Prenatal Ethanol Exposure Persistently Alters Endocannabinoid Signaling and Endocannabinoid-Mediated Excitatory Synaptic Plasticity in Ventral Tegmental Area Dopamine Neurons

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Prenatal ethanol exposure (PE) leads to increased addiction risk which could be mediated by enhanced excitatory synaptic strength in ventral tegmental area (VTA) dopamine (DA) neurons. Previous studies have shown that PE enhances excitatory synaptic strength by facilitating an anti-Hebbian form of long-term potentiation (LTP). In this study, we investigated the effect of PE on endocannabinoid-mediated long-term depression (eCB-LTD) in VTA DA neurons. Rats were exposed to moderate (3 g/kg/d) or high (6 g/kg/d) levels of ethanol during gestation. Whole-cell recordings were conducted in male offspring between 4 and 10 weeks old.

We found that PE led to increased amphetamine self-administration. Both moderate and high levels of PE persistently reduced low-frequency stimulation-induced eCB-LTD. Furthermore, action potential-independent glutamate release was regulated by tonic eCB signaling in PE animals. Mechanistic studies for impaired eCB-LTD revealed that PE downregulated CB1 receptor function. Interestingly, eCB-LTD in PE animals was rescued by metabotropic glutamate receptor I activation, suggesting that PE did not impair the synthesis/release of eCBs. In contrast, eCB-LTD in PE animals was not rescued by increasing presynaptic activity, which actually led to LTP in PE animals, whereas LTD was still observed in controls. This result shows that the regulation of excitatory synaptic plasticity is fundamentally altered in PE animals. Together, PE leads to impaired eCB-LTD at the excitatory synapses of VTA DA neurons primarily due to CB1 receptor downregulation. This effect could contribute to enhanced LTP and the maintenance of augmented excitatory synaptic strength in VTA DA neurons and increased addiction risk after PE.

Key words: addiction risk; CB1 receptors; endocannabinoid-mediated long-term depression; long-term potentiation; synaptic homeostasis; ventral tegmental area dopamine neurons

Significance Statement

Prenatal ethanol exposure (PE) is among many adverse developmental factors known to increase drug addiction risk. Increased excitatory synaptic strength in VTA DA neurons is a critical cellular mechanism for addiction risk. Our results show that PE persistently alters eCB signaling and impairs eCB-LTD at the excitatory synapses, an important synaptic plasticity that weakens synaptic strength. These effects combined with PE-induced anti-Hebbian long-term potentiation reported in a previous study could result in the maintenance of enhanced excitatory synaptic strength in VTA DA neurons, which in turn contributes to PE-induced increase in addiction risk. Our findings also suggest that restoring normal eCB signaling in VTA DA neurons could be a useful strategy for treating behavioral symptoms caused by PE.

Introduction

Adverse environmental factors during the prenatal period, such as exposure to drugs of abuse (e.g., ethanol, psychostimulants,

opiates, cannabinoids, nicotine) and stress (Malanga and Kosofsky, 2003) could lead to increased addiction risk. The effects of prenatal ethanol exposure (PE) on increased addiction risk are well documented in clinical studies (Famy et al., 1998; Baer et al.,

Received Dec. 21, 2016; revised March 15, 2017; accepted April 5, 2017.

Author contributions: K.H., Y.-L.S., R.-X.W., S.H.-D., and R.-Y.S. designed research; K.H., Y.-L.S., R.-X.W., and R.-Y.S. performed research; K.H. and R.-Y.S. analyzed data; S.H.-D. and R.-Y.S. wrote the paper.

This work is funded by NIH/NIAAA Grant AA019482.

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.3894-16.2017

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2003; Alati et al., 2006). In animal studies, PE leads to behavioral phenotypes associated with increased addiction risk, such as enhanced locomotor activity to novelty (Bond, 1981; Abel, 1984; Kelly et al., 1988; Thomas et al., 2008), anxiety/depression-like behavior (Hellems et al., 2008; Weinberg et al., 2008), behavioral sensitization to stimulants (Blanchard et al., 1987; Barbier et al., 2009), and augmented hypothalamic-pituitary-adrenal (HPA) axis reactivity (Weinberg et al., 2008). Furthermore, PE directly facilitates addictive behaviors including the learning of drug cues (Spear and Molina, 2005; Barbier et al., 2009) and psychostimulant self-administration (Hausknecht et al., 2015).

Many lines of evidence support that increased excitatory synaptic strength onto dopamine (DA) neurons located in the ventral tegmental area (VTA) could be a critical cellular mechanism for PE-induced increase in addiction risk. These neurons are the origin of the mesolimbic/cortical DA systems, which are also the major targets of drugs of abuse (Koob and Le Moal, 1997). Direct administration of psychostimulants in the VTA facilitates future cocaine self-administration (Suto et al., 2003). Importantly augmenting and blocking excitatory neurotransmission in the VTA can increase responding to (Carlezon et al., 2000) and prevent psychostimulant self-administration (Suto et al., 2003), respectively. Furthermore, several studies report increased excitatory synaptic strength in VTA DA neurons after cocaine, ethanol, or stress exposure, conditions known to increase addiction risk. Increased excitatory synaptic strength in VTA DA neurons after drug or stress exposure appears to be mediated by elevated calcium-permeable AMPA receptor (CP-AMPA) expression in VTA DA neurons (Ortiz et al., 1995; Bellone and Lüscher, 2006; Argilli et al., 2008), which have larger conductance than calcium-impermeable AMPARs (Cull-Candy et al., 2006).

We have previously demonstrated that PE can facilitate amphetamine self-administration and persistently increase the expression of CP-AMPA receptors in VTA DA neurons (Hausknecht et al., 2015). This effect indeed leads to increased excitatory synaptic strength as well as a CP-AMPA-dependent, anti-Hebbian form of long-term potentiation (LTP), which can promote further synaptic strengthening even in the absence of postsynaptic neuronal activity. Therefore, we propose that the anti-Hebbian form of LTP plays a critical role in the maintenance of increased excitatory synaptic strength and overexcitation in VTA DA neurons in PE rats, which in turn leads to augmented behavioral sensitivities to drugs of abuse and addiction risk (Hausknecht et al., 2015).

Normal synapses undergo homeostasis by multiple forms of synaptic plasticity including LTP and long-term depression (LTD). Excitatory synapses at VTA DA neurons can be weakened by endocannabinoids (eCBs; Haj-Dahmane and Shen, 2010; Melis and Pistis, 2012; Wang and Lupica, 2014), which are readily synthesized and released by concomitant glutamate release and membrane depolarization/increased activity of VTA DA neurons. They then function as retrograde signals to activate presynaptic type 1 cannabinoid receptors (CB1 receptors) and reduce glutamate release. This effect can lead to short-term suppression (Melis et al., 2004; Lupica and Riegel, 2005), and/or LTD of excitatory synapses (Haj-Dahmane and Shen, 2010). The persistence of PE-induced increase in excitatory synaptic strength suggests that PE could impair eCB-LTD and result in abnormal synaptic homeostasis to allow a persistent increase in excitatory synaptic strength (Hausknecht et al., 2015). Furthermore, many lines of evidence show eCB signaling plays an important role in addictive behaviors (Volkow et al., 2017). Therefore, we investigated whether PE led to impaired eCB signaling including re-

duced eCB-LTD in VTA DA neurons and the underlying mechanisms.

Materials and Methods

All experimental procedures involving animals were performed in accordance with the University at Buffalo Institutional Animal Care and Use Committee guidelines.

