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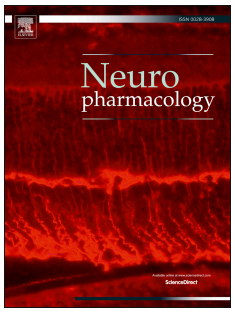

An *Aplysia*-like synaptic switch for rapid protection against ethanol-induced synaptic inhibition in a mammalian habit circuit

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-A novel mammalian synaptic plasticity mechanism protects against LTD in the DLS

-Burst-dependent protection against LTD is Ca2+ and PDZ domain-dependent

-This protection occurs in *Aplysia californica* and is possibly evolutionarily conserved

An *Aplysia*-like synaptic switch for rapid protection against ethanol-induced synaptic inhibition in a mammalian habit circuit

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Key words: striatum, FSI, MSN, parvalbumin, LTD, alcohol, PKC, PKA, delta opioid receptor

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Abstract

Decades of work in *Aplysia californica* established the general rule that principles of synaptic plasticity and their molecular mechanisms are evolutionarily conserved from mollusks to mammals. However, an exquisitely sensitive, activity-dependent homosynaptic mechanism that protects against the depression of neurotransmitter release in *Aplysia* sensory neuron terminals has, to date, not been uncovered in other animals, including mammals. Here, we discover that depression at a mammalian synapse that is implicated in habit formation and habit learning acceleration by ethanol, the fast-spiking interneuron (FSI) to medium spiny principal projection neuron (MSN) synapse of the dorsolateral striatum, is subject to this type of synaptic protection. We show that this protection against synaptic depression is calcium- and PDZ domain interaction-dependent. These findings support activity dependent protection against synaptic depression as an *Aplysia*-like synaptic switch in mammals that may represent a leveraging point for treating alcohol use disorders.

Key words: striatum, parvalbumin, LTD, alcohol, PKC, delta opioid receptor

1. Introduction

Synaptic plasticity operates over a broad range of temporal domains, from hundreds of milliseconds to weeks or months. Typically, brief activity, such as short bursts of action potentials, trigger very transient plasticity lasting less than a second to tens of seconds. In contrast, persistent synaptic changes typically require more extended sequences of activity or prolonged modulatory input for initiation. These stable synaptic changes mediate various forms of learning and memory that occur in response to experience. Moreover, these persistent forms of plasticity are modulated by drugs of abuse, such as ethanol.

A novel form of synaptic plasticity was recently identified in *Aplysia* in which very brief bursts of only two to four presynaptic action potentials protect the sensory neuron (SN)-to-motor neuron (MN) synapse from homosynaptic depression (HSD) (Wan et al., 2012). This mechanism, "burst-dependent protection against synaptic depression" (BDP) is unusual in that, rather than activating a short-lived faciliatory process, bursts of only a few action potentials entirely block the development of stable HSD; thus, BDP affects synaptic strength for tens of minutes. The only comparably sensitive, conventional form of synaptic plasticity is paired-pulse facilitation (PPF), which lasts at most hundreds of milliseconds. BDP at these afferent synapses reduces the development of behavioral habituation, and was suggested to contribute to a simple attention-like process in these marine snails (Wan et al., 2012). A substantial number of molecular mechanisms that contribute importantly to synaptic plasticity were initially identified in *Aplysia* and *Drosophila*, such as CREB and calmodulin-sensitive adenylyl cyclase (Dash et al., 1990); these molecular mechanisms were later found to play critical roles in plasticity in mammals (Wang et al., 2004). In contrast, there are a plethora of diverse pre- and postsynaptic mechanisms of plasticity in *Aplysia* and mammalian central nervous systems (Atwood et al., 2014; Calabresi et al., 1992; Dolen et al., 2013; Gerdeman et al., 2002; Huang et al., 1999; Huang et al., 2013; Kobayashi et al., 1999; Lovinger et al., 1993; Malenka and Bear, 2004; Mathur et al., 2011; Mathur et al., 2013; Robbe et al., 2002; Shen et al., 2008). Thus, it is not surprising that BDP has not yet been observed at mammalian synapses.

The mechanism by which BDP blocks the development of persistent depression at the SN-MN synapse involves Ca^{2+} influx and activation of the classical, Ca^{2+}-dependent isoform of PKC in *Aplysia*, Apl-I. This appeared paradoxical because with only two action potentials, the increase in global Ca^{2+} at synaptic terminals is dramatically lower than the levels required to translocate and activate PKC Apl-I (de Juan-Sanz et al., 2017; Eliot et al., 1993). The surprising ability of PKC to respond to minimal Ca^{2+} influx and initiate BDP depends on the interaction between the kinase and Protein Interacting with C-Kinase 1 (PICK1), mediated by binding of Apl-I to the PDZ domain of PICK1 (Wan et al., 2012). This scaffolding protein, PICK1, presumably functions to localize the kinase to Ca^{2+} nanodomains proximal to presynaptic Ca^{2+} channels in the active zone.

Decreases in the probability of neurotransmitter release leading to both short-term and long-term synaptic depression (LTD) are commonly initiated by the activation of presynaptically localized $G_{i/o}$-coupled receptors (Atwood et al., 2014). These forms of plasticity are prevalent across the central nervous system of mammals (Huang et al., 1999; Huang et al., 2013; Kobayashi et al., 1999; Mathur et al., 2011; Patton et al., 2016; Robbe et al., 2002), and are also found in mollusks (Dunn et al., 2018; Volterra and Siegelbaum, 1988). Indeed, several neurotransmitter systems employ presynaptic $G_{i/o}$-coupled receptors to homosynaptically or heterosynaptically decrease neurotransmitter release probability (Atwood et al., 2014; Calabresi et al., 1992; Dolen et al., 2013; Gerdeman et al., 2002; Lovinger et al., 1993; Mathur et al., 2011; Mathur et al., 2013; Patton et al., 2016; Robbe et al., 2002; Shen et al., 2008). In the mammalian dorsolateral striatum (DLS), a region critical for habit learning (Packard, 1999; Packard and McGaugh, 1996; Yin et al., 2004), activation of the presynaptic, $G_{i/o}$-coupled delta opioid receptor induces LTD (DOR-LTD) at a key inhibitory synapse: the fast-spiking interneuron (FSI)-to-medium spiny projection neuron (MSN) synapse (Patton et al., 2016). FSIs are critical for habit formation (O'Hare et al., 2017), which is facilitated by drugs of abuse such as ethanol (Corbit et al., 2012; Dickinson et al., 2002; Lesscher et al., 2010). Ethanol induces DOR-LTD at the FSI-MSN synapse (Patton et al., 2016) to disinhibit the DLS and, accordingly, intrastriatal infusion of a DOR antagonist dampens ethanol drinking

(Nielsen et al., 2012). As such, DOR-LTD at the mammalian FSI-MSN synapse is a presynaptically-expressed form of synaptic plasticity with translational potential.

