in WT mice that received oxygen CON exposure (fig. 5A top, 5B; median, IQ: 123, 119 to 147) or inactive PDZ2MUT peptide (fig. 5A bottom, 5B; 125, 119 to 131) 55-60 min after HFS. Expression of LTP was impaired in ISO (fig. 5A top, 5B; WT ISO, 101, 96 to 118; PSD93KO ISO, 107, 97 to 117), PDZ2WT peptide (fig. 5A bottom, 5B; 104, 97 to 107) and PSD93KO CON (fig. 5A middle, 5B; 102, 94 to 112) groups. Kruskal-Wallis test indicated a significant effect of treatment (WT CON, WT ISO, PSD93KO CON, PSD93 ISO, p=0.009). Post-hoc Dunn's analysis showed significant differences between WT CON vs WT ISO (fig. 5B; p=0.049) and WT CON vs PSD93KO ISO (fig. 5B; p=0.026). No differences were observevd in the following comparisons (WT CON vs PSD93KO CON, p=0.056; WT ISO vs PSD93KO CON, p>0.999; WT ISO vs PSD93KO ISO, p>0.999; PSD93KO CON vs PSD93KO ISO, p>0.999). PDZ2MUT vs PDZ2WT (fig. 5B; p=0.029) groups differed during the last 5 min of recording after HFS.

ISO 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 ISO, PSD93 deficiency, and disrupting PSD-95 PDZ2 domain-mediated protein-protein interactions on memory by assessing hippocampal dependent object recognition in PND21 mice two weeks

after exposure at PND7. In the majority of conditions, 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: WT Naïve (25±11 vs 6±4), p<0.0001; WTCON (34±19 vs 9±6), p<0.0001; WT ISO (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 ISO (42±20 vs 29±13), p=0.098; PDZ2WT+ISO (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 x 100) plot (fig. 6B). Kruskal-Wallis test indicates a significant effect of exposure treatment (fig. 6B; median, IQ: WT NAÏVE (85, 78 to 87), WT CON (79, 72 to 88), WT ISO (63, 55 to 72), PSD93KO CON (78, 65 to 87), PSD93KO ISO (57, 47 to 73), p=0.0006). Post-hoc Dunn's comparisons across groups shows that ISO exposed WT animals have a subtle but significant decrement in recognition memory as compared to controls (WT NAÏVE vs WT ISO, p=0.023 and WT CON vs WT ISO, p= 0.0.044). PSD93 deficiency did not have a significant effect on recognition memory (WT NAÏVE vs PSD93KOCON, p>0.999 and WT CON vs PSD93KOCON, p>0.999). ISO exposure did not cause detectable impairment in PSD93 deficient animals (PSD93KO CON vs PSD93KO ISO, p=0.177). Kruskal-Wallis test indicates a significant effect of peptide treatment on memory (PDZ2MUT (81, 69 to 84), PDZ2WT (67, 57 to 77), PDZ2WT+ISO (56, 51 to 64), p=0.001). PDZ2WT exposed animals have a subtle but significant decrement in recognition memory as compared to PDZ2MUT controls (PDZ2MUT vs PDZ2WT, p=0.0.039 and PDZ2MUT vs PDZ2WT+ISO, p=0.001). ISO did not cause further significant decrement in recognition memory in PDZ2WT exposed animals (PDZ2WT vs PDZ2WT+ISO, p=0.386).

Treatment with NO donor prevents the negative effects of ISO and PDZ2WT peptide on hippocampal LTP

Treatment with NO donor prevents the impairment in LTP caused by ISO or PDZ2WT peptide as indicated by the renewed expression of LTP (fig. 7A, 7B; median, IQ: WT CON + NO (129, 123 to 130), ISO + NO (136, 125 to 146), PDZ2MUT + NO (134, 128 to 142), PDZ2WT + NO (139, 130 to 147). There is no longer a significant difference between WT CON and WT ISO when NO donor is added (fig. 7B; WT CON + NO vs WT ISO + NO, p=0.284) or between PDZ2MUT and PDZ2WT (fig. 7B; PDZ2MUT + NO vs PDZ2WT + NO, p=0.662). Data were analyzed with Mann-Whitney test. .

Treatment with NO donor prevents ISO or PDZ2WT induced impairment in acute recognition memory

Treatment with NO donor prevents the impairment in NOR caused by ISO or PDZ2WT peptide as indicated by the increased discrimination in NOR (fig. 8; median, IQ: WT CON + NO (71, 61 to 82), WT ISO + NO (87, 73 to 93), PDZ2MUT + NO (84, 73 to 86), PDZ22WT + NO (79, 73 to 85). There is no longer a significant difference between WT CON and WT ISO when NO donor is added (WT CON + NO vs ISO + NO, p=0.073) or between PDZ2MUT and PDZ2WT (PDZ2MUT + NO vs PDZ2WT + NO, p=0.779). Data were analyzed with Mann-Whitney test.