Prenatal ethanol exposure and cross fostering. The procedures of PE and cross fostering were described in detail in a previous study (Choong and Shen, 2004). Briefly, breeding of Sprague-Dawley rats (Harlan) was performed to strictly control the prenatal environment. Ethanol (15% w/v) was administered via intragastric intubation from gestation days 8–20 at 0, 3, or 6 g/kg/d (2 intubations at 0, 1.5, or 3 g/kg ethanol/d) during weekdays. A single dose (0, 2, 4 g/kg) was given during weekends. The ethanol exposure treatment corresponded to moderate and heavy prenatal ethanol exposure (Eckardt et al., 1998; Shen et al., 1999). The control dams received sucrose solution (22.5% w/v in 0.9% saline) to equate for ethanol's calories with the 6 g/kg group. Both the control and the 3 g/kg dams were pair-fed with the 6 g/kg dams. All dams received thiamine injections twice/week (8 mg/kg, i.m.) to compensate ethanol intake-induced thiamine deficiency or pair-feeding, which could cause dysfunction in neurons. To control for postnatal rearing conditions, such as disrupted maternal behaviors in PE dams due to ethanol withdrawal, on postnatal day 1, pups from the ethanol groups were culled to 10/litter and transferred to dams that did not receive any treatment during pregnancy and gave birth 2 d earlier. Pups from the control litters were cross-fostered with each other (switching dams). One to two male offspring/litter were used in each experiment. Seventy-two dams (26 control, 23 PE, and 23 foster) were used. There were no differences in litter size (control: 14.2 ± 0.5 ; PE: 13.8 ± 0.4), pup number by gender (control male: 7.6 ± 0.4 ; control female 6.6 ± 0.4 ; PE male: 6.8 ± 0.4 , PE female: 7.0 ± 0.5), or pup weight (means from each litter) on postnatal day 1 (control male: 6.64 ± 0.08 g, PE male: 6.88 ± 0.28 g; control female: 6.31 ± 0.09 g, PE female: 6.21 ± 0.12 g).

Amphetamine self-administration. Results from a previous study show that only 10% of control rats acquired amphetamine self-administration at 0.02 mg/kg/infusion (Hausknecht et al., 2015). Therefore, we conducted the pretraining procedure to train rats (7 weeks old) to press bars. Water scheduling started 3 d before and during the pretraining. Rats were on a fixed ratio (FR) 1 schedule for water (0.02 ml) until they obtained 150 rewards (3 ml water/d) for 4 d. Rats had 30 min *ad libitum* access to water after daily sessions.

Rats were then anesthetized with ketamine/xylazine (60 mg/kg/5 mg/kg, i.p.) following buprenorphine (0.025 mg/kg, s.c.; see Hausknecht et al., 2015 for details of surgery and amphetamine self-administration). The right external jugular vein was implanted with a cannula (Instech). Five to 7 d after surgery, animals were trained daily to self-administer amphetamine intravenously (0.02 mg/kg/infusion, salt weight) under the FR 1 schedule for 3 h/d for 2 d. A priming dose of amphetamine was delivered. An additional nine infusions per day were allowed. Rats were trained under an FR 2 schedule for 1 d and then tested under a progressive ratio (PR) schedule (ratio values: $5 \times \text{EXP}^{(0.25 \times \text{infusion number}-5)}$; maximal infusions/d: 14; Richardson and Roberts, 1996) without priming infusion for 6 d. Data analysis was conducted only in rats with patent cannula at the end of the self-administration experiments.

Brain slices preparation. Midbrain slices containing the VTA were prepared from 4- to 10-week-old male rats ($n = 120$) using a standard method (Hausknecht et al., 2015). Rats were deeply anesthetized with isoflurane and killed by decapitation. The brain was quickly removed and placed in cold modified artificial CSF (ACSF) containing the following (in mM): 110 choline-Cl, 2.5 KCl, 0.5 CaCl₂, 7 MgSO₄, 1.25 NaH₂PO₄, 26.2 NaHCO₃, 11.6 sodium L-ascorbate, 3.1 sodium pyruvate, 25 glucose and saturated with 95% O₂/5% CO₂. Midbrain horizontal slices (200–250 μm) were cut using a vibratome (VT1200S; Leica Biosystems). Slices were then incubated for 45–60 min at 35°C in standard ACSF containing the following (in mM): 119 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1 NaH₂PO₄, 26.2 NaHCO₃, 11 glucose saturated with 95% O₂/5% CO₂. Slices were allowed to recover at room temperature for at least 1 h before transferred to the recording chamber and continuously perfused with

standard ACSF saturated with 95% O₂/5% CO₂ and maintained at 30 ± 1°C. In animals >6-week-old, slices were incubated in ACSF containing kynurenic acid (0.5 mM) to improve the viability of DA neurons. This manipulation did not alter the excitatory synaptic neurotransmission in VTA DA neurons (Hausknecht et al., 2015).

Whole-cell recordings. Neurons in the VTA were visualized using an upright microscope (Olympus BX 51) equipped with a differential interference contrast/infrared imaging system. Recordings were obtained with patch electrodes (3–5 MΩ) filled with a solution containing the following (in mM): 120 potassium gluconate, 10 Na₂-phosphocreatine, 10 KCl, 10 HEPES, 1 MgCl₂, 1 EGTA, 2 Na₂-ATP, and 0.25 Na-GTP, pH 7.3. In some experiments examining the LTD, potassium gluconate was substituted with cesium methanesulfonate. Putative DA neurons adjacent to the accessory optic track were identified by the presence of I_h current. Using this criterion leads to ~90% of positive identification (Hausknecht et al., 2015). Membrane potentials reported were corrected for the liquid junction potentials which were ~+5 and +8 mV for potassium and cesium-based internal solution, respectively.

Stimulation and recordings. All recordings were performed in the presence of picrotoxin (100 μM) to block GABA_A receptors. A patch pipette (3–5 MΩ) filled with ACSF was placed (50–100 μm) rostral to the recording sites to stimulate afferents to VTA neurons. Excitatory postsynaptic currents (EPSCs) were evoked with single square-pulses (duration = 100–200 μs, 0.1 Hz) in neurons voltage-clamped at –70 mV. Stimulation intensity was adjusted to evoke 50–70% of maximal response with EPSC amplitude ranging from 150 to 300 pA. Membrane currents were amplified with an Axoclamp 2B or Multiclamp 700 B amplifier (Molecular Devices), filtered at 3 kHz, digitized at 20 kHz with Digidata 1440A, and acquired using the pClamp 10 software (Molecular Devices). Access resistance (10–20 MΩ) was monitored online using 5 mV hyperpolarizing voltage steps (200 ms duration). Recordings with series resistance increased by >10% were discarded.

Induction of LTD was made by pairing postsynaptic depolarization to –30 mV with 2 Hz afferent stimulation for 5 min in the voltage-clamp mode. In some neurons, attempt to induce LTD was conducted at 5 Hz stimulation. To measure miniature action potential-independent EPSCs (mEPSCs), tetrodotoxin (TTX, 1 μM) was added in the bath and mEPSCs were recorded for 150 s. To investigate tonic eCB signaling, the effects of AM251, a CB1 receptor antagonist/inverse agonist were examined on the paired-pulse ratio (PPR) and frequency and amplitude of mEPSCs. To determine the PPR, pairs of stimuli with an interstimulus interval of 50 ms were delivered at 0.1 Hz. The function of CB1 receptors was assessed by examining the magnitude of the inhibition of EPSCs induced by the CB1 receptor agonist, WIN 55,212-2 (10–30 μM).

Data analysis and statistics. The amplitude of EPSCs was determined by the difference between peak EPSC and the baseline current immediately before the stimulus artifact. EPSC amplitudes were normalized to mean baseline amplitude recorded for at least 10 min before pairing. The PPR (EPSC₂/EPSC₁) was averaged for 30 trials and normalized to the mean PPR. To determine the coefficient of variation (CV), the SD and the mean amplitude of EPSCs were obtained for 30 consecutive trials and CV was calculated as SD/mean. Miniature EPSCs were analyzed with Mini analysis software using the criteria: amplitude threshold (5 pA), rise time (1 ms), and area threshold (30 fc). All selected events were further inspected to prevent noise from compromising the analysis. Results in the text and figures are presented as mean ± SEM. Kolmogorov–Smirnov (K–S) test was used to examine group differences in mEPSC amplitude and frequency. Statistical analyses were conducted using ANOVA or *t* test for comparisons between groups. The level of significance was set at 0.05 for all tests.

Chemicals and drugs. Most chemicals were obtained from Sigma-Aldrich. Drugs such as picrotoxin, (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN 55,212-2), *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251), 1-Naphthyl acetyl spermine trihydrochloride (NASPM), and (S)-3,5-dihydroxyphenylglycine (DHPPG) were purchased from R and D Systems.