We report here a BDP-like form of plasticity in the mammalian DLS: elicitation of only two presynaptic FSI action potentials (at 20 Hz) blocks the development of DOR-LTD, as induced by a DOR agonist or ethanol. We found that the mechanism underlying BDP of DOR-LTD at the FSI-MSN synapse depends on Ca^{2+} influx, classical PKC, and a PDZ domain interaction. Thus, striatal BDP represents a mechanism for the regulation of synaptic depression in mammals by an exquisitely sensitive homosynaptic activity-dependent mechanism. The gating of DOR-LTD at these striatal synapses by BDP may provide a novel site for therapeutic intervention for habitual alcohol consumption.

2. Methods

2.1 Animals and Animal Care. All procedures and experiments performed were approved by both the United States Public Health Service Guide for Care and Use of Laboratory and the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine. Mice were housed under a 12h light/dark cycle (lights on from 0700 hours to 1900 hours) with 2-5 littermates and given *ad libitum* access to food and water. Female and male mice were used for all experiments and no effect of sex was found. *Aplysia californica* weighing 70 to 120 g were obtained from Alacrity Marine Biological Services (Redondo Beach, CA, USA)

2.2 Stereotaxic Surgery and Channelrhodopsin (ChR2) Expression. Parvalbumin (PV)-cre transgenic mice (> 2 months old, C57BL/6J background) were injected stereotaxically with an AAV vector driven by an EF1a promoter containing a DIO-ChR2-mCherry or eYFP construct flanked by loxP sites into the DLS (coordinates from bregma: A/P +0.6, M/L ±2.25, D/V -2.4 from brain) at a rate of 20 nL/minute with a total injection volume of 300-450 nL per striatal side. Animals were given at least 3 weeks post-surgery to allow for viral expression before use in *ex vivo* electrophysiological experiments.

2.3 Brain Slice Preparation. Following deep isoflurane anesthetization, brains were dissected and kept in an ice cold cutting solution (194 mM sucrose, 30 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 26 mM

$NaHCO_3$, 1.2 mM NaH_2PO_4, and 10 mM D-glucose) bubbled with 95% oxygen, 5% carbon dioxide (carbogen). 250 μm coronal sections were sliced via vibratome (Leica VT 1200) and allowed to incubate at approximately 32.4°C in carbogen-bubbled artificial cerebral spinal fluid (aCSF; 124 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, and 10 mM D-glucose) for 30 minutes. Slices were then stored at room temperature for the remainder of the day before being hemisected and transferred to the recording chamber. Slices were constantly bathed in temperature-control, carbogen-bubbled aCSF (29-31°C) via a gravity perfusion system for the duration of experiments.

2.4 Whole-Cell Voltage-Clamp Recordings. Expression of ChR2-mCherry/eYFP at injection sites in the DLS was assessed with an epifluorescence light path using a mercury bulb lamp (X-Cite series 120Q). MSNs within the DLS receiving inputs from FSIs expressing ChR2 were targeted for whole cell recordings (Mathur et al., 2013). To elicit optogenetically-evoked inhibitory postsynaptic currents (oIPSC), blue light (473 nm) was delivered via LED field illumination (Lumen Dynamics, XLED1). Single or paired (20 Hz) blue-light evoked test pulses (pulse duration of 2-4 ms) were delivered every 20 s. For a subset of experiments, local electrical stimulation was delivered through a concentric bipolar stimulating electrode (World Precision Instruments). Borosilicate glass pipettes (resistance range of 2-5 MΩ; Narishige, PC-100) were used to record oIPSCs and were filled with a CsCl-based internal solution (150 mM CsCl, 10 mM HEPES, 2 mM $MgCl_2$, 0.3 mM Na-GTP, 5 mM QX-314, 3 mM Mg-ATP, and 0.2 mM BAPTA, osmolarity ranging from 307 to 313 mOsm, pH approximately 7.3). A Multiclamp 700B amplifier (Molecular Devices) was used to voltage clamp MSNs at -60 mV throughout the duration of all experiments. Recordings were filtered at 2 kHz and digitized at 10 kHz. If the series resistance changed by more than 15% throughout the duration of the experiment, the recording was discarded from further analysis.

2.5 *Aplysia californica* electrophysiology. Following an injection of isotonic $MgCl_2$, *Aplysia* abdominal ganglia were dissected and superfused with high-divalent saline at 22-24°C (6 × normal Ca^{2+}, 1.6 × normal Mg^{2+}) to reduce spontaneous synaptic activity: 328 mM NaCl, 10 mM KCl, 66 mM $CaCl_2$, 88

mM $MgCl_2$, 10 mM Na-HEPES, pH 7.6, supplemented with nutrients (7 mM glucose, MEM essential and non-essential amino acids (0.2 × normal concentration, Invitrogen), and MEM vitamin solution (0.7 × normal concentration, Invitrogen)) (Jiang and Abrams, 1998; Wan et al., 2012). 12-20 MΩ microelectrodes containing 2 M potassium acetate and 0.4 M KCl were used to record from or stimulate siphon sensory and motor neurons, respectively. Action potentials in siphon neurons were elicited by injected 2 ms depolarizing current pulses.

2.6 Statistical Analysis. Mean amplitudes of oIPSCs were measured in Clampfit 10.4.1.4 and analyzed via GraphPad Prism 6.01. The mean of the amplitudes of the first oIPSC event in the pair were averaged per minute and expressed as a percentage relative to the average baseline amplitude. A two-tailed paired *t* test was used to compare the average baseline IPSC amplitudes and IPSC amplitudes from the last 5 minutes of recording. A two-tailed unpaired *t* test was used to compare changes in IPSC amplitudes between experiments. A repeated measure ANOVA was performed in SPSS to compare trial number x burst stimulation parameter for *Aplysia* experiments. All data are represented as mean ± SEM.

2.7 Drugs. Where indicated, aCSF with 2 mM $SrCl_2$ (2 mM) replacing $CaCl_2$ was used. In a subset of experiments, slices were incubated in TAT-PKC-CT or control peptides (1 µM) for 30 minutes before being transferred to the recording chamber and bathed in regular aCSF for the duration of the experiment. All other drugs were dissolved into the aCSF and constantly perfused over the slice. DPDPE, Go 6976, and Bisindolylmaleimide I (BIM-1) were purchased from Tocris Bioscience. BAPTA was purchased from Molecular Probes. TAT-PKC-CT (RQIKIWFQNRRMKWKKGFVHPILQSAV) and TAT-PKCdeltaCT (RQIKIWFQNRRMKWKKGFVHPIL) were purchased from BioSynthesis Inc. All other drugs were purchased from Sigma-Aldrich.