Discussion

To better understand the mechanisms mediating ISO 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 nNOS (fig. 1) [25, 27]. Here, we determined the effects of this disruption *in vivo* in the context of ISO exposure by specifically mimicking this isolated action of anesthesia with the PDZ2WT peptide which disrupts PSD-PDZ2-mediated protein interactions by binding to interaction partners such as NMDA receptor NR2. In contrast to earlier work [3], we found that ISO exposure did not induce a detectable increase in the level of apoptosis nor did PDZ2WT peptide (fig. 2). However, our findings are consistent to recent findings of others that showed brief exposures to anesthesia did not induce apoptosis [44-46]. Further investigation into the sublethal effects of ISO, PSD-93 deficiency, and disruption of PSD-95 PDZ2 domain-mediated protein-protein interactions revealed changes in spine morphology, impairments in LTP induction, and impairments in memory. Introduction of NO donor at the time of exposure prevents impairment in LTP and recognition memory further implicating the involvement of the NMDAR NR2-PSD-95 PDZ2-nNOS signaling pathway in early anesthetic exposure-produced cognitive impairment.

Alterations in spine number and shape are associated with cognitive and developmental dysfunction in various neurological disorders [47]. Anesthesia exposure, nNOS activity, and modulation of MAGUK 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 <2 microns and width <0.6 microns) in ISO exposed, PSD-93 null mutants, and PDZ2WT peptide exposed animals compared to controls that persists 14 days after exposure. Others have also shown selective loss of spines from anesthesia such as the persistent decrease (up to 90 days) in spine density of spines with head diameter between 0.3-0.4 microns [50]. Length-shortening of dendritic protrusions following sevoflurane anesthesia [51] and reduction of long protrusions following ISO anesthesia [52, 53] in culture has been reported. Platholi specifically showed that ISO reduces F-actin concentration in spines suggesting a role in spine shrinkage and loss [54]. Thus the loss of long thin spines we observe in our study could be the result of length-shortening following disruption of PDZ-domain mediated interactions that lead to F-actin depolymerization. Experiments are underway to determine if the specific PDZ-domain mediated interaction involves NMDAR NR2-PSD-95 PDZ2-nNOS or other 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 following ISO 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 two-weeks later at PND21. Our findings are consistent with the lack of

synaptic density change in the single 2-h anesthesia exposure observed in the Amrock study [55] and lack of synaptic density loss in PSD-95 null mutants [56]. Follow up studies are underway to determine whether synaptic density changes occur following our exposure paradigm in mice allowed to recover for longer periods (i.e. >PND21).

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 LTP, 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 LTP in the hippocampus of adolescent rats [3]. We found suppression of LTP in ISO exposed WT animals. Similar to Carlisle et al (2008) we found LTP to be impaired in PSD93 mutant mice. Like ISO exposed animals PDZ2WT peptide exposed mice showed impaired LTP as compared to controls. Thus disrupting PDZ domain-mediated protein interactions mimicked to the effect of ISO on LTP. These results suggest 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.

Over a decade ago Jevtovic-Todorovic et al (2003) first reported persistent impairments in learning and memory following 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 contribute to impairments in learning and memory after early anesthetic exposure we should see deficits in recognition memory performance. We found WT mice exposed to ISO exhibited reduced recognition memory performance as compared to controls as we expected. Injection of PDZ2WT peptide mimicked the effect of ISO reducing recognition memory performance as compared to 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 nNOS [25], PSD-95 and Shaker-type potassium channel Kv1.4 [26], and between AMPAR 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 ISO or PDZ2WT peptide exposure may prevent deficits in LTP and memory. Indeed, treatment with the NO donor, Molsidomine, prevents impairment in LTP and recognition memory caused by ISO or PDZ2WT peptide suggesting the effect of NO is downstream of the disrupted PDZ interactions. These results support the involvement of the NMDAR NR2-PSD-95 PDZ2-nNOS signaling pathway in ISO mediated impairment in LTP and recognition memory. A better understanding of how NO signaling affects changes in LTP 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 is 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 N we took multiple measurements per animal in an effort to lower variation (each animal provided one data point; 6 dendritic fields were averaged per animal in the spine analysis and 10 images were averaged per animal in the EM 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-hr exposure of infant mice to ISO (~1 MAC) or targeted disruption of PSD-PDZ2-mediated protein interactions (i.e. a specific molecular target of ISO) with PDZ2WT peptide results in spine morphological changes, impairments in LTP induction, and impairments in memory in mice without inducing apoptosis or changes in synaptic density. The observed impairments in LTP and object recognition memory can be prevented by introduction of an NO donor suggesting the involvement of NMDAR NR2-PSD-95 PDZ2-nNOS signaling pathway in these processes.

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What We Already Know about This Topic

- Certain general anesthetics have been shown to have adverse effects on neuronal development including spinogenesis and synaptogenesis that affect neural function and cognitive behavior.
- Clinically relevant concentrations of inhalational anesthetics inhibit the PDZ domain-mediated protein-protein interaction between PSD-95 or PSD-93 and NMDA receptors or nNOS.

What This Article Tells Us That Is New

- Neonatal PSD-95 PDZ2WT peptide exposure mimics ISO (~1 MAC) by altering dendritic spine morphology, neural plasticity (i.e. LTP), and object recognition memory without inducing detectable increases in apoptosis or changes in synaptic density.
- The results indicate a sublethal effect of a single dose of ISO (~1MAC) or PSD-95 PDZ2WT peptide on the developing brain that alters dendritic spine architecture and function important to cognition and that this impairment can be prevented by NO donor, Molsidomine.

