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Endocannabinoid-Dependent Long-Term Depression of Ventral Tegmental Area GABA Neurons

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Endocannabinoid-Dependent Long-Term Depression
of Ventral Tegmental Area GABA Neurons

Jared Mark Weed

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science

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ABSTRACT

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Master of Science

GABA neurons in the ventral tegmental area of the midbrain are important components in the brain's reward circuit. Long term changes in this circuit occur through the process of synaptic plasticity. It has been shown that high frequency stimulation, as well as treatment with endocannabinoids, can cause GABA neurons in the ventral tegmental area to undergo long term depression, a form of synaptic plasticity that decreases excitability of cells. The present study elaborates on the mechanism whereby high frequency stimulation can result in long term depression of ventral tegmental area GABA neurons. Using the whole cell patch clamp technique in acute brain slices, we recorded excitatory currents from ventral tegmental area GABA neurons in GAD-GFP expressing CD1 mice and observed how the excitatory currents changed in response to different treatments. We confirm that high frequency stimulation causes long term depression, and the cannabinoid type 1 receptor antagonist AM-251 blocks this effect. Long term depression is also elicited by treatment with the cannabinoid type 1 receptor agonist 2-arachidonylglycerol. It is inconclusive whether treatment with 2-arachidonylglycerol occludes further long term depression by high frequency stimulation. We also demonstrate that activation of group I metabotropic glutamate receptors by DHPG produces long term depression. These results support the model that at these excitatory synapses, high frequency stimulation causes the release of glutamate from presynaptic terminals, activating group I metabotropic glutamate receptors, causing production of 2-arachidonylglycerol. 2-arachidonylglycerol in turn acts on presynaptic cannabinoid type 1 receptors to decrease release of glutamate onto GABA neurons. This model can be tested by further research, which should include cannabinoid type 1 receptor knockout mice. This study provides more insight into how drugs of abuse such as tetrahydrocannabinol, the active component of marijuana that activate cannabinoid type I receptors, can corrupt the natural reward mechanisms of the brain.

Key words: VTA, GABA, plasticity, LTD, electrophysiology, endocannabinoids, 2-AG

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INTRODUCTION

Addiction is a disorder in which an organism's natural reward system is usurped by substances or behaviors. The effects of addiction have a negative impact on both society and individuals. As of 2010, an estimated 23.1 million Americans were in need of treatment related to drug or alcohol abuse (Substance Abuse and Mental Health Services Administration, 2010). In cases of severe addiction, drug abusers may neglect their basic survival needs and even behave recklessly in order to obtain drugs. Indeed, a large proportion of crimes in the United States involve drugs directly, are committed in order to obtain drugs, or are committed while under the influence of psychoactive substances (Dorsey, Zawitz, & Middleton, 2005; Reedt & Reimer, 2012). The ramifications of addictive behavior highlight the need for the reward system to be understood at neural network, cellular, and molecular levels.

The Mammalian Reward System

Highly evolved organisms have developed sophisticated reward systems to enhance the chances for the survival of their species. For humans, this system promotes goal-directed behaviors that meet dietary and sexual needs. It also allows humans to adapt to an environment by attaching salience to novel rewarding stimuli and by mediating feelings of pleasure. The exact neural correlates for these various functions of the reward system are debatable, but the mesocorticolimbic dopamine system is key to our current understanding of how reward is processed in the brain (Robinson & Berridge, 2008; R. Wise, 2008).

The Mesocorticolimbic System

The mesocorticolimbic system is comprised of the ventral tegmental area (VTA) of the midbrain and its principal targets: the nucleus accumbens, the prefrontal cortex, the amygdala, and the hippocampus (Kent C Berridge, 2007; Kelley, 2004). The rodent VTA consists of cells

that produce dopamine (DA) (55%), cells that produce gamma-amino butyric acid (GABA) (23%), and cells that produce glutamate (up to 22%)(Margolis, Toy, Himmels, Morales, & Fields, 2012). In response to rewarding stimuli, VTA DA cells release DA into the VTA and its projections. This increase of DA can be elicited by natural reward stimuli as well as by drugs of abuse (Fields, Hjelmstad, Margolis, & Nicola, 2007; Schultz, 1998). Rats will compulsively self-administer drugs of abuse directly into the VTA, and goal-directed behaviors can be blocked by intracranial VTA DA depletion via 6-hydroxydopamine (K C Berridge & Robinson, 1998; McBride, Murphy, & Ikemoto, 1999). Furthermore, rats will also self-stimulate by pushing a lever that causes current to pass through an electrode intracranially placed into the VTA (R. a Wise, 1996).

Cellular Mechanisms of Reward

At the cellular level, different drugs of abuse have different mechanisms of action, but they share the common effect of increasing the amount of DA present at synapses of dopaminergic cells. Cocaine acts by blocking DA transporters (DATs), preventing re-uptake of DA and keeping it in the synaptic cleft. Methamphetamine more potently increases synaptic DA levels by causing DATs to reverse transport DA into the synapse itself. Nicotine acts on all cells in the VTA to increase dopaminergic neuron firing rates. Ethanol has broad effects in the CNS, so its mechanism in the DA system is not as defined. Δ 9-tetrahydrocannabinol (THC), the active component in marijuana, acts on cannabinoid receptor 1 (CB1) in the VTA to attenuate the inhibitory inputs of GABA neurons on DA neurons. (Pierce & Kumaresan, 2006) This releases dopamine neurons from inhibitory regulation, or disinhibits them, allowing them to release more dopamine.