3. Results

3.1 Paired-pulse stimulation prevents delta-opioid receptor-induced long-term depression (DOR-LTD). Expressing channelrhodopsin (ChR2) in FSIs and recording from postsynaptic MSNs, we first delivered a single pulse of light every 20 s to elicit optogenetically-evoked inhibitory postsynaptic

currents (oIPSCs) to probe FSI-MSN synaptic strength (**Figure 1a**). We found that a 10 min application of the delta opioid receptor (DOR) agonist DPDPE (500 nM) induced LTD (DOR-LTD) of FSI-MSN oIPSC amplitude (70.29 ± 7.87% of baseline, t=3.77, df=4, p=0.02, paired t test, **Figure 1b**), as we have previously reported (Patton et al., 2016). However, when we delivered a paired light pulse (50 ms inter-pulse interval (IPI)) every 20 s to track FSI-MSN oIPSC amplitude over time, we found that DPDPE (500 nM) application no longer induced DOR-LTD (oIPSC amplitude = 104.7 ± 9.19% of baseline, t=0.51, df=6, p=0.63, paired t test, **Figure 1b**). This was significantly different from the single pulse stimulation condition: t=2.69, df=10, p=0.02, unpaired t test (**Figure 1b**). To confirm these effects were not due to activation of the nonspecific cation channel, ChR2, we repeated these experiments while delivering local electrical stimulation instead of optogenetic stimulation. We found that DPDPE (500 nM) depressed inhibitory inputs onto MSNs when single electrical pulses were delivered (electrically evoked IPSC (eIPSC) amplitude = 71.41 ± 7.75% of baseline, t=3.69, df=11, p=0.0036, paired t test, **Figure 1c**), and this DOR-LTD was eliminated when paired electrical pulses were delivered (50 ms IPI every 20 s; eIPSC amplitude = 105.7 ± 10.67% of baseline, t=0.53, df=11, p=0.61, paired t test, **Figure 1c**). There was a significant attenuation of DOR-LTD magnitude with paired electrical pulse stimulation: t=2.6, df=22, p=0.017, unpaired t test (**Figure 1c**). It should be noted that optically and electrically-induced IPSC paired pulse ratios may be different, likely due to the recruitment of additional synapses in the electrical induction paradigm compared to the synapse-specific optogenetic interrogation of the FSI-MSN synapse.

A similar electrical stimulation protocol (two to four pulses delivered with a 15 or 60 s inter-trial interval (ITI)) protects against synaptic depression in the sea mollusk *Aplysia californica* (Wan et al., 2012). To examine whether burst dependent protection against synaptic depression (BDP) also occurs at the same ITI at which we observe protection against DOR-LTD, we tested whether two or four action potentials at 20 Hz would also induce BDP with a 20 s ITI. Delivering single pulse stimulation induced homosynaptic depression (HSD) of the siphon SN-MN synapse (**Figure 1d**). Bursts of two action potentials in the sensory neuron attenuated this depression, and bursts of four action potentials largely

prevented development of HSD ($F(28, 140) = 2.45$, $p<0.001$, Burst x Trial Number interaction, repeated measures ANOVA, **Figure 1d**).

To explore the possibility that paired pulse stimulation of FSI terminals potentiates the FSI-MSN synapse to nullify DPDPE-induced synaptic depression, we delivered paired pulse light stimulation (50 ms IPI, every 20 s) after a baseline of single pulse light stimulation (every 20 s). Paired pulse stimulation did not, on its own, cause a lasting change in oIPSC amplitude (oIPSC amplitude = 91.89 ± 8.58% of baseline, t=0.39, df=5, p=0.39, paired t test, **Figure 2a**). To verify that this lack of an observed synaptic potentiation is not due to desensitization of ChR2, we repeated this experiment with electrical stimulation. Delivery of paired electrical stimulation after a baseline of single pulse electrical stimulation did not cause a lasting change in oIPSC amplitude (eIPSC amplitude = 116.6 ± 15.26% of baseline, t=1.09, df=11, p=0.29, paired t test, **Figure 2b**).

3.2 Paired pulse stimulation protection against DOR-LTD requires Ca^{2+}-dependent PKC isoforms.

At the *Aplysia* sensory neuron to motor neuron synapse, BDP requires the activity of a Ca^{2+}-sensitive isoform of PKC in the presynaptic terminal (Wan et al., 2012). To test if our paired stimulation paradigm in mouse slices also requires PKC, we incubated slices in the PKC inhibitor bisindolylmaleimide I (2 µM, BIM-1). In the presence of BIM-1, DPDPE (500 nM) application induced synaptic depression at the FSI-MSN synapse during paired pulse light delivery (oIPSC amplitude = 72.31 ± 8.85% of baseline, t=3.13, df=4, p=0.04, paired t test, **Figure 3a**). Investigating the necessity for Ca^{2+} in striatal BDP against synaptic depression, we replaced Ca^{2+} in the artificial cerebrospinal fluid (aCSF) with 2 mM strontium (Sr^{2+}), which supports neurotransmitter release but in a Ca^{2+}-independent, asynchronous manner (Choi and Lovinger, 1997). Replacing extracellular Ca^{2+} with Sr^{2+} blocked the ability of paired light pulse stimulation to protect against DOR-LTD, and thus, DPDPE application (500 nM) depressed the FSI-MSN synapse (oIPSC amplitude = 62.02 ± 6.34% of baseline, t=5.99, df=9, p=0.0002, paired t test, **Figure 3b**). We have previously shown that DPDPE application during single light pulse delivery in the presence of 2 mM Sr^{2+} (with 0 mM Ca^{2+}) induces FSI-MSN DOR-LTD (Patton et al., 2016). We next tested whether

paired light pulse stimulation protects against DOR-LTD by selectively recruiting Ca^{2+} sensitive PKC isoforms by incubating slices in the selective Ca^{2+} sensitive PKC inhibitor Go 6976 (3 µM) (Martiny-Baron et al., 1993). This inhibitor blocked the paired light pulse protection against oIPSC amplitude depression after DPDPE application (oIPSC amplitude = 70.48 ± 10.90% of baseline, t=2.71, df=6, p=0.04, paired t test, **Figure 3c**).