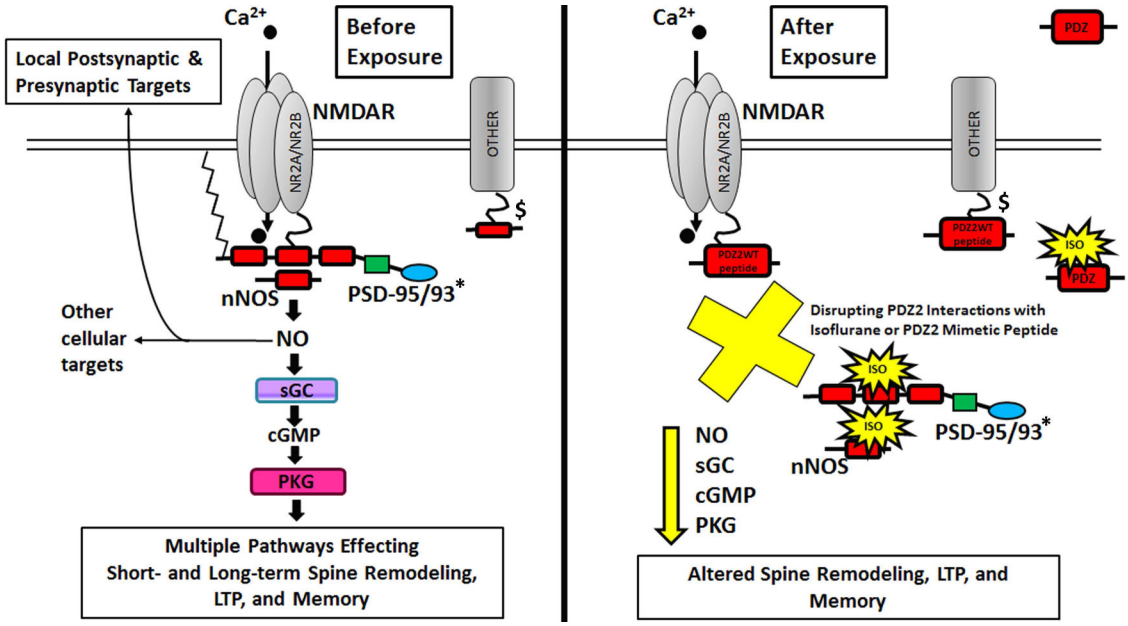

Fig. 1.

Diagram illustrating dissociation of NMDAR-PSD95/93*-nNOS interaction by ISO or PDZ2WT (active) peptide. *For simplicity, not all PSD-95 family members are represented. Before Exposure, The NMDA receptor is linked to downstream molecules such as nNOS through PSD-95: through its first and second PDZ domain, PSD-95 forms a ternary complex by binding to both the tSXV motif of NMDAR NR2 subunit and to the PDZ domain in nNOS [63]. Disrupting NMDAR-PSD-95/93-nNOS complexes can reduce the efficiency by which calcium ions activate the signaling molecule nNOS. 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 NMDAR 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].

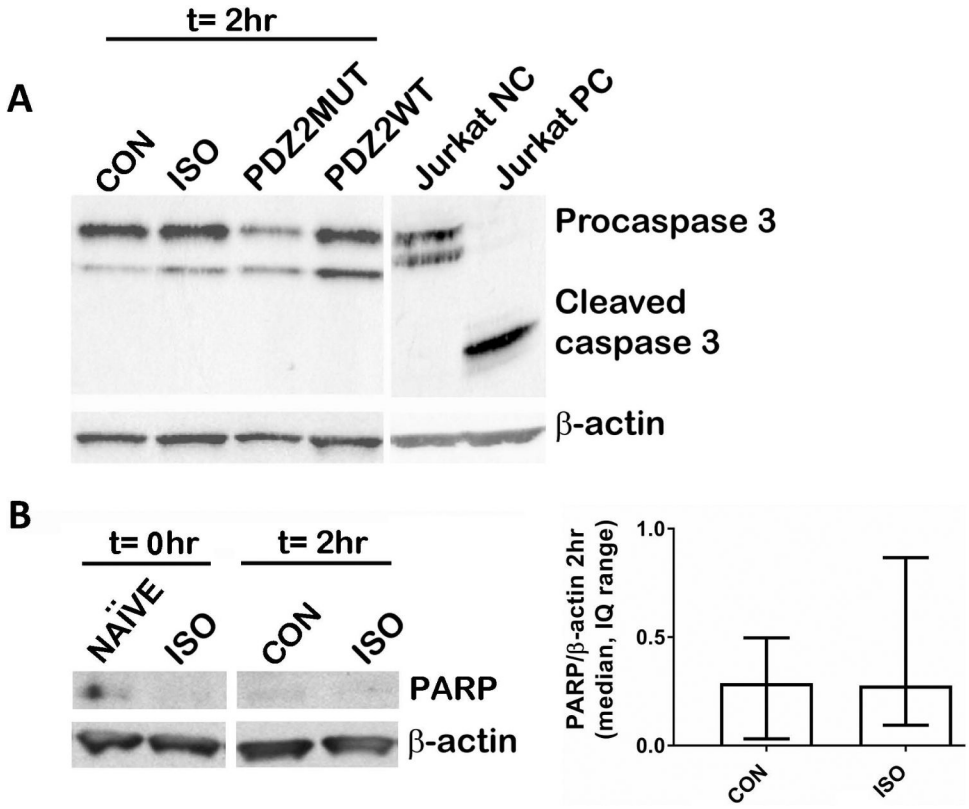

Fig. 2.