Figure 1. Number of amphetamine infusions and responses (lever presses) in control and PE animals during six progressive ratio schedule self-administration sessions. Animals were first pretrained for lever pressing and went through fixed ratio training for amphetamine self-administration at 0.02 mg/kg/infusion. Increased infusions (**A**) and lever presses (**B**) were observed in PE animals indicating increased work output to obtain more amphetamine. Left, The number of infusions and lever presses at each session. Right, The average across 6 d. * $p < 0.05$, *** $p < 0.001$ indicate differences between control and PE animals.

Results

Prenatal ethanol exposure enhances amphetamine self-administration

No group differences were found during the pretraining or acquisition phase under the FR schedule. Under the PR schedule, PE animals self-administered more amphetamine than controls indicated by the number of infusions ($F_{(1,10)} = 7.7$; $p < 0.05$; two-way ANOVA with repeated measures; Fig. 1A). A significant session main effect was also found ($F_{(5,50)} = 3.9$; $p < 0.01$). A significant group and session interaction effect ($F_{(5,50)} = 2.7$; $p < 0.05$) was found in the number of lever presses. Rats in PE group responded (lever pressed) significantly more than control animals (planned comparison following two-way ANOVA; $p < 0.05$; Fig. 1B). These results show that PE can lead to enhanced work output and drug intake under a PR schedule of reinforcement (Fig. 1B). The results replicate our previous finding (Hausknecht et al., 2015) and show that the current PE paradigm consistently produces strong behavioral effects of increased drug taking and support that PE could place individuals at increased risk for drug addiction. The difference is that in the present study, a pretraining procedure was introduced to allow us to evaluate all animals in both groups for the acquisition of amphetamine self-administration compared with only 10% of control animals acquiring amphetamine self-administration in the previous study.

Prenatal ethanol exposure impairs eCB-LTD

Results from previous behavioral studies have established that eCB signaling regulates addiction-related behaviors and pharmacological manipulations which alter eCB signaling could influence addictive behaviors (Pava and Woodward, 2012; Volkow et al., 2017). To test whether the PE-induced increase in amphetamine self-administration was associated with alterations of eCB signaling in the VTA, we examined the effect of PE on eCB-LTD. This form of synaptic plasticity was induced by pairing low-frequency presynaptic stimulation (LFS; 2 Hz) with postsynaptic membrane depolarization (–30 mV; Haj-Dahmane and Shen, 2010). We found that PE profoundly reduced the magnitude of the eCB-LTD ($F_{(2,21)} = 14.70$; $p < 0.001$; two-way ANOVA with repeated measures; Fig. 2). This effect was observed in animals with either

Figure 2. Prenatal ethanol exposure leads to reduced eCB-LTD of AMPA receptor-mediated EPSCs in VTA DA neurons. **A**, The induction of eCB-LTD via pairing low-frequency stimulation (2 Hz) and moderate membrane depolarization (−30 mV) led to a prominent reduction in evoked EPSC amplitude in control (○), but significantly less reduction in either moderate PE (3 g/kg/d ethanol; ▲), or high PE animals (6 g/kg/d ethanol; ●). **B**, Summary bar graph of the magnitude of eCB-LTD as percentage of baseline EPSC amplitude at time points indicated in **A**. The magnitude of eCB-LTD was profoundly reduced in both PE groups as indicated by reduced inhibition of EPSC amplitude. There were no differences between the moderate and high PE groups. Upper traces depict representative EPSC recordings in control (left) and PE animals before and after LTD induction at time points indicated in **A**. *** $p < 0.001$ difference between control and PE animals; # $p < 0.05$ difference before and after LTD induction.

moderate (3 g/kg/d; $p < 0.001$; planned comparison following ANOVA) or high (6 g/kg/d; $p < 0.001$) PE and no differences were observed between these two groups. On average, the EPSC amplitude measured 29–30 min after induction was $56.4 \pm 11.3\%$ of baseline in controls ($n = 5$; $p < 0.001$ vs baseline). In contrast, the EPSC amplitude was $86.7 \pm 3.7\%$ ($n = 9$; $p < 0.01$ vs baseline) and $86.2 \pm 3.5\%$ ($n = 10$; $p < 0.001$ vs baseline; Fig. 2) of baseline in the moderate and high PE groups, respectively. There were no differences in the baseline EPSC amplitude between the control and PE groups (control: 142.77 ± 19.02 pA; PE 3 g/kg/d: 133.46 ± 16.27 pA; PE 6 g/kg/d: 132.57 ± 20.85 pA). Therefore the PE-induced impairment of eCB-LTD could not be attributed to bias caused by the normalization procedure. These results show that eCB-LTD is sensitive to ethanol exposure; even a moderate level of PE could significantly impair eCB-LTD.

The role of CB1 receptors in PE-induced impairment of eCB-LTD

Several distinct mechanisms could mediate the PE-induced impairment of eCB-LTD. These include downregulation of presynaptic CB1 receptors, occlusion of the eCB-LTD, inhibition of eCB synthesis and release, or a combination of these effects. To investigate these possibilities, we first examined whether the impaired eCB-LTD was caused by CB1 receptor downregulation. To that end, we assessed the effects of PE on the inhibition of EPSC amplitude induced by CB1 receptor agonist WIN 55,212-2. We found at $10 \mu\text{M}$, the WIN 55,212-2-induced inhibition of EPSCs was significantly reduced in PE rats compared with control (two-way ANOVA with repeated measures, $F_{(1,12)} = 10.75$, $p < 0.01$; Fig. 3A). The amplitude of EPSCs was $48.8 \pm 6.8\%$ of baseline ($p < 0.001$, planned comparison following two-way ANOVA, $n = 8$) and $82.3 \pm 7.8\%$ of baseline ($p = 0.051$, $n = 6$; Fig. 3A) in control and PE rats, respectively. However, when a higher concentration ($30 \mu\text{M}$) of WIN 55,212-2 was applied, the WIN 55,212-2-induced inhibition of EPSCs in PE rats was comparable to controls (control: $57.6 \pm 6.5\%$ of baseline, $p < 0.001$ vs baseline, $n = 6$, planned comparison following two-way ANOVA

with WIN 55,212-2 main effect; $F_{(1,10)} = 67.4$, $p < 0.001$; PE: $64.6 \pm 6.5\%$ of baseline, $p < 0.001$ vs baseline; $n = 6$, planned comparison; Fig. 3B). We also observed that the inhibitory effect of WIN 55,212-2 at both 10 and $30 \mu\text{M}$ was completely blocked by $10 \mu\text{M}$ of AM251 applied at least 20 min before WIN 55,212-2 in several naive animals, confirming that the inhibitory effect of WIN 55,212-2 was indeed mediated by CB1 receptor activation. The above observations support that PE leads to downregulation of CB1 receptors and such an effect could underlie PE-induced reduction in eCB-LTD.

The role of occlusion in PE-induced impairment of eCB-LTD

Because eCB-LTD is mediated by decreased presynaptic glutamate release (Haj-Dahmane and Shen, 2010), it is possible that PE could reduce eCB-LTD by an occlusion effect. To test this possibility, we investigated the effect of PE on glutamate release. The evoked glutamate release was assessed by the PPR and CV of evoked EP-

SCs, two measures of release probability. The results showed that PE significantly increased the PPR (control: 1.00 ± 0.06 , $n = 21$; PE: 1.41 ± 0.12 , $n = 22$; t test, $t_{(41)} = 3.2$, $p < 0.01$; Fig. 4A). There was also a strong trend toward increased CV (control: 0.26 ± 2.8 ; PE: 0.36 ± 3.9 ; $t_{(41)} = 1.91$, $p = 0.06$; Fig. 4A), supporting that PE reduces the probability of glutamate release.

Previous studies have shown that postsynaptic factors, such as increased expression of CP-AMPA receptors, can lead to increased PPR due to removal of the polyamine block of CP-AMPA receptors induced by the first EPSC (Toth et al., 2000). Because PE has been shown to increase the expression of CP-AMPA receptors in VTA DA neurons (Hausknecht et al., 2015), it is possible that the PE-induced increase in PPR may not reflect a decrease in glutamate release per se, but rather an increase in CP-AMPA receptors. To test this possibility, we assessed the effect of PE on PPR in the absence or presence of a selective CP-AMPA receptor antagonist, NASPM ($20 \mu\text{M}$) in the same neuron. The result showed that the increase in PPR induced by PE persisted in the presence of NASPM (control baseline: 0.97 ± 0.10 , NASPM: 0.97 ± 0.12 , $n = 10$; PE baseline: 1.39 ± 0.09 , NASPM: 1.28 ± 0.11 , $n = 12$; group main effect: $F_{(1,20)} = 7.90$, $p < 0.05$, two-way ANOVA with repeated measures; Fig. 4B) and NASPM did not alter the PPR in either control or PE animals. Similarly, NASPM did not affect CV. These results strongly support that the increased PPR in PE animals is due to decreased evoked glutamate release.