In BDP in *Aplysia* sensory neurons, Apl-I is localized near Ca^{2+} channels through an interaction with the PDZ binding domain on the scaffolding protein PICK1 (Wan et al., 2012). To test if an interaction between PKC and a PDZ binding domain plays a role in paired light pulse protection against DOR-LTD, we developed a peptide corresponding to the C terminus sequence of PKC alpha, designed to block PKC-PDZ domain interactions, following the approach of Wan et al (2012). Whereas these authors injected the C terminus peptide presynaptically, we used a TAT-conjugated membrane-permeant peptide. Slices were incubated in the TAT-PKC-CT peptide (1 µM) for 30 min prior to experiments or in a control truncated peptide, TAT-PKCdeltaCT. TAT-PKC-CT restored DOR-LTD during paired light pulse stimulation (oIPSC amplitude = 65.41 ± 9.96 % of baseline, t=3.47, df=7, p=0.01, paired t test, **Figure 3d**), whereas incubating in the truncated peptide lacking the PDZ binding domain, did not (1 µM, oIPSC amplitude = 100.7 ± 11.23% of baseline, t=0.06, df=5, p=0.95, paired t test, **Figure 3d**). The experimental and control peptide conditions were significantly different from each other (t=2.34, df=12, p=0.04, unpaired t test, **Figure 3d**). Finally, to verify the TAT-PKC-CT peptide alone does not depress FSI-MSN synaptic transmission, we delivered paired light pulses after incubating slices in the TAT-tagged peptide and measured oIPSC amplitude changes without applying DPDPE. Incubation in TAT-PKC-CT without DPDPE application did not result in synaptic depression (oIPSC amplitude = 106.4 ± 7.95% of baseline, t=0.8, df=5, p=0.46, paired t test, **Figure 3e**).

3.3 Paired pulse light stimulation blocks ethanol-induced LTD at the FSI-MSN synapse. While assaying FSI-MSN synaptic strength with single pulse light stimulation every 20 s, application of ethanol (50 mM) induces LTD of this synapse in a DOR-dependent manner (Patton et al., 2016). We replicated

these findings by applying 50 mM ethanol for 10 min to striatal slices and observed an ethanol-induced LTD (EtOH-LTD) of the FSI-MSN synapse (oIPSC amplitude = 66.49 ± 12.32% of baseline, t=2.72, df=8, p=0.03, paired t test, **Figure 4a**). However, delivering paired pulse light stimulation (50 ms IPI) every 20 s prevented EtOH-LTD (oIPSC amplitude = 110.2 ± 13.06% of baseline, t=0.78, df=6, p=0.47, paired t test, **Figure 4a**). There was a significant attenuation of EtOH-LTD magnitude with paired pulse stimulation (t=2.41, df=14, p=0.03, unpaired t test, **Figure 4a**). Finally, similar to our DPDPE application findings, incubating slices in the global PKC inhibitor, BIM-1 (2 µM), eliminated the paired light pulse stimulation protection against EtOH-LTD (oIPSC amplitude = 71.71 ± 10.93% of baseline, t=2.59, df=8, p=0.03, paired t test, **Figure 4b**).

4. Discussion

These data support the existence of a highly sensitive, activity-dependent mechanism for protection against synaptic depression initiated by a presynaptically expressed, $G_{i/o}$-coupled receptor in a mammalian synapse. Delivering paired, rather than a single, stimulation pulses to evoke GABA release from DLS FSIs eliminates LTD induced either by DOR agonist application or by ethanol, which was previously shown to induce DOR-LTD (Patton et al., 2016). This protection against presynaptic LTD produced by short bursts of two action potentials shares key features of BDP at *Aplysia* sensory neuron synapses: a dependence on Ca^{2+} influx and a Ca^{2+}-sensitive PKC isoform, and the involvement of a PDZ domain interaction (**Figure 5**). Thus, it is possible that this exquisitely Ca^{2+} sensitive synaptic mechanism may, in part, be conserved across 600 million years of evolution.

Two unusual characteristics of BDP in *Aplysia* are exhibited by our observed activity-dependent protection against depression at FSI-MSN synapses. First, brief bursts of action potentials block the development of stable synaptic depression, rather than initiating an independent synaptic potentiation process that simply masks the depression. Second, this form of plasticity is exceptionally sensitive to modest amounts of activity. In slight contrast to what is observed in *Aplysia*, striatal BDP is initiated by bursts of only two action potentials; in *Aplysia* fully effective BDP typically requires bursts of four action

potentials, and pairs of action potentials result only in partial BDP (Wan et al., 2012). Single action potentials increase global Ca^{2+} at synaptic terminals of hippocampal neurons by only ~10 nM (de Juan-Sanz et al., 2017), similar to increases in synaptic terminals of *Aplysia* sensory neurons (Eliot et al., 1993). In contrast, effective activation of classical PKC requires approximately 1 µM Ca^{2+}, a 10-fold increase above resting levels (Kohout et al., 2002). Thus, the PKC isoform that mediates protection against depression at FSI synapses must be localized where it can respond to highly localized increases in intraterminal Ca^{2+} concentrations, in Ca^{2+} nanodomains. Binding of classical PKC to PICK1 is a possible mechanism for this localization, as was concluded for BDP in *Aplysia* sensory neurons (Wan et al., 2012). Whereas in *Aplysia*, there is a single form of Ca^{2+}-activated PKC, in mammals there are four isoforms α, βI, βII and γ; these share approximately equal sequence similarity to PKC Apl-I in *Aplysia* (Sossin, 2007). Though evidence exists for an interaction of PKCα with the PDZ domain of PICK1 (Leitges et al., 2004; Staudinger et al., 1997), future studies are required to determine whether striatal BDP is mediated by PKCα.

How exactly striatal BDP interacts with the DOR-LTD mechanisms requires further exploration. There is a fundamental difference between the two forms of synaptic depression in how they are initiated: whereas HSD at *Aplysia* SN-MN synapses is initiated effectively by Ca^{2+} influx during single action potentials, LTD at FSI synapses is initiated by activation of presynaptic DOR, which is coupled to $G_{i/o}$ and inhibits adenylyl cyclase (Al-Hasani and Bruchas, 2011; Patton et al., 2016). Like DOR, group II metabotropic glutamate receptors reside presynaptically, couple to Gi/o, and upon activation reduce neurotransmitter release probability (Baskys and Malenka, 1991; Lovinger, 1991). Synaptic depression induced by group II metabotropic receptors is inhibited by activation of PKC in hippocampus and striatum (Swartz et al., 1993; Tyler and Lovinger, 1995), and evidence suggests that PKC decouples metabotropic glutamate receptors from GTP-binding proteins (Macek et al., 1998, 1999; Zhang and Schmidt, 1999). Alternatively, $G_{i/o}$-coupled receptor activation leads to direct inhibition of Ca^{2+} channel activity (Zamponi et al., 2015), which decreases neurotransmitter release probability. Therefore, paired

pulse stimulation may eliminate the expression of presynaptic LTD through an increase in intraterminal Ca^{2+} concentration.