Western blot assays did not show any significant differences in apoptosis in our exposure paradigm. PND7 mice were exposed for 4-hours and harvested 2-hours 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 CON (O_2), ISO (1.5% ISO in O_2), PDZ2MUT (8 mg/kg inactive peptide), PDZ2WT (8 mg/kg active peptide). Negative and positive Jurkat control proteins were run to demonstrate sensitivity of the caspase-3 antibody. B, Western blot analysis for PARP did not reveal significant differences between CON (O_2) and ISO (1.5% ISO in O_2). Left, representative blot. Right, Densitometry data are plotted as median, interquartile range. CON (n=6) vs ISO (n=6), p=0.818. Data were analyzed using Mann Whitney test. Gender was not determined.

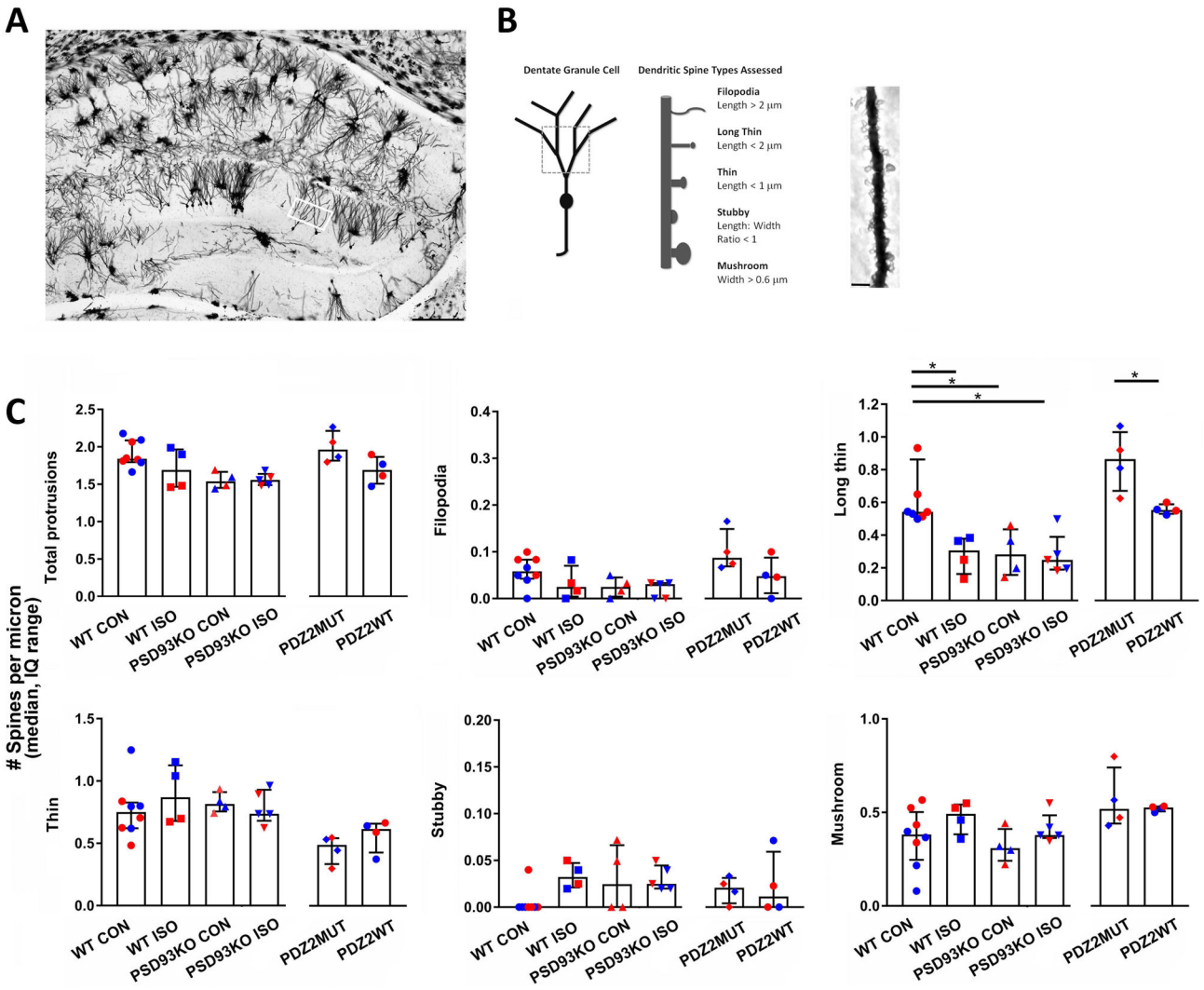

Fig. 3.

Neonatal exposure to 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 (DG) 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 DG among exposed groups assessed at PND21. WT and PSD-93 KO PND7 mice were exposed to O_2 (control, 100% O_2) or ISO (1.5% ISO in O_2); WT PND7 mice were also injected with PDZ2MUT (8 mg/kg inactive peptide) or PDZ2WT (8 mg/kg active peptide). (WT CON = 8, WT ISO = 4, PSD93KO CON = 4, PSD93KO ISO = 5, PDZ2MUT = 4, PDZ2WT = 4). Data from individual animals are plotted and color coded by gender (red=female and blue=male). Data are plotted as median number of spines per micron with interquartile range). Data were analyzed with Kruskal-Wallis followed by Dunn's multiple comparison correction and Mann-Whitney tests. A *$p < 0.05$ was considered significant.