We next examined the effect of PE on the frequency and amplitude of mEPSCs to assess action potential-independent glutamate release. The result shows a significant reduction in the frequency of mEPSCs in PE animals ($n = 27$) compared with control ($n = 24$) as indicated by a significant right shift in the cumulative probability of inter-event intervals (K–S test, $p < 0.001$; Fig. 5A,B). The difference was also detected by t test ($t_{(49)} = 2.45$, $p = 0.05$) on mean frequency (Hz; Fig. 5B). Prenatal ethanol exposure also induced an increase in the mEPSC amplitude (K–S test, $p < 0.01$; Fig. 5C). This finding is consistent with the PE-induced increase in CP-AMPA receptors (Hausknecht et al., 2015), which exhibit larger unitary conductance (Cull-Candy et al.,

2006). Together, these observations show that PE induces a persistent decrease in both the evoked and action potential-independent glutamate release in VTA DA neurons and support the possibility that occlusion can also contribute to impaired eCB-LTD in PE animals.

Does tonic eCB signaling contribute to occlusion of eCB-LTD?

Results from previous studies in other brain areas have reported that tonic (constitutive) eCB signaling controls glutamate and GABA release at central synapses (Castillo et al., 2012). Consequently, the PE-induced reduction in glutamate release could be mediated by an increase in tonic eCB signaling leading to the occlusion of eCB-LTD. To investigate this possibility, we examined the effects of CB1 receptor antagonist/inverse agonist AM251 on both evoked EPSCs and mEPSCs. The results showed that bath application of AM251 (3 μ M, 20 min) did not alter the amplitude or the PPR of evoked EPSCs in either control or PE animals (control: $n = 9$; PE: $n = 11$; two-way ANOVA with repeated measures; Fig. 6A). These results suggest that evoked glutamate release is not under the control of tonic eCB signaling in VTA DA neurons recorded *in vitro*.

In contrast, PE led to tonic eCB signaling on action-potential independent glutamate release. We found that in control animals, AM251 (3 μ M) did not alter the amplitude or frequency of mEPSCs ($n = 13$; K-S test; Fig. 6C). Whereas in PE animals, AM251 increased the frequency of mEPSCs indicated by a right shift in the cumulative probability of mEPSC interevent interval ($n = 11$; K-S test $p < 0.001$; Fig. 6B) but did not change the amplitude (Fig. 6C). There was also a strong trend of reduced mean mEPSC frequency detected by t test in PE animals ($t_{(10)} = 2.08$; $p = 0.06$; Fig. 6B). This result supports that PE enhances tonic eCB signaling, which exerts a persistent inhibition on action potential-independent glutamate release in VTA DA neurons.

Role of eCB synthesis in PE-induced impairment of eCB-LTD

Although the results described above suggest that CB1 receptor downregulation contributes to the impaired eCB-LTD in PE rats, they do not exclude possible involvement of reduced eCB synthesis/release. We have previously shown that activation of phospholipase C (PLC) and the subsequent synthesis of the eCB species, 2-arachidonyl glycerol (2-AG), mediate the induction eCB-LTD in VTA DA

Figure 3. Prenatal ethanol exposure leads to presynaptic CB1 receptor downregulation in VTA DA neurons. **A**, Left, PE profoundly reduced the inhibitory effect of CB1 receptor agonist WIN 55,212-2 at 10 μ M on evoked EPSC amplitude. Right, The summary graph of WIN 55,212-2 effect as percentage of baseline at time points indicated on the left. Upper traces depict representative EPSCs recorded at time points indicated on the left. **B**, Left, At a higher WIN 55,212-2 concentration (30 μ M), there were no differences between control and PE animals in WIN 55,212-2-induced inhibitory effect. Right, Summary graph of WIN 55,212-2 effect as percentage of baseline at time points indicated on the left. Upper traces depict representative EPSCs recorded at time points indicated on the left. $**p < 0.01$ difference between control and PE animals.

Figure 4. Prenatal ethanol exposure decreases evoked glutamate release in VTA DA neurons. **A**, Prenatal ethanol exposure increased the PPR (left) and CV (right) in VTA DA neurons, indicating a decrease in evoked glutamate release. **B**, Neither PPR (left) nor CV (right) was influenced by CP-AMPA activation. These parameters were evaluated in a subset of neurons before and after bath application of NASPM (20 μ M). NASPM did not alter PPR or CV in control or PE animals, indicating that CP-AMPA-dependent short-term plasticity did not influence the values of PPR or CV. Increased PPR and CV in PE animals should be attributed to a reduction in evoked glutamate release. Upper traces depict representative EPSC recordings in conditions described in the bar graph below. $*p < 0.05$; $**p < 0.01$ indicate difference between control and PE animals.

Figure 5. Prenatal ethanol exposure decreases action potential-independent glutamate release in VTA DA neurons. **A**, Representative mEPSCs recorded in the presence of TTX in VTA DA neurons in control and PE animals. **B**, Left, The mEPSC frequency was significantly lower in PE animals, indicated by a significant right shift in the cumulative probability curve of inter-event intervals (K–S test). Right, Bar graph showing group difference in mean mEPSC frequency can also be detected by *t* test. These results indicate that action potential-independent glutamate release was reduced in PE animals. **C**, The mEPSC amplitude was greater in VTA DA neurons recorded from PE animals, indicated by a significant right shift in the cumulative probability curve of mEPSC amplitude (K–S test). This effect is consistent with increased expression of CP-AMPA receptors which have larger conductance in PE animals. Right, Summary bar graph showing no group differences in mean amplitude were detected using *t* test. ** $p < 0.01$; *** $p < 0.001$ indicate differences between control and PE animals.

neurons (Haj-Dahmane and Shen, 2010). Thus, to test whether PE impaired PLC-driven 2-AG signaling, we examined the effect of PE on the LTD induced by stimulating type I metabotropic glutamate receptors (mGluR-LTD), which is also signaled by activation of PLC pathway. We found administration of DHPG (50 μ M) induced LTD in both control and PE animals (two-way ANOVA with repeated measures DHPG main effect; $F_{(1,13)} = 9.87$, $p < 0.01$; control: $78.2 \pm 7.7\%$ of baseline, $n = 9$; PE: $84.3 \pm 9.5\%$ of baseline, $n = 6$; Fig. 7A). However, there were no group differences found in mGluR-LTD amplitude. The effect of lower concentrations of DHPG (20–30 μ M) was not investigated because no effects were found on EPSC amplitude in control animals (data not shown). The LTD was blocked by AM251 (3 μ M), confirming DHPG-induced LTD was signaled by PLC-driven eCB synthesis/release (Fig. 7B). An absence of group differences in the magnitude of the LTD induced by DHPG does not support that PLC-dependent eCB synthesis is impaired in PE animals.

Regulation of synaptic plasticity is altered in PE animals

Because the induction of eCB-LTD requires pairing of presynaptic stimulation with postsynaptic membrane depolarization (Haj-Dahmane and Shen, 2010), we investigated whether augmenting presynaptic activity by increasing stimulation frequency could overcome the CB1 receptor downregulation and rescue eCB-LTD. We increased stimulation frequency during eCB-LTD induction from 2 to 5 Hz, whereas the postsynaptic depolarization remained at -30 mV. The results showed a significant group and LTD induction interaction effect ($F_{(1,13)} = 17.68$; $p < 0.01$; two-way ANOVA with repeated measures). We observed that

LTD was still induced in control animals as indicated by reduced EPSC amplitude 29–30 min after the onset of induction ($74 \pm 10\%$ of baseline, $n = 8$, $p < 0.05$ vs baseline, planned comparison following ANOVA; Fig. 8). To our surprise, in PE animals, a strong LTP was observed ($143 \pm 11\%$ of baseline, $n = 7$, $p < 0.01$ vs baseline, planned comparison; Fig. 8B). The above observation indicates that impaired eCB-LTD in PE animals cannot be rescued by increasing presynaptic activity. Importantly, these findings indicate that PE profoundly alters the rules regulating synaptic plasticity of VTA DA neurons, making excitatory synapses more prone to exhibiting LTP than LTD.