Although similar Ca^{2+} channel modulation occurs in *Aplysia* (Dunn et al., 2018), there is no evidence that HSD in *Aplysia* involves reductions in Ca^{2+} influx. Thus, it is possible that Ca^{2+}-dependent PKC blocks persistent synaptic depression by phosphorylating proteins in distinct pathways in mouse and *Aplysia*. In hippocampus and at lamprey giant synapses, LTD involves binding of $G_{\beta\gamma}$ to the C terminus of SNAP-25 (Gerachshenko et al., 2005; Photowala et al., 2006). Notably, opioids result in persistent dephosphorylation of SNAP-25 at a PKC phosphorylation site, Ser187. Zhang and colleagues (2011) speculated that persistent dephosphorylation at this site could play a key role in the switch to stable LTD. It is possible that SNAP-25 is the locus at which PKC prevents DOR-LTD induction during BDP. Alternatively, RIM1α, which is necessary for LTD at GABAergic synapses in both hippocampus and basolateral amygdala (Chevaleyre et al., 2007), could be the site of interaction between PKC and the depression mechanism.

In light of the widespread expression of Gi/o-coupled receptors that are presynaptically localized to negatively modulate neurotransmitter (Atwood et al., 2014), the BDP-like mechanism described herein is positioned to serve as an exquisitely sensitive activity-dependent switch protecting against synaptic depression at multiple synapse types, thereby holding the potential to affect a wide range of behaviors. BDP in *Aplysia* is necessary to protect against HSD that occurs following repeated biologically innocuous stimuli (Wan et al., 2012), which is argued to allow *Aplysia* to remain attentive to behaviorally relevant environmental information. In contrast, within the context of the striatal FSI-MSN synapse, burst-dependent protection against DOR-LTD under physiological conditions may serve to shape inhibitory control of DLS MSN output. Given that *in vivo* ethanol exposure depresses inhibitory transmission onto DLS MSNs (Cuzon Carlson et al., 2011; Wilcox et al., 2014) through a DOR-dependent mechanism (Patton et al., 2016), ethanol-induced DOR-LTD may manifest at some FSI-MSN synapses while activity-dependent protection against DOR-LTD may exist at others. In light of the critical role of FSIs in habit formation (O'Hare et al., 2017) and the enhancement of habit learning by ethanol (Corbit et al.,

2012; Dickinson et al., 2002; Lesscher et al., 2010), a complex interplay between ethanol-induced DOR-LTD and activity-dependent protection against DOR-LTD may shape MSN ensemble activity (Barbera et al., 2016; Cui et al., 2013; Klaus et al., 2017) in favor of habitual action expression.

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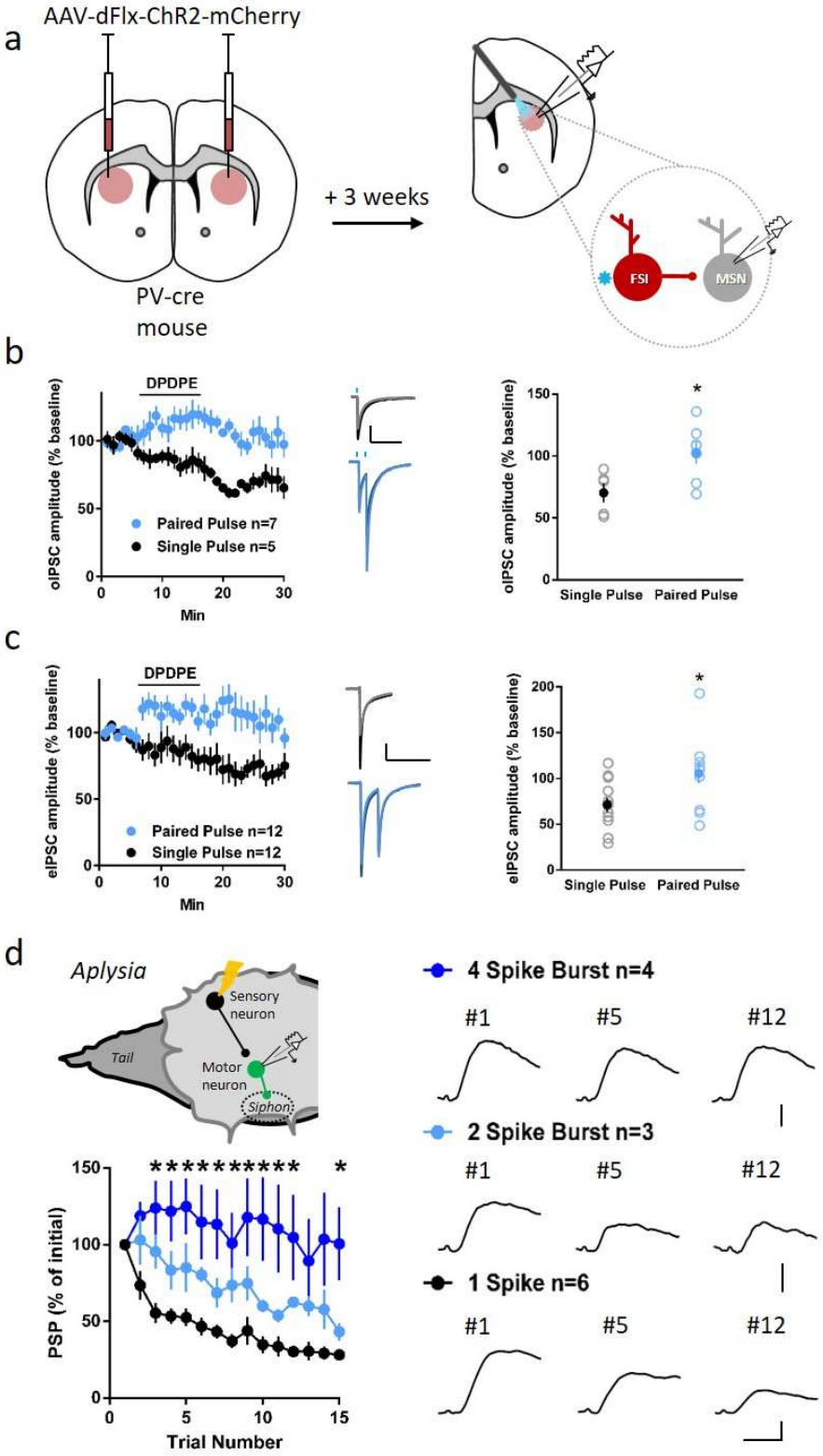
a
AAV-dFlx-ChR2-mCherry
PV-cre
mouse
+ 3 weeks
FSI
MSN
b
DPDPE
oIPSC amplitude (% baseline)
Paired Pulse n=7
Single Pulse n=5
Min
Single Pulse
Paired Pulse
c
DPDPE
eIPSC amplitude (% baseline)
Paired Pulse n=12
Single Pulse n=12
Min
Single Pulse
Paired Pulse
d
Aplysia
Sensory neuron
Tail
Motor neuron
Siphon
PSP (% of initial)
Trial Number
4 Spike Burst n=4
2 Spike Burst n=3
1 Spike n=6
#1
#5
#12

Figure 1.