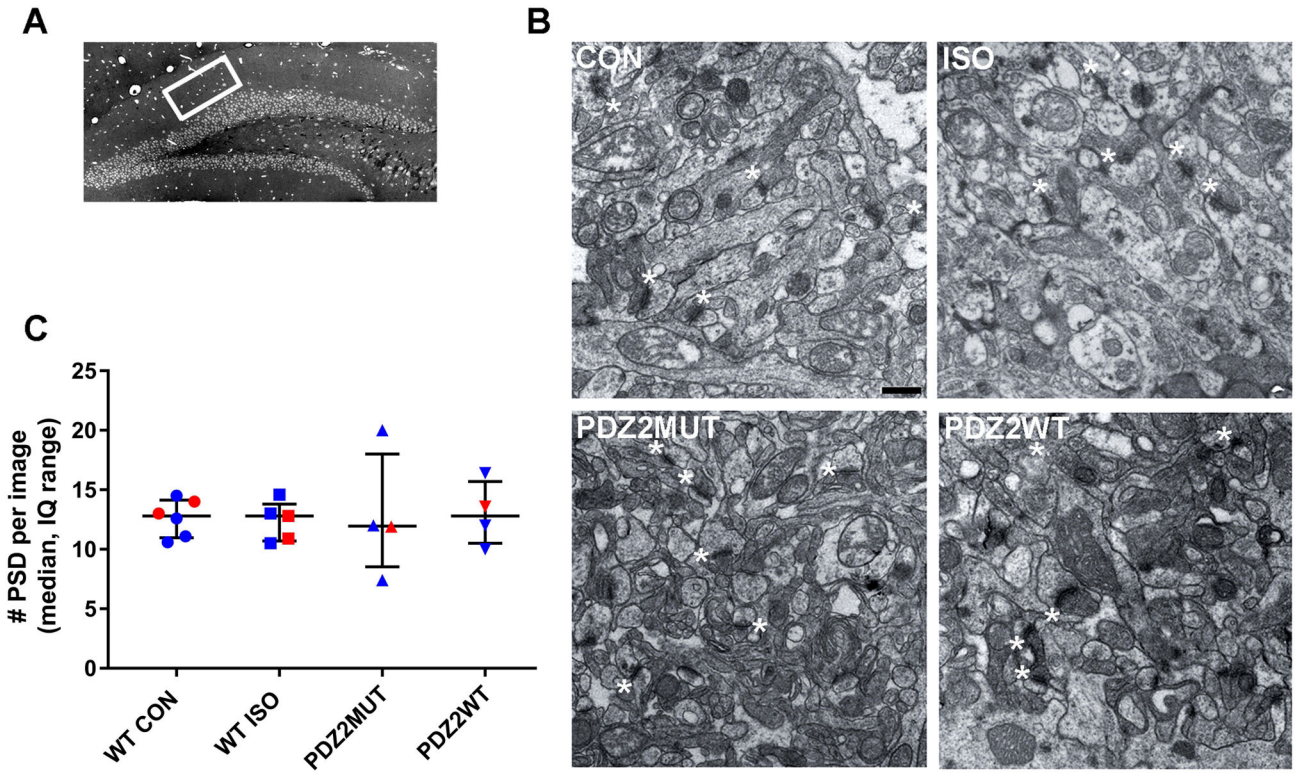

Fig. 4.

Neonatal exposure to 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 DG subregion of interest (white box). **B**, representative ultrastructure images from PND21 mice exposed at PND7 to O_2 (WT CON, 100% O_2), WT ISO (1.5% ISO 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 PSDs (not all are labeled). **C**, plots showing the median with interquartile range number of PSD's. WT CON (n=6) vs WT ISO (n=5), p=0.829; PDZ2MUT (n=4) vs PDZ2WT (n=4), p=0.742. Data from individual animals are plotted and color coded by gender (red=female and blue=male). Data were analyzed with Mann-Whitney.