Discussion

The results from the present study show that PE increases amphetamine intake by enhanced work output to acquire amphetamine in a self-administration task. We also find that PE persistently (in 4- to 10-week-old rats) reduces LFS-induced eCB-LTD at the excitatory synapses in VTA DA neurons. The reduced eCB-LTD is observed after a moderate or high level of PE, indicating that eCB signaling in VTA DA neurons is vulnerable to ethanol exposure during gestation. The results also show a clear association between enhanced drug-taking behavior and reduced eCB-LTD in PE animals.

The PE-induced impairment of eCB-LTD could be attributed, at least in part, to CB1 receptor downregulation. This conclusion is supported by the observation that PE reduces the inhibition of evoked EPSCs by CB1 receptor agonist WIN 55,212-2 at a moderate (10 μ M), but not a high concentration (30 μ M). Such an observation is consistent with several studies showing that chronic ethanol exposure during early development and adulthood downregulates CB1 receptors (Basavarajappa and Hungund, 1999; Ortiz et al., 2004; Vinod et al., 2006; Mittrirattanakul et al., 2007; Pava and Woodward, 2012; Varodayan et al., 2016). One study also shows that ethanol exposure leads to decreased CB1 receptor function without reducing its protein content in GABAergic synapses in the prefrontal cortex (Pava and Woodward, 2014). Instead, the reduced function is mediated by decoupling of CB1 receptors from Gi/o subunits of G proteins (Basavarajappa and Hungund, 1999; Vinod et al., 2010). On the other hand, a recent study has shown CB1 receptor downregulation after chronic Δ^9 -tetrahydrocannabinol exposure could be mediated by reduced receptor numbers and receptor internalization (Dudok et al., 2015). This study also shows that the efficacy of CB1 receptors in inhibiting presynaptic neurotransmitter release is positively correlated with axon terminal size with larger terminals containing more CB1 receptors and higher efficacy. Therefore, other than decoupling of CB1 receptors from Gi/o, PE could cause CB1 receptor downregulation by decreased excitatory axon terminal size, decreased receptor numbers, and/or increased receptor internalization.

Both acute and chronic ethanol can increase brain eCB levels including 2-AG (Caillé et al., 2007; Mittrirattanakul et al., 2007),

the eCB species used in VTA DA neurons (Melis et al., 2004; Haj-Dahmane and Shen, 2010). Downregulation of CB1 receptor is also observed after acute and chronic Δ^9 -tetrahydrocannabinol exposure (Mato et al., 2004; Dudok et al., 2015). Therefore, it was suggested that the PE-induced downregulation of CB1 receptors is caused by a compensatory mechanism in response to ethanol-induced increase in eCB levels (Pava and Woodward, 2012). This notion is supported by the observations that CB1 receptors and 2-AG are present prenatally and CB1 receptors are already coupled to Gi/o (Berrendero et al., 1998).

One difference between PE and chronic ethanol effects on CB1 receptor downregulation is that the effect of chronic ethanol exposure is temporary (Vinod et al., 2006; Mitrirattanakul et al., 2007), whereas PE-induced effect is persistent (4- to 10-week-old rats). In addition to controlling synaptic strength and plasticity, CB1 receptors play important roles in neurite growth and branching, synaptogenesis, and neuronal differentiation during development (Mulder et al., 2008; Galve-Roperh et al., 2013; Monory et al., 2015). It is tempting to speculate that PE-induced CB1 receptor downregulation could contribute to abnormal dendritic branching, alteration of cell body size, and impaired synaptic functions in VTA DA neurons reported previously (Shetty et al., 1993; Hausknecht et al., 2015).

In addition to CB1 receptor downregulation, PE-induced impairment of eCB-LTD could also be attributed to occlusion. Namely, eCB-LTD has already taken place and therefore cannot be fully expressed. This possibility is supported by reduced glutamate release observed in the present study and the previous finding that ethanol can increase the level of 2-AG (Caillé et al., 2007; Mitrirattanakul et al., 2007). Furthermore, occlusion can also be caused by an increase in tonic eCB release which is demonstrated in VTA DA neurons in developing rats (14- to 21-d-old; Melis et al., 2004). Therefore, we investigate whether PE could enhance tonic eCB release. We find that although in control animals there is an absence in tonic eCB signaling, in PE animals tonic eCB signaling can be demonstrated. These results support that an occlusion effect could be mediated by tonic eCB signaling and contribute to PE-induced impairment of eCB-LTD.

Interestingly, PE-induced tonic eCB release is observed to only modulate action potential-independent but not evoked glutamate release. The simplest explanation for this effect is that the stimulation protocol used for evoked glutamate release only activates a subset of excitatory synapses which are not impacted by tonic eCB release in our *in vitro* condition. Another possibility is that tonic eCB signaling controlling glutamate release in PE animals is segregated. Indeed, strong evidence from the literature has shown that evoked and action potential-independent neurotransmitter release are segregated functionally and physically (Melom et al., 2013; Peled et al., 2014; Kavalali, 2015) and regulated differentially by tonic eCB. For example, tonic eCB signaling is shown to control only

Figure 6. Prenatal ethanol exposure induces tonic eCB signaling to regulate action potential-independent but not evoked glutamate release. **A**, A lack of tonic eCB signaling on evoked EPSCs. Bath applied AM251, a CB1 receptor antagonist, did not alter evoked EPSC amplitude in either control or PE animals, indicating a lack of tonic eCB signaling regulating evoked glutamate release. Left, Normalized evoked EPSC amplitude before and after AM251 administration. Middle, Summary bar graph of normalized evoked EPSC after AM251 application. Right, PPR as percentage baseline was not altered by AM251 in control or PE animals. Upper traces depict recordings of PPR at time points indicated on the left. **B**, Left, mEPSC frequency was not altered by AM251 in control animals but increased in PE animals, indicated by a significant left shift in the cumulative probability of inter-event interval of mEPSC (K-S test). Upper traces depict representative mEPSC recordings. Right, Summary bar graph showing increased mean mEPSC frequency by AM251 in PE animals could also be detected by *t* test. **C**, Left, mEPSC amplitude was not altered by AM251 in control or PE animals indicated by a lack of shift in the cumulative probability of inter-event interval of mEPSCs. Right, Summary bar graph showing average mEPSC amplitude before and after AM251. ****p* < 0.001 indicate difference before and after AM251 administration.

a specific subset of GABAergic synapses in hippocampal pyramidal neurons (Lee et al., 2015). Chronic ethanol exposure has been shown to alter tonic eCB signaling which controls action potential-independent but not evoked glutamate release at the excitatory synapses in the amygdala (Varodayan et al., 2016). These findings support evoked and action potential-independent glutamate release could be differentially regulated by tonic eCB signaling.

The source of tonic eCB signaling in PE animals is unclear at the present time. Tonic eCB synthesis/release can arise from post-synaptic neurons (Castillo et al., 2012) or microglia in a proinflammatory state (Stella, 2009). It is demonstrated that PE can induce proinflammatory microglia activation (Drew et al., 2015), supporting the possibility of tonic eCB release from activated microglia in PE animals. Tonic eCB signaling can also be mediated by constitutively active CB1 receptors (Pertwee, 2005). Future studies are required to delineate the mechanisms underlying PE-induced tonic eCB signaling.

We have also examined whether PE could impair eCB-LTD via reducing eCB synthesis/release. To that end, we investigate how PE influences the eCB-mediated mGluR-LTD induced by DHPG. Similar to LFS-induced eCB-LTD, mGluR-LTD is induced by the activation of PLC pathway and 2-AG production (Castillo et al., 2012; Melis and Pistis, 2012). However, unlike LFS-induced eCB-LTD, which requires increase in intracellular calcium via low threshold calcium channels (Haj-Dahmane and Shen, 2010), mGluR-LTD relies on the activation of the PLC pathway through $G_{q/11}$. We find that PE does not alter mGluR-

Figure 7. Prenatal ethanol exposure does not impair metabotropic glutamate receptor 1 (mGluR1) activation-induced eCB-LTD. **A**, Left, Bath administration of mGluR1 agonist DHPG led to LTD induction, indicated by reduced amplitude of evoked EPSCs in control and PE animals. Right, Summary graph shows the averaged magnitude of mGluR-LTD did not differ between control and PE animals. Upper traces depict the representative EPSCs recorded at time points indicated on the left. **B**, Left, eCB signaling is required for mGluR-LTD. LTD induced by DHPG was completely blocked in the presence of CB1 receptor antagonist AM251. Right, Summary graph showing the average EPSC amplitude did not change after DHPG administration in the presence of AM251. Top, The representative EPSCs recorded at time points indicated on the left.