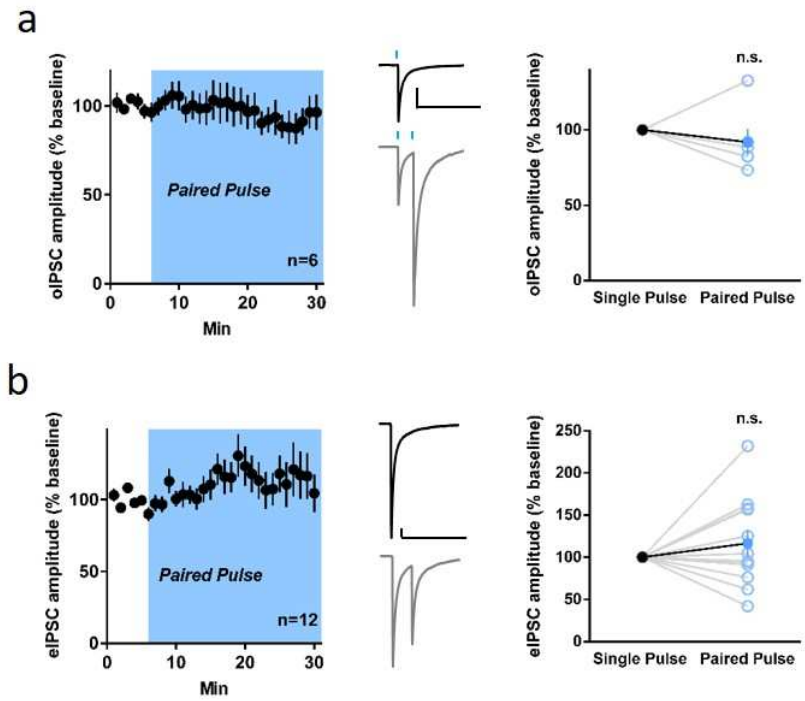
a
oIPSC amplitude (% baseline)
100
50
0
Paired Pulse
n=6
0
10
20
30
Min
n.s.
100
50
0
Single Pulse
Paired Pulse
b
eIPSC amplitude (% baseline)
100
50
0
Paired Pulse
n=12
0
10
20
30
Min
n.s.
250
200
150
100
50
0
Single Pulse
Paired Pulse

Figure 2.

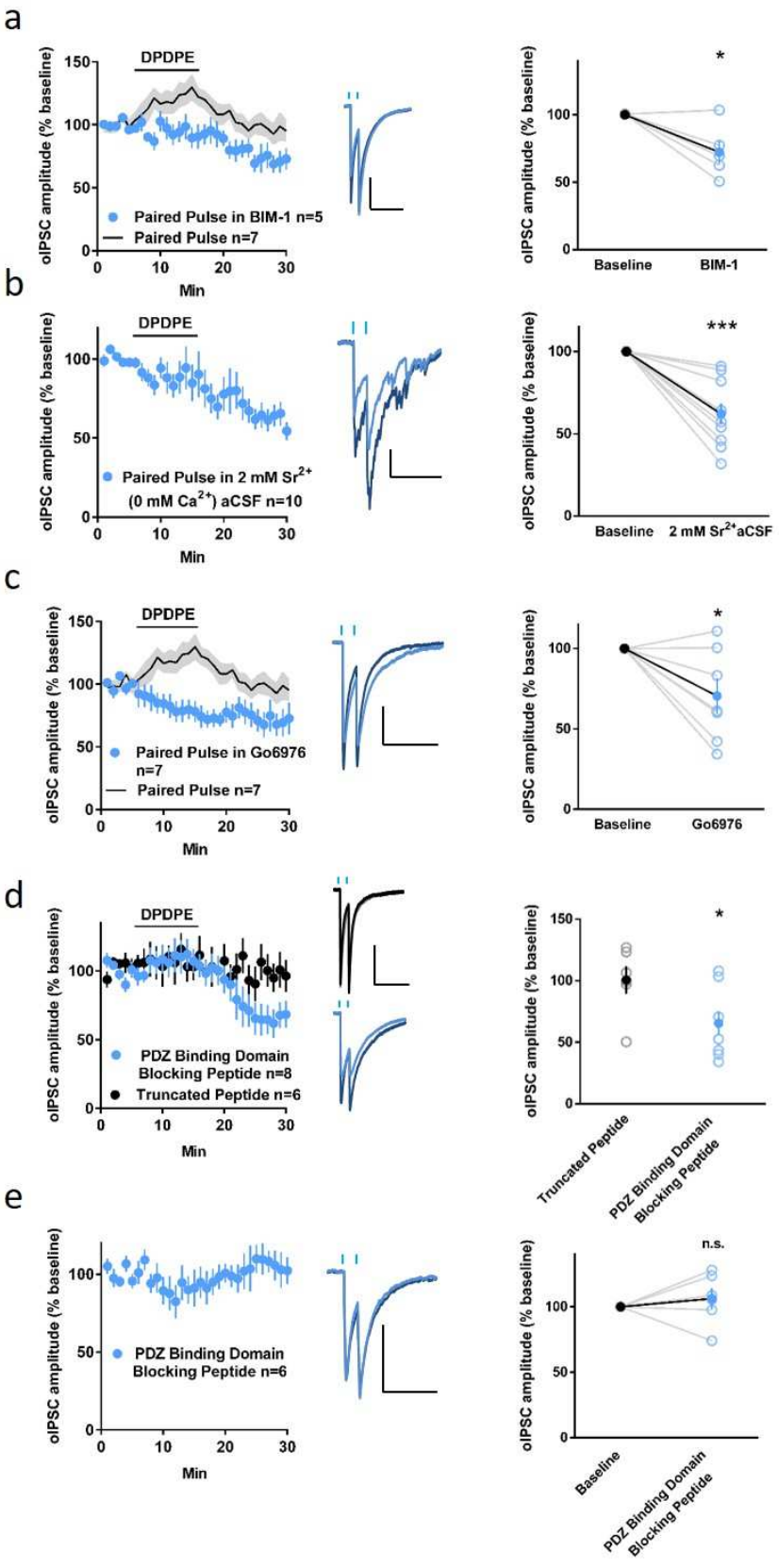
a
DPDPE
oIPSC amplitude (% baseline)
Paired Pulse in BIM-1 n=5
Paired Pulse n=7
Min
Baseline
BIM-1
b
DPDPE
Paired Pulse in 2 mM Sr2+
(0 mM Ca2+) aCSF n=10
Min
Baseline
2 mM Sr2+aCSF
c
DPDPE
Paired Pulse in Go6976
n=7
Paired Pulse n=7
Min
Baseline
Go6976
d
DPDPE
PDZ Binding Domain
Blocking Peptide n=8
Truncated Peptide n=6
Min
Truncated Peptide
PDZ Binding Domain
Blocking Peptide
e
PDZ Binding Domain
Blocking Peptide n=6
Min
n.s.
Baseline
PDZ Binding Domain
Blocking Peptide