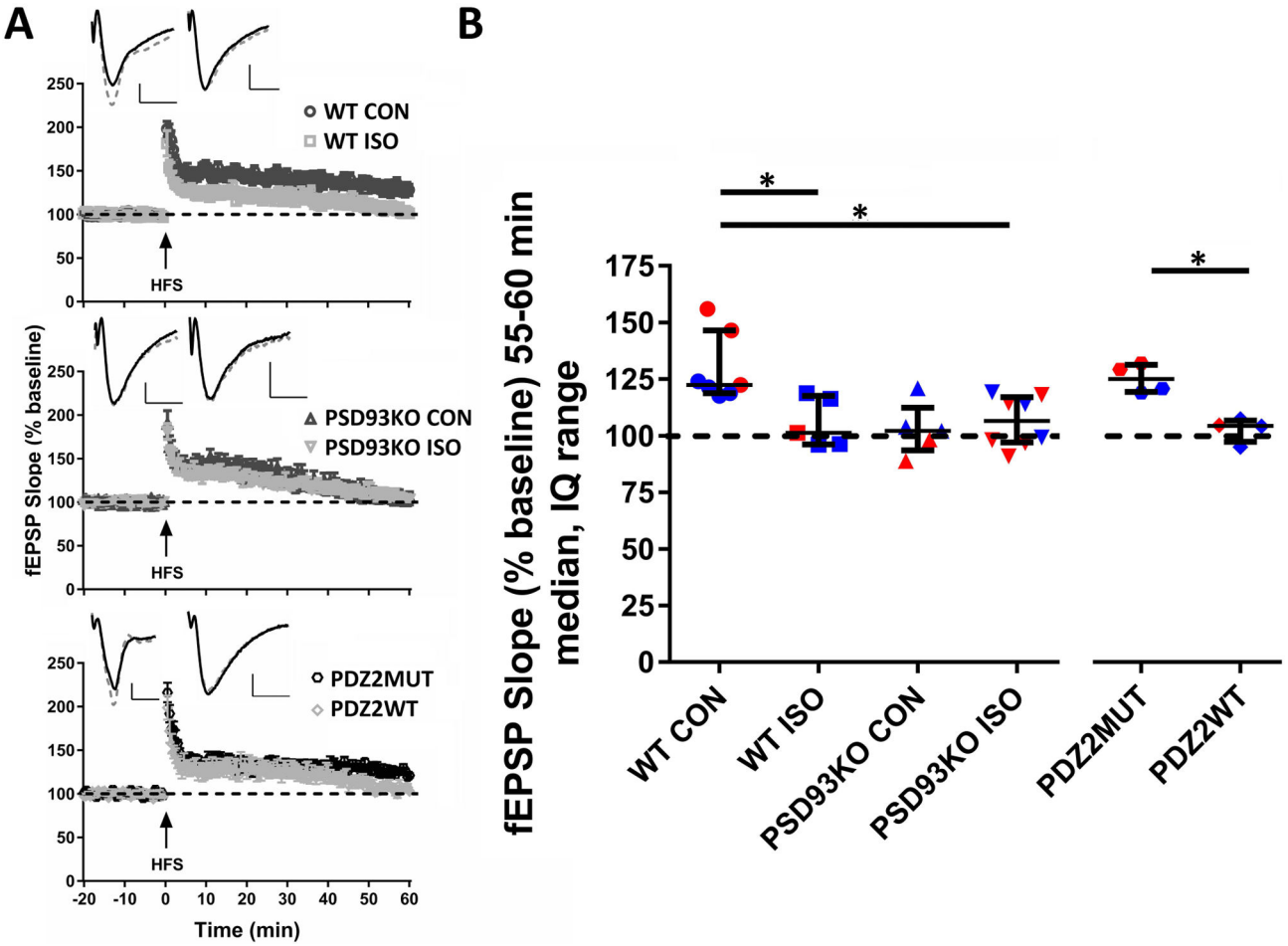

Fig. 5.

Neonatal exposure to ISO or PDZ2WT peptide impairs long-term potentiation (LTP) in hippocampal CA1 at PND21. **A**, high frequency stimulation (HFS) induced robust LTP in WT CON (top; WT CON, 100% O_2) and inactive PDZ2MUT (bottom) treated groups in hippocampal Schafer collateral to CA1 pathway. ISO (top) and PDZ2WT (bottom) exposure as well as PSD93 deficiency (middle) impaired the expression of LTP. Example traces are shown in upper left quadrants of fEPSP plots. WT CON and WT ISO (top), PSD93KO CON and PSD93KO ISO (middle), and PDZ2MUT and PDZ2WT (bottom) treated groups at baseline before HFS (solid line trace) and the average of 55-60 min after HFS (dashed line trace). **B**, the median of normalized fEPSP 55-60min after HFS showed significant differences between WT CON (n=7) vs WT ISO (n=5), p=0.049 and PDZ2MUT (n=4) vs PDZ2WT (n=4), p=0.028 treated groups. Significant differences were not observed between WT CON vs PSD93KO CON (n=5), p=0.056 but were observed between WT CON vs PSD93KO ISO (n=8), p=0.025 groups. Data from individual animals are plotted and color coded by gender (red=female and blue=male). Data were analyzed with Kruskal-Wallis followed by post-hoc Dunn's test and Mann Whitney tests. Values were considered significant at *$p < 0.05$ or less. Data were plotted as median and interquartile range. Scale bar: 10ms, 0.25mV.