Figure 8. Prenatal ethanol exposure promotes LTP in VTA DA neurons. **A**, When presynaptic stimulation frequency was increased from 2 to 5 Hz during eCB-LTD induction, LTD indicated by reduced EPSC amplitude was still observed in control animals, whereas robust LTP was observed in PE animals. This observation shows increasing presynaptic activity cannot rescue eCB-LTD. In addition, the excitatory synapses were prone to further strengthening in PE animals. **B**, Summary bar graph depicting the average EPSC amplitude collected at time points indicated in **A**. Upper traces show representative EPSCs at time points indicated in **A**. # $p < 0.05$, difference between two time points indicated in **A**; ** $p < 0.01$, difference between control and PE groups.

LTD compared with controls. Thus, it is unlikely that PE-induced impairment of eCB-LTD is caused by an inhibition of PLC mediated 2-AG synthesis. This observation also indicates that eCB-LTD could be rescued by mGluR activation.

During eCB-LTD induction, other than CB1 receptor activation, presynaptic activity is also required (Haj-Dahmane and Shen, 2010; Castillo et al., 2012). We then investigate whether increasing presynaptic activity (i.e., stimulation frequency) is increased, whereas LTD is still expressed in controls; LTP is observed in PE animals. This result is consistent with a previous study showing a switch from LTD to LTP in the cerebellum after PE (Servais et al., 2007). It also demonstrates that in PE animals, the rules governing the excitatory synaptic plasticity is fundamentally changed and favors potentiation. At the present time, the underlying mechanisms for altered regulation of excitatory synaptic plasticity are not clear. Excitatory synapses in VTA DA neurons in PE animals express a form of anti-Hebbian LTP induced by the activation of CP-AMPA receptors which are increased in PE animals (Hausknecht et al., 2015). This form of plasticity does not require concomitant presynaptic and postsynaptic activities; only presynaptic glutamate release leading to CP-AMPA activation and the subsequent calcium entry through CP-AMPA receptors is required (Lamsa et al., 2007). It is possible that in PE animals, increasing stimulation frequency during LTD induction promotes the anti-Hebbian LTP, which competes and masks possible enhancement in eCB-LTD. This notion is supported by previous findings that eCB-LTD competes with different forms of LTP. For example, eCB signaling can suppress NMDA-dependent LTP generation (Kortleven et al., 2011) and blocking eCB-LTD enhances LTP (Sjöström et al., 2007). Another possible mechanism underlying PE-induced switch from LTD to LTP is that increased presynaptic activity leads to increased eCB synthesis/release and induces eCB-mediated LTP. Indeed, a recent study has shown that spike-time-dependent LTP can be induced in corticostriatal synapses. This form of plasticity requires enhanced eCB mobilization that leads to concomitant activation of presynaptic CB1 receptors and postsynaptic transient receptor potential vanilloid-1 receptors (Cui et al., 2015).

Together, the results from the present study show PE-induced impairment of eCB-LTD is primarily due to a persistent downregulation of CB1 receptors. However, occlusion effect due to increased eCB level during/after ethanol exposure or tonic eCB signaling could also contribute to impaired eCB-LTD in PE ani-

mals. In contrast, the results do not support impaired eCB-LTD in PE animals is caused by reduced eCB synthesis and release. In fact, the observation that mGluR-LTD is not altered by PE would suggest that there is enhanced eCB synthesis and release that could overcome CB1 receptor downregulation. Another interesting finding is that PE enhances tonic eCB release, which decreases action-potential-independent glutamate release. Results from previous studies have shown that decreased action potential-independent glutamate release often leads to “scaling up” of postsynaptic receptors, such as the expression of immature CP-AMPA (Cull-Candy et al., 2006). In PE animals, we indeed observe a persistent increase in CP-AMPA contributing to an overall increase in excitatory synaptic strength (Hausknecht et al., 2015), raising the possibility that PE-induced tonic eCB signaling could paradoxically contribute to an overall increase in excitatory synaptic strength in VTA DA neurons. Furthermore, impaired eCB-LTD could promote LTP by leaving CP-AMPA-mediated anti-Hebbian LTP unopposed and allow the maintenance and even further facilitation of the already enhanced excitatory synaptic strength in VTA DA neurons (Hausknecht et al., 2015). We have suggested that increased excitatory synaptic strength in VTA DA neurons could ultimately lead to overexcitation/depolarization block and the cessation of impulse activity in VTA DA neurons in PE animals recorded *in vivo* (Hausknecht et al., 2015). We show that this effect can be alleviated by hyperpolarization agents, such as psychostimulants, and allow the impulse activity of VTA DA neurons to resume, leading to impulse-dependent DA release (Xu and Shen, 2001). We suggest this mechanism mediates increased behavioral sensitivity to drugs of abuse and increased addiction risk in PE animals. The current findings supports that rescuing eCB-LTD (e.g., by mGluR activation) and normalizing tonic eCB signaling could contribute to the functional recovery of excitatory synapses and ameliorate increased addiction risk in PE animals.

We would like to caution that the present study only investigates PE-induced changes in eCB signaling regulating excitatory synapses. However, strong evidence shows the GABAergic synapses onto VTA DA neurons can also be modulated by eCB signaling and this mechanism can be modified by drugs of abuse (Cheer et al., 2000; Szabo et al., 2002; Riegel and Lupica, 2004; Mátyás et al., 2008; Pan et al., 2008). At the present time, it is unclear how PE alters the function of these GABAergic synapses and whether eCB signaling regulating GABAergic synapses is altered by PE. An important future direction is to investigate these possibilities and integrate the results with PE-induced changes in excitatory synapses and overexcitation in VTA DA neurons observed *in vivo*.

In summary, PE leads to increased drug intake and multiple persistent changes in eCB signaling at the excitatory synapses onto VTA DA neurons, including CB1 receptor downregulation, impaired eCB-LTD, and enhanced tonic eCB signaling. Such effects could interact with PE-induced overexpression of CP-AMPA and anti-Hebbian LTP to qualitatively change the homeostasis of the excitatory synapses. Consequentially, a persistent increase in excitatory synaptic strength is maintained or even further enhanced. This effect in turn might contribute to enhanced sensitivity to drugs of abuse and increased addiction risk. Therefore, restoring eCB signaling in VTA DA neurons in PE animals could be a critical step in normalizing the function of excitatory synapses in these neurons, leading to behavioral recovery.