Figure 3.

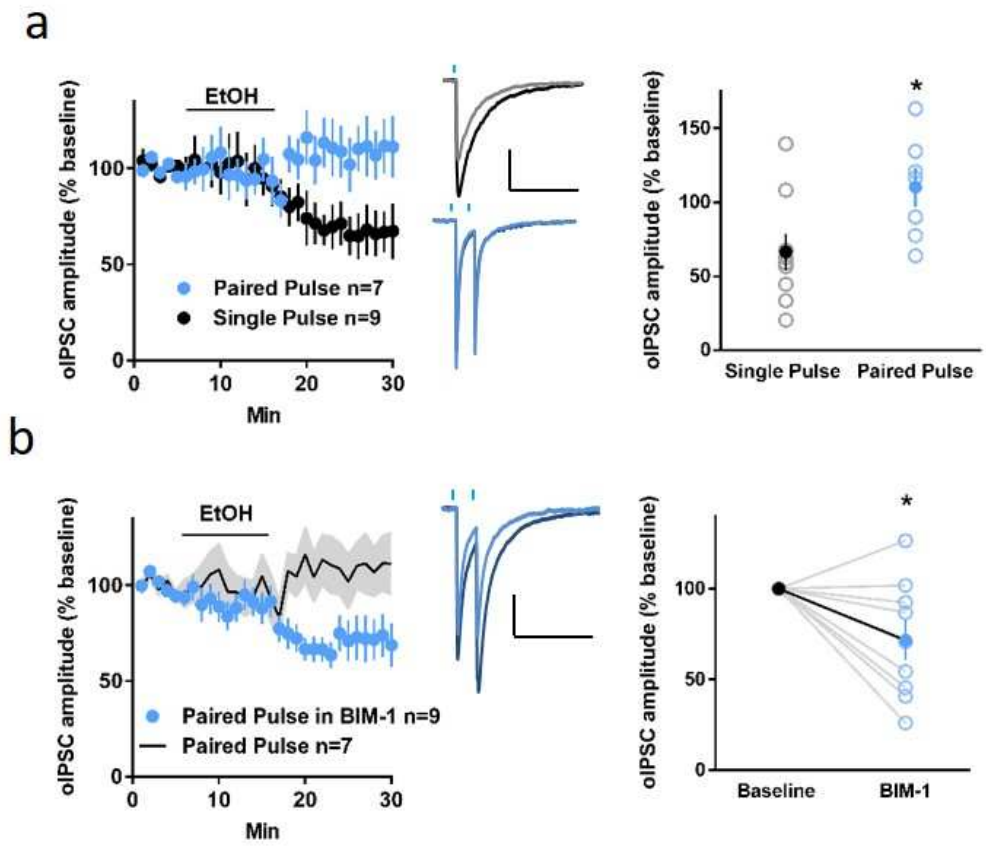
a
EtOH
oIPSC amplitude (% baseline)
100
50
0
0
10
20
30
Min
Paired Pulse n=7
Single Pulse n=9
150
100
50
0
*
Single Pulse
Paired Pulse
b
EtOH
Paired Pulse in BIM-1 n=9
Paired Pulse n=7
Baseline
BIM-1

Figure 4.

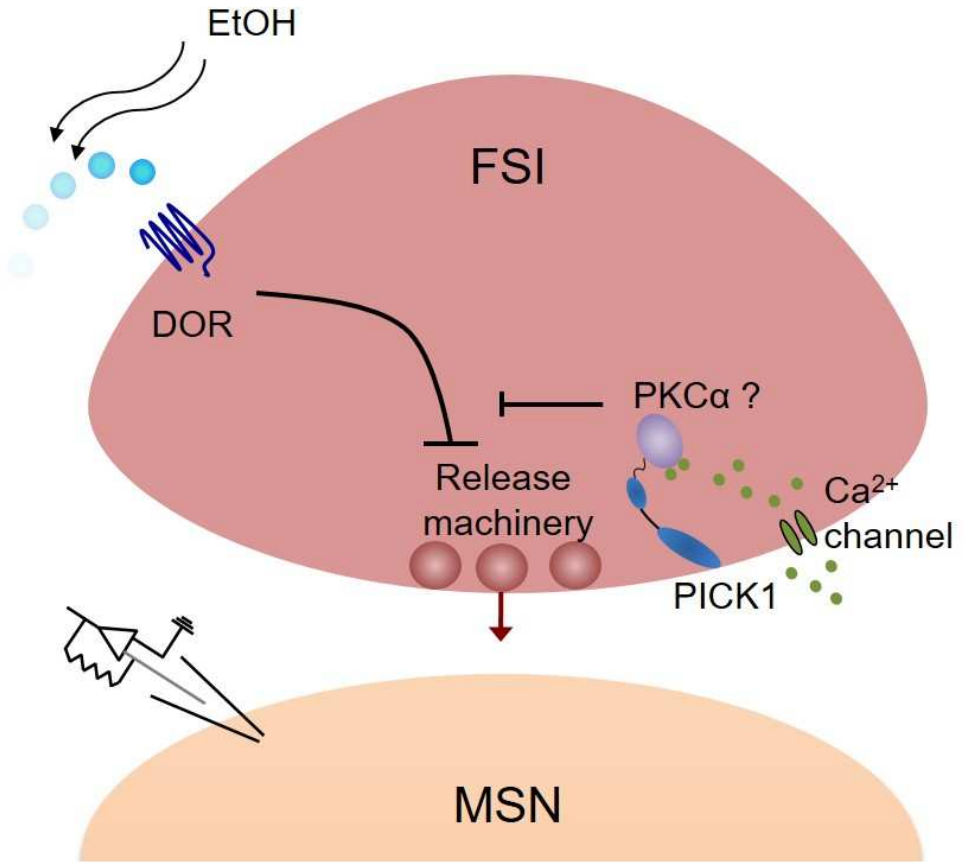

Figure 5.