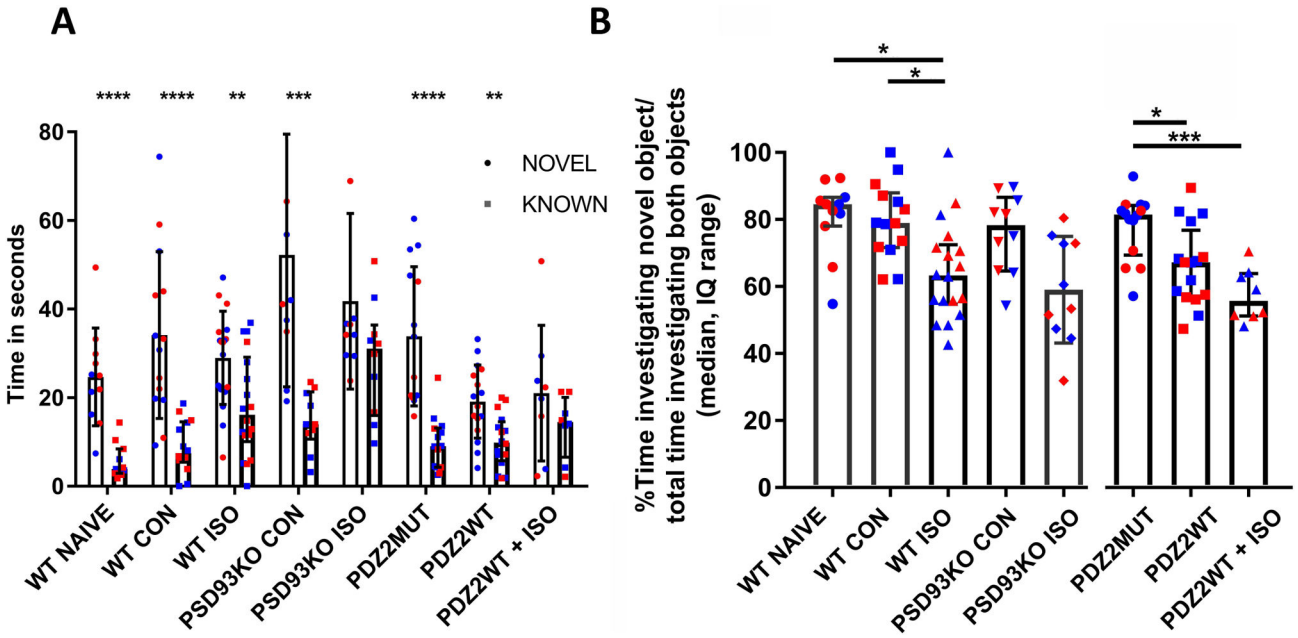

Fig. 6.

Neonatal exposure to 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(WT Naïve, n=11, p<0.0001; WTCON, n=14, p<0.0001; WT ISO, 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 ISO, n=10, p=0.098; PDZ2WT+ISO, n=8, p=0.227). Data were plotted as mean and SD. Data were analyzed with two-tailed t-tests known vs. 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. ISO exposed WT animals have a subtle but significant decrement in recognition memory as compared to controls (WT NAÏVE vs WT ISO, p = 0.022; WT CON vs WT ISO, p = 0.043; WT NAÏVE vs WTCON, 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). ISO exposed PSD93 KO animals were not significantly different from PSD93 KO controls (PSD93KO CON vs PSD93KO ISO, p=0.176). ISO exposed PSD93KO animals differed from WT controls (WT NAÏVE vs PSD93KO ISO, p=0.011; WT CON vs PSD93KO ISO, p=0.022). Active peptide exposed animals have a subtle but significant decrement in recognition memory as compared to inactive peptide controls (PDZ2MUT vs PDZ2WT, p=0.038; PDZ2MUT vs PDZ2WT + ISO, p<0.001) ISO exposure did not further significantly impair peptide exposed animals (PDZ2WT vs PDZ2WT + ISO, p=0.385). Data from individual animals are plotted and color coded by gender (red=female and blue=male). Data were analyzed with Kruskal-Wallis followed by post-hoc Dunn's test. Data were plotted as median and interquartile range. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

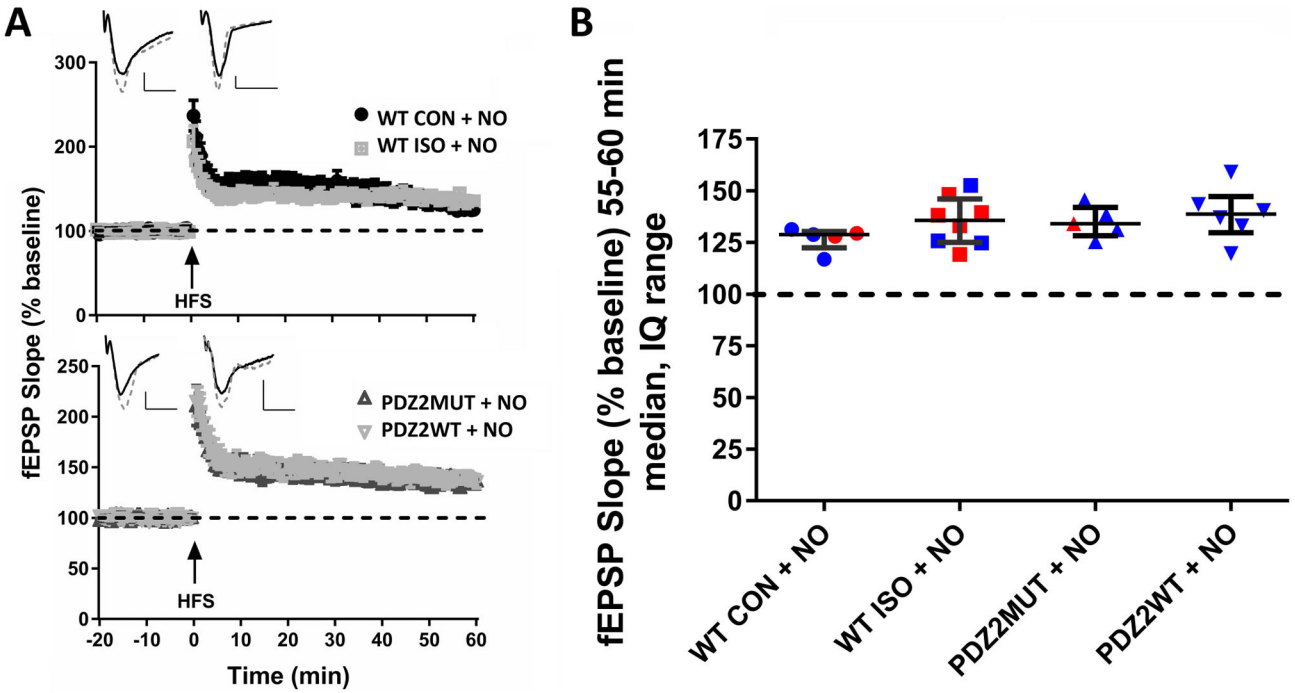

Fig.7.