References

- Abel EL (1984) Prenatal effects of alcohol. *Drug Alcohol Depend* 14:1–10. [CrossRef Medline](#)
- Alati R, Al Mamun A, Williams GM, O’Callaghan M, Najman JM, Bor W (2006) *In utero* alcohol exposure and prediction of alcohol disorders in early adulthood: a birth cohort study. *Arch Gen Psychiatry* 63:1009–1016. [CrossRef Medline](#)
- Argilli E, Sibley DR, Malenka RC, England PM, Bonci A (2008) Mechanism and time course of cocaine-induced long-term potentiation in the ventral tegmental area. *J Neurosci* 28:9092–9100. [CrossRef Medline](#)
- Baer JS, Sampson PD, Barr HM, Connor PD, Streissguth AP (2003) A 21-year longitudinal analysis of the effects of prenatal alcohol exposure on young adult drinking. *Arch Gen Psychiatry* 60:377–385. [CrossRef Medline](#)
- Barbier E, Houchi H, Warnault V, Pierrefiche O, Daoust M, Naassila M (2009) Effects of prenatal and postnatal maternal ethanol on offspring response to alcohol and psychostimulants in Long–Evans rats. *Neuroscience* 161:427–440. [CrossRef Medline](#)
- Basavarajappa BS, Hungund BL (1999) Down-regulation of cannabinoid receptor agonist-stimulated [35 S]GTP- γ S binding in synaptic plasma membrane from chronic ethanol exposed mouse. *Brain Res* 815:89–97. [CrossRef Medline](#)
- Bellone C, Lüscher C (2006) Cocaine triggered AMPA receptor redistribution is reversed *in vivo* by mGluR-dependent long-term depression. *Nat Neurosci* 9:636–641. [CrossRef Medline](#)
- Berrendero F, García-Gil L, Hernández ML, Romero J, Cebeira M, de Miguel R, Ramos JA, Fernández-Ruiz JJ (1998) Localization of mRNA expression and activation of signal transduction mechanisms for cannabinoid receptor in rat brain during fetal development. *Development* 125:3179–3188. [Medline](#)
- Blanchard BA, Hannigan JH, Riley EP (1987) Amphetamine-induced activity after fetal alcohol exposure and undernutrition in rats. *Neurotoxicol Teratol* 9:113–119. [CrossRef Medline](#)
- Bond NW (1981) Prenatal alcohol exposure in rodents: a review of its effects on offspring activity and learning ability. *Australian J Psychol* 33:331–344. [CrossRef](#)
- Caillé S, Alvarez-Jaimes L, Polis I, Stouffer DG, Parsons LH (2007) Specific alterations of extracellular endocannabinoid levels in the nucleus accumbens by ethanol, heroin, and cocaine self-administration. *J Neurosci* 27:3695–3702. [CrossRef Medline](#)
- Carlezon WA Jr, Haile CN, Coppersmith R, Hayashi Y, Malinow R, Neve RL, Nestler EJ (2000) Distinct sites of opiate reward and aversion within the midbrain identified using a herpes simplex virus vector expressing glur1. *J Neurosci* 20:RC62. [Medline](#)
- Castillo PE, Younts TJ, Chávez AE, Hashimoto-dani Y (2012) Endocannabinoid signaling and synaptic function. *Neuron* 76:70–81. [CrossRef Medline](#)
- Cheer JF, Marsden CA, Kendall DA, Mason R (2000) Lack of response suppression follows repeated ventral tegmental cannabinoid administration: an *in vitro* electrophysiological study. *Neuroscience* 99:661–667. [CrossRef Medline](#)
- Choong K, Shen R (2004) Prenatal ethanol exposure alters the postnatal development of the spontaneous electrical activity of dopamine neurons in the ventral tegmental area. *Neuroscience* 126:1083–1091. [CrossRef Medline](#)
- Cui Y, Paillé V, Xu H, Genet S, Delord B, Fino E, Berry H, Venance L (2015) Endocannabinoids mediate bidirectional striatal spike-timing-dependent plasticity. *J Physiol* 593:2833–2849. [CrossRef Medline](#)
- Cull-Candy S, Kelly L, Farrant M (2006) Regulation of Ca^{2+} -permeable AMPA receptors: synaptic plasticity and beyond. *Curr Opin Neurobiol* 16:288–297. [CrossRef Medline](#)
- Drew PD, Johnson JW, Douglas JC, Phelan KD, Kane CJ (2015) Pioglitazone blocks ethanol induction of microglial activation and immune responses in the hippocampus, cerebellum, and cerebral cortex in a mouse model of fetal alcohol spectrum disorders. *Alcohol Clin Exp Res* 39:445–454. [CrossRef Medline](#)
- Dudok B, Barna L, Ledri M, Szabó SI, Szabadits E, Pintér B, Woodhams SG, Henstridge CM, Balla GY, Nyilas R, Varga C, Lee SH, Matolcsi M, Cervenak J, Kacsiovics I, Watanabe M, Sagheddu C, Melis M, Pistis M, Soltesz I, et al. (2015) Cell-specific storm super-resolution imaging reveals nanoscale organization of cannabinoid signaling. *Nat Neurosci* 18:75–86. [CrossRef Medline](#)
- Eckardt MJ, File SE, Gessa GL, Grant KA, Guerri C, Hoffman PL, Kalant H, Koob GF, Li TK, Tabakoff B (1998) Effects of moderate alcohol con-