Figure 1. Paired pulse light stimulation eliminates delta-opioid receptor-induced long-term depression (DOR-LTD). (A) To optogenetically target fast-spiking interneurons (FSI) in the dorsolateral striatum (DLS), an adeno-associated viral (AAV) vector containing a double loxP flanked (dFlx)-channelrhodopsin (ChR2) construct was injected into the DLS of parvalbumin (PV)-cre mice. Optogenetically-evoked inhibitory postsynaptic currents (oIPSC) arising from FSIs were recorded from medium spiny neurons (MSNs). (B) Left: Delivering paired light pulses (50 ms inter-pulse interval) blocked DOR-LTD induced by DPDPE (500 nM) (blue) compared to single light pulse interrogation of the synapse (black). Scale bars: 200 pA, 200 ms. Right: Scatterplot depicting the change in oIPSC strength from minutes 25-30 for each cell in single (black) and paired (blue) light pulse delivery experiments. (C) Left: Delivering paired electrical pulses (blue) abolished DOR-LTD compared to single electrical pulse stimulation (black). Scale bars: 200 pA, 200 ms. Right: Scatterplot depicting the change in electrically-evoked IPSC (eIPSC) strength from minutes 25-30 for each cell in single (black) and paired (blue) electrical pulse delivery experiments. (D) Left top: Schematic of the recording configuration in *Aplysia californica*. Left bottom: Single electrical pulse interrogation of the *Aplysia* siphon sensory neuron-to-motor neuron synapse resulted in synaptic depression (black) that attenuated with 2 bursts of action potentials (light blue) and eliminated with bursts of 4 action potentials (dark blue) (50 ms inter-pulse interval). Right: Representative postsynaptic potentials (PSPs) from each condition. Scale bars: 400 pA, 200 ms. * $p < 0.05$. B, C: representative traces are dark from the first 5 minutes and light from the last 5 minutes of the experiments. B, C scatterplots: open circles are individual cells, filled circles are mean ± SEM. D: data are represented as mean ± SEM.

Figure 2. Paired pulse stimulation does not induce long-term changes in synaptic strength. (A) Right: Paired light pulse stimulation (blue shaded region) following a 5 min baseline of single light pulse stimulation did not produce lasting synaptic changes. Right: Scatterplot depicting no significant effect of pulse number on oIPSC amplitude (single pulse, black; paired pulse, blue). (B) Left: Paired electrical pulse stimulation (blue shaded region) following a 5 min baseline of single electrical pulse stimulation had no effect on eIPSC amplitude. Right: Scatterplot depicting no effect of electrical stimulation pulse number on eIPSC amplitude (single pulse, black; paired pulse, blue). All scale bars: 200 pA, 200 ms. n.s.: not significant. All representative traces are dark from the first 5 minutes and light from the last 5 minutes of the experiment. Scatterplots: open circles are individual cells, filled circles are mean ± SEM.

Figure 3. Paired pulse protection against DOR-LTD requires a Ca^{2+}-sensitive PKC isoform. (A) Left: Incubating slices in BIM-1 (2 µM) eliminated the paired light pulse-induced protection against DOR-LTD (blue). Control paired pulse data from (Figure 1B) are represented as mean ± SEM in black. Right: Scatterplot depicting the change in oIPSC amplitude from baseline (black) following DPDPE wash in each neuron recorded (blue). (B) Left: Delivering paired light pulses in artificial cerebrospinal fluid (aCSF) containing 0 mM Ca^{2+} and 2 mM Sr^{2+} eliminated the protection against DOR-LTD. Right: Scatterplot depicting the change in oIPSC amplitude from baseline (black) following DPDPE wash (blue) in each neuron recorded. (C) Left: In the presence of the PKC inhibitor Go 6976 (3 µM) the paired light pulse protection against DOR-LTD was eliminated. Control paired pulse data from (Figure 1B) are represented as mean ± SEM in black. Right: Scatterplot showing the change in oIPSC amplitude from baseline (black) following DPDPE wash (blue) in each neuron recorded. (D) Left: Incubating slices in the blocking peptide TAT-PKC-CT (1 µM; blue) eliminated paired pulse protection against DOR-LTD. This protection was intact following incubation in a truncated control peptide TAT-PKCdeltaCT (1 µM; black). Right: Scatterplot depicting the change in oIPSC strength from minutes 25-30 for each cell in both

experimental conditions (control peptide, black; blocking peptide, blue). (E) Left: Incubation in TAT-PKC-CT did not lead to synaptic depression in the absence of DPDPE. Right: Scatterplot showing no change in oIPSC amplitude from baseline (black) and minutes 25-30 (blue) in each neuron recorded. Scatterplots: all open circles are individual cells and filled circles are mean ± SEM. All dark representative traces are from the first 5 minutes and light traces are from the last 5 minutes of the experiment. All scale bars: 200 pA, 200 ms. * $p < 0.05$, *** $p < 0.0005$, n.s.: not significant.

Figure 4. Paired light stimulation eliminates ethanol (EtOH)-induced LTD at the FSI-MSN synapse. (A) Left: Delivering paired light pulse stimulation eliminated EtOH-induced LTD (blue) compared to delivering single pulse light stimulation (black). Right: Scatterplot depicting the change in oIPSC strength from minutes 25-30 for each cell in single (black) and paired (blue) light pulse delivery experiments. (B) Left: BIM-1 (2 µM) in the aCSF eliminated EtOH-induced LTD while delivering a paired light pulse stimulation (blue). Control paired pulse data from (A) is represented as mean ± SEM (black). Right: Scatterplot showing the change in oIPSC amplitude from baseline (black) following EtOH wash (blue) in each neuron recorded. All scale bars: 200 pA, 200 ms. * $p < 0.05$. All representative traces are dark from the first 5 minutes and light from the last 5 minutes of the experiment. Scatterplots: open circles are individual cells, filled circles are mean ± SEM.

Figure 5. Schematic depicting the proposed mechanism of paired pulse protection against DOR-LTD. The activation of a Ca^{2+} sensitive PKC isoform such as PKCα by paired pulse-facilitated additional Ca^{2+} influx interacts with downstream effectors of DOR to eliminate FSI-MSN DOR-LTD. We hypothesize that this PKC isoform is localized close to Ca^{2+} channels by interacting with the scaffolding protein, PICK1. Because EtOH produces LTD at the FSI-MSN synapse through the activation of DOR, PKC activity also eliminates EtOH-induced LTD.