Treatment with NO donor prevents the negative effect of ISO and PDZ2WT peptide on hippocampal LTP. **A,** robust LTP was induced by HFS in all groups. In upper left quadrants example traces of WT CON+NO and WT ISO+NO (top), PDZ2MUT+NO and PDZ2WT +NO (bottom) treated groups before HFS (solid line trace) and 55-60min after HFS (dashed line trace) are shown. **B**, the median of normalized fEPSP 55-60min after HFS no longer shows a significant difference between WT CON and WT ISO when NO donor is added (WT CON + NO (n=5) vs ISO + NO (n=8), p=0.284) or between PDZ2MUT and PDZ2WT (PDZ2MUT + NO (n=5) vs PDZ2WT + NO (n=6), p=0.662). Data were analyzed with Mann-Whitney tests. Data from individual animals are plotted and color coded by gender (red=female and blue=male). Data were plotted as median and interquartile range. Values were considered significant at *p < 0.05 or less. Scale bar: 10ms, 0.25mV.

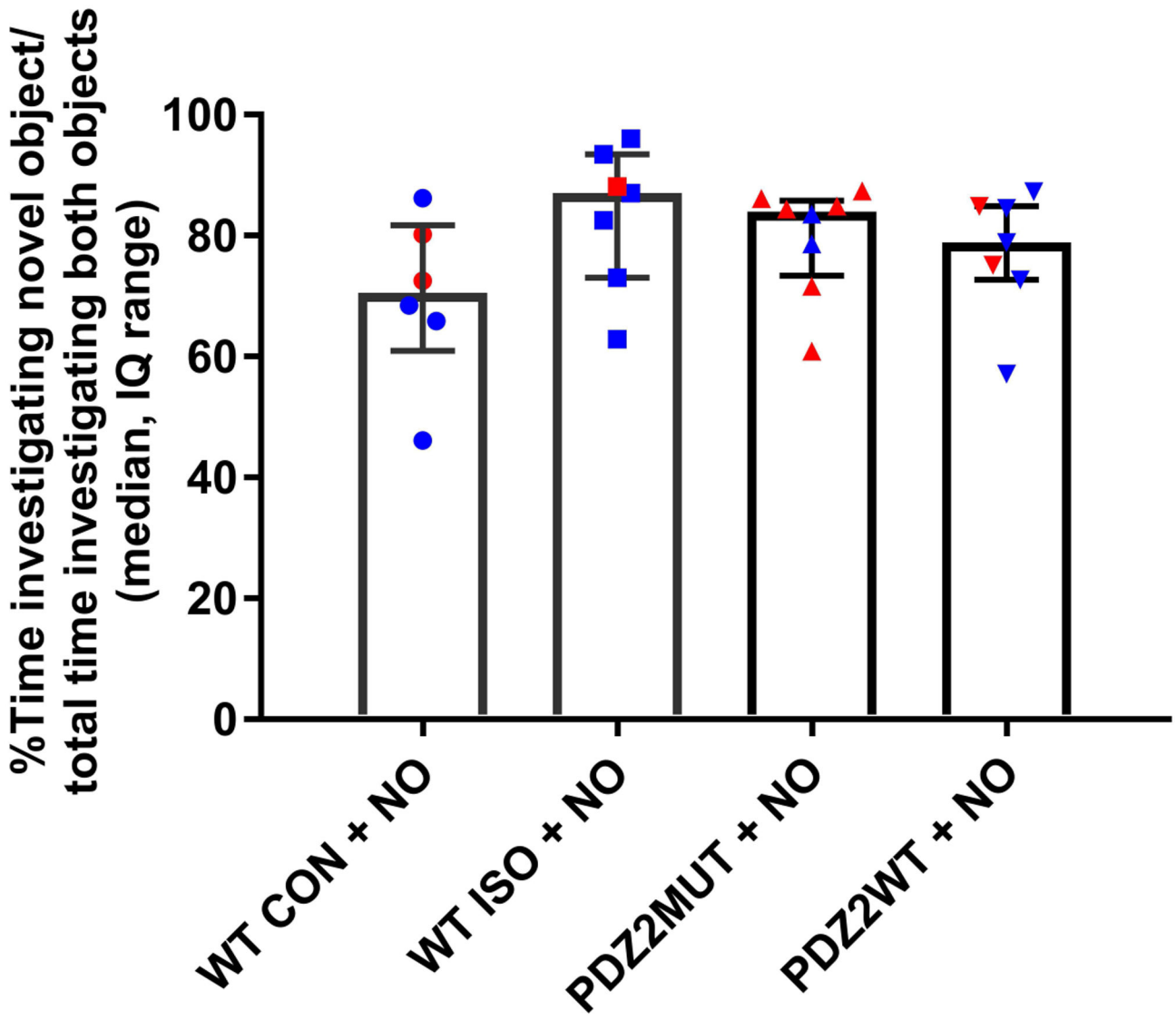

Fig. 8.

Treatment with NO donor prevents ISO or PDZ2WT peptide induced impairment in acute recognition memory. 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. There is no longer a significant difference between WT CON and WT ISO when NO donor is added (WT CON + NO (n=6) vs ISO + 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 gender (red=female and blue=male). Data were plotted as median and interquartile range. Data were analyzed with Mann-Whitney tests. Values were considered significant at *p < 0.05 or less.