- sumption on the central nervous system. *Alcohol Clin Exp Res* 22:998–1040. [CrossRef Medline](#)
- Famy C, Streissguth AP, Unis AS (1998) Mental illness in adults with fetal alcohol syndrome or fetal alcohol effects. *Am J Psychiatry* 155:552–554. [CrossRef Medline](#)
- Galve-Roperch I, Chiurchiù V, Diaz-Alonso J, Bari M, Guzmán M, Maccarrone M (2013) Cannabinoid receptor signaling in progenitor/stem cell proliferation and differentiation. *Prog Lipid Res* 52:633–650. [CrossRef Medline](#)
- Haj-Dahmane S, Shen RY (2010) Regulation of plasticity of glutamate synapses by endocannabinoids and the cyclic-AMP/protein kinase A pathway in midbrain dopamine neurons. *J Physiol* 588:2589–2604. [CrossRef Medline](#)
- Hausknecht K, Haj-Dahmane S, Shen YL, Vezina P, Dlugos C, Shen RY (2015) Excitatory synaptic function and plasticity is persistently altered in ventral tegmental area dopamine neurons after prenatal ethanol exposure. *Neuropsychopharmacology* 40:893–905. [CrossRef Medline](#)
- Hellemans KG, Verma P, Yoon E, Yu W, Weinberg J (2008) Prenatal alcohol exposure increases vulnerability to stress and anxiety-like disorders in adulthood. *Ann N Y Acad Sci* 1144:154–175. [CrossRef Medline](#)
- Kavalali ET (2015) The mechanisms and functions of spontaneous neurotransmitter release. *Nat Rev Neurosci* 16:5–16. [CrossRef Medline](#)
- Kelly SJ, Goodlett CR, Hulsether SA, West JR (1988) Impaired spatial navigation in adult female but not adult male rats exposed to alcohol during the brain growth spurt. *Behav Brain Res* 27:247–257. [CrossRef Medline](#)
- Koob GF, Le Moal M (1997) Drug abuse: Hedonic homeostatic dysregulation. *Science* 278:52–58. [CrossRef Medline](#)
- Kortleven C, Fasano C, Thibault D, Lacaille JC, Trudeau LE (2011) The endocannabinoid 2-arachidonoylglycerol inhibits long-term potentiation of glutamatergic synapses onto ventral tegmental area dopamine neurons in mice. *Eur J Neurosci* 33:1751–1760. [CrossRef Medline](#)
- Lamsa KP, Heeroma JH, Somogyi P, Rusakov DA, Kullmann DM (2007) Anti-hebbian long-term potentiation in the hippocampal feedback inhibitory circuit. *Science* 315:1262–1266. [CrossRef Medline](#)
- Lee SH, Ledri M, Tóth B, Marchionni I, Henstridge CM, Dudok B, Keneszi K, Barna L, Szabó SI, Renkecz T, Oberoi M, Watanabe M, Limoli CL, Horvai G, Soltesz I, Katona I (2015) Multiple forms of endocannabinoid and endovanilloid signaling regulate the tonic control of GABA release. *J Neurosci* 35:10039–10057. [CrossRef Medline](#)
- Lupica CR, Riegel AC (2005) Endocannabinoid release from midbrain dopamine neurons: a potential substrate for cannabinoid receptor antagonist treatment of addiction. *Neuropharmacology* 48:1105–1116. [CrossRef Medline](#)
- Malanga CJ, Kosofsky BE (2003) Does drug abuse beget drug abuse? Behavioral analysis of addiction liability in animal models of prenatal drug exposure. *Brain Res Dev Brain Res* 147:47–57. [CrossRef Medline](#)
- Mato S, Chevalere V, Robbe D, Pazos A, Castillo PE, Manzoni OJ (2004) A single *in-vivo* exposure to δ 9THC blocks endocannabinoid-mediated synaptic plasticity. *Nat Neurosci* 7:585–586. [CrossRef Medline](#)
- Mátyás F, Urbán GM, Watanabe M, Mackie K, Zimmer A, Freund TF, Katona I (2008) Identification of the sites of 2-arachidonoylglycerol synthesis and action imply retrograde endocannabinoid signaling at both GABAergic and glutamatergic synapses in the ventral tegmental area. *Neuropharmacology* 54:95–107. [CrossRef Medline](#)
- Melis M, Pistis M (2012) Hub and switches: endocannabinoid signalling in midbrain dopamine neurons. *Philos Trans R Soc Lond B Biol Sci* 367:3276–3285. [CrossRef Medline](#)
- Melis M, Pistis M, Perra S, Muntoni AL, Pillolla G, Gessa GL (2004) Endocannabinoids mediate presynaptic inhibition of glutamatergic transmission in rat ventral tegmental area dopamine neurons through activation of CB1 receptors. *J Neurosci* 24:53–62. [CrossRef Medline](#)
- Melom JE, Akbergenova Y, Gavornik JP, Littleton JT (2013) Spontaneous and evoked release are independently regulated at individual active zones. *J Neurosci* 33:17253–17263. [CrossRef Medline](#)
- Mittrattanakul S, López-Valdés HE, Liang J, Matsuka Y, Mackie K, Faull KF, Spigelman I (2007) Bidirectional alterations of hippocampal cannabinoid 1 receptors and their endogenous ligands in a rat model of alcohol withdrawal and dependence. *Alcohol Clin Exp Res* 31:855–867. [CrossRef Medline](#)
- Monory K, Polack M, Remus A, Lutz B, Korte M (2015) Cannabinoid CB1 receptor calibrates excitatory synaptic balance in the mouse hippocampus. *J Neurosci* 35:3842–3850. [CrossRef Medline](#)
- Mulder J, Aguado T, Keimpema E, Barabás K, Ballester Rosado CJ, Nguyen L, Monory K, Marsicano G, Di Marzo V, Hurd YL, Guillemot F, Mackie K, Lutz B, Guzmán M, Lu HC, Galve-Roperch I, Harkany T (2008) Endocannabinoid signaling controls pyramidal cell specification and long-range axon patterning. *Proc Natl Acad Sci USA* 105:8760–8765. [CrossRef Medline](#)
- Ortiz J, Fitzgerald LW, Charlton M, Lane S, Trevisan L, Guitart X, Shoemaker W, Duman RS, Nestler EJ (1995) Biochemical actions of chronic ethanol exposure in the mesolimbic dopamine system. *Synapse* 21:289–298. [CrossRef Medline](#)
- Ortiz S, Oliva JM, Pérez-Rial S, Palomo T, Manzanera J (2004) Chronic ethanol consumption regulates cannabinoid CB1 receptor gene expression in selected regions of rat brain. *Alcohol Alcohol* 39:88–92. [CrossRef Medline](#)
- Pan B, Hillard CJ, Liu QS (2008) Endocannabinoid signaling mediates cocaine-induced inhibitory synaptic plasticity in midbrain dopamine neurons. *J Neurosci* 28:1385–1397. [CrossRef Medline](#)
- Pava MJ, Woodward JJ (2012) A review of the interactions between alcohol and the endocannabinoid system: implications for alcohol dependence and future directions for research. *Alcohol* 46:185–204. [CrossRef Medline](#)
- Pava MJ, Woodward JJ (2014) Chronic ethanol alters network activity and endocannabinoid signaling in the prefrontal cortex. *Front Integr Neurosci* 8:58. [CrossRef Medline](#)
- Peled ES, Newman ZL, Isacoff EY (2014) Evoked and spontaneous transmission favored by distinct sets of synapses. *Curr Biol* 24:484–493. [CrossRef Medline](#)
- Pertwee RG (2005) Inverse agonism and neutral antagonism at cannabinoid CB1 receptors. *Life Sci* 76:1307–1324. [CrossRef Medline](#)
- Richardson NR, Roberts DC (1996) Progressive ratio schedules in drug self-administration studies in rats: a method to evaluate reinforcing efficacy. *J Neurosci Methods* 66:1–11. [CrossRef Medline](#)
- Riegel AC, Lupica CR (2004) Independent presynaptic and postsynaptic mechanisms regulate endocannabinoid signaling at multiple synapses in the ventral tegmental area. *J Neurosci* 24:11070–11078. [CrossRef Medline](#)
- Servais L, Hourez R, Bearzatto B, Gall D, Schiffmann SN, Cheron G (2007) Purkinje cell dysfunction and alteration of long-term synaptic plasticity in fetal alcohol syndrome. *Proc Natl Acad Sci USA* 104:9858–9863. [CrossRef Medline](#)
- Shen RY, Hannigan JH, Kapatos G (1999) Prenatal ethanol reduces the activity of adult midbrain dopamine neurons. *Alcohol Clin Exp Res* 23:1801–1807. [CrossRef Medline](#)
- Shetty AK, Burrows RC, Phillips DE (1993) Alterations in neuronal development in the substantia nigra pars compacta following in utero ethanol exposure: immunohistochemical and Golgi studies. *Neuroscience* 52:311–322. [CrossRef Medline](#)
- Sjöström PJ, Turrigiano GG, Nelson SB (2007) Multiple forms of long-term plasticity at unitary neocortical layer 5 synapses. *Neuropharmacology* 52:176–184. [CrossRef Medline](#)
- Spear NE, Molina JC (2005) Fetal or infantile exposure to ethanol promotes ethanol ingestion in adolescence and adulthood: a theoretical review. *Alcohol Clin Exp Res* 29:909–929. [CrossRef Medline](#)
- Stella N (2009) Endocannabinoid signaling in microglial cells. *Neuropharmacology* 56:244–253. [CrossRef Medline](#)
- Suto N, Tanabe LM, Austin JD, Creekmore E, Vezina P (2003) Previous exposure to VTA amphetamine enhances cocaine self-administration under a progressive ratio schedule in an NMDA, AMPA/kainate, and metabotropic glutamate receptor-dependent manner. *Neuropsychopharmacology* 28:629–639. [CrossRef Medline](#)
- Szabo B, Siemes S, Wallmichrath I (2002) Inhibition of GABAergic neurotransmission in the ventral tegmental area by cannabinoids. *Eur J Neurosci* 15:2057–2061. [CrossRef Medline](#)
- Thomas JD, Sather TM, Whinery LA (2008) Voluntary exercise influences behavioral development in rats exposed to alcohol during the neonatal brain growth spurt. *Behav Neurosci* 122:1264–1273. [CrossRef Medline](#)
- Toth K, Soares G, Lawrence JJ, Philips-Tansey E, McBain CJ (2000) Differential mechanisms of transmission at three types of mossy fiber synapse. *J Neurosci* 20:8279–8289. [CrossRef Medline](#)

- Varodayan FP, Soni N, Bajo M, Luu G, Madamba SG, Schweitzer P, Parsons LH, Roberto M (2016) Chronic ethanol exposure decreases CB1 receptor function at GABAergic synapses in the rat central amygdala. *Addict Biol* 21:788–801. [CrossRef Medline](#)
- Vinod KY, Yalamanchili R, Xie S, Cooper TB, Hungund BL (2006) Effect of chronic ethanol exposure and its withdrawal on the endocannabinoid system. *Neurochem Int* 49:619–625. [CrossRef Medline](#)
- Vinod KY, Kassir SA, Hungund BL, Cooper TB, Mann JJ, Arango V (2010) Selective alterations of the CB1 receptors and the fatty acid amide hydrolase in the ventral striatum of alcoholics and suicides. *J Psychiatr Res* 44:591–597. [CrossRef Medline](#)
- Volkow ND, Hampson AJ, Baler RD (2017) Don't worry, be happy: endocannabinoids and cannabis at the intersection of stress and reward. *Annu Rev Pharmacol Toxicol* 57:285–308. [CrossRef Medline](#)
- Wang H, Lupica CR (2014) Release of endogenous cannabinoids from ventral tegmental area dopamine neurons and the modulation of synaptic processes. *Prog Neuropsychopharmacol Biol Psychiatry* 52:24–27. [CrossRef Medline](#)
- Weinberg J, Sliwowska JH, Lan N, Hellems KG (2008) Prenatal alcohol exposure: foetal programming, the hypothalamic-pituitary-adrenal axis and sex differences in outcome. *J Neuroendocrinol* 20:470–488. [CrossRef Medline](#)
- Xu C, Shen RY (2001) Amphetamine normalizes the electrical activity of dopamine neurons in the ventral tegmental area following prenatal ethanol exposure. *J Pharmacol Exp Ther* 297:746–752. [Medline](#)