Synaptic Plasticity: A mechanism for long-term changes in brain circuitry

These aforementioned mechanisms help to explain the immediate actions of drugs of abuse on reward, but fail to account for the long-term addictive effects of drug use. The study of synaptic plasticity, the neuronal basis for long-term connection changes in the brain, has helped to elucidate how these long-term changes happen in the mesocorticolimbic system. Synaptic signaling can be strengthened through processes of long term potentiation (LTP) or weakened through processes of long term depression (LTD). Synaptic plasticity can occur by high levels of calcium entry via activated N-Methyl-D-Aspartate glutamate receptors (NMDARs), resulting in post-synaptic LTP, in which non-NMDA glutamate receptors are trafficked to the synapse. Alternatively, either low levels of calcium entry via activated NMDARs or activation of mGluR1 can result in post-synaptic LTD, in which non-NMDA receptors are internalized (Malenka & Bear, 2004). Presynaptic LTD can also occur, most commonly via presynaptic cannabinoid (CB) receptors. In response to stimulation of mGluR1/5 receptors, the postsynaptic neuron manufactures endocannabinoids (eCBs) such as anandamide or 2-arachidonoylglycerol (2-AG), which retrogradely activate CB receptors and cause less presynaptic neurotransmitter release (Kauer & Malenka, 2007). Another type of presynaptic LTD, which has been shown to operate in the hippocampus, occurs via the TRPV1 channel. Unlike CB receptors, which are metabotropic, the TRPV1 channel is ionotropic and conducts Ca^{2+} ions which can act as second messengers in the presynaptic terminal to decrease neurotransmitter release (Gibson, Edwards, Page, Van Hook, & Kauer, 2008; Jensen & Edwards, 2012).

Synaptic Plasticity in the Mesocorticolimbic System

NMDAR-mediated synaptic plasticity has been well studied in the mesocorticolimbic system, and especially on dopaminergic cells. Early evidence that synaptic plasticity was

involved in the mesocorticolimbic system was that co-administration of the NMDA receptor antagonist MK-801 with amphetamine in the ventral striatum of rats failed to induce the behavioral sensitization to amphetamine that the control rats displayed (Wolf, White, & Hu, 1994). In the VTA, nicotine-induced LTP of glutamatergic-dopaminergic synapses was blocked by the NMDAR antagonist APV (Mansvelder & McGehee, 2000). Soon after, it was shown that mouse VTA Dopaminergic cells undergo potentiation that lasts 5 days after a single *in vivo* exposure to cocaine, and this potentiation can be prevented by co-administration of MK-801 (Ungless, Whistler, Malenka, & Bonci, 2001). It was further demonstrated that this form of plasticity required the action of the endogenous molecule orexin, which is elsewhere involved with feeding behavior (Borgland, Taha, Sarti, Fields, & Bonci, 2006). These results together demonstrate that NMDAR-mediated LTP operates in the mesocorticolimbic system in both normal reward function and in the addictive state.

Synaptic activity in VTA DA neurons can also be modulated by eCBs. eCBs work in conjunction with D2 receptor activation to produce depolarization-induced suppression of excitation (DSE) at glutamatergic synapses on DA cells (Melis *et al.*, 2004). Consistent with this observation, it was recently found that eCB signaling inhibited induction of LTP of DA cells, and this inhibition was blocked in the presence of CB1 antagonists (Kortleven, Fasano, Thibault, Lacaille, & Trudeau, 2011). It has also been shown that LTD at inhibitory synapses can be elicited by cocaine administration paired with 10 Hz stimulation, and this inhibitory LTD (I-LTD) can be blocked by the CB1 receptor antagonist AM-251 or in CB1 knockout mice. This eCB-mediated I-LTD depended on both D2 receptor and group I mGluR activation, as would be expected for an eCB-dependent pathway (Pan, Hillard, & Liu, 2008a). Further study showed that D2 receptor activation blocks neurotransmitter release via presynaptic protein kinase A (PKA)

signaling. PKA signaling occurs downstream of CB1 activation, so somehow D2 activation is able to modulate CB1 receptor activity to facilitate the increase in presynaptic PKA signaling (Pan, Hillard, & Liu, 2008b). These findings collectively show that both excitatory and inhibitory transmission onto VTA dopaminergic cells can be modified via eCB activity.

Involvement of VTA GABA Neurons in the Mesocorticolimbic System

However, while VTA DA neurons have been abundantly researched, VTA GABA neurons have been largely ignored until recently. As excitement about researching these neurons has increased, researchers have discovered how they play an important part in the mesocorticolimbic reward system. Compelling evidence suggests that VTA GABA neurons are responsible for the brain stimulation reward response seen in the self-stimulation paradigm in rats (Lassen *et al.*, 2007). It was further found that optogenetic activation of VTA GABA neurons inhibits VTA DA neurons and precludes attenuates reward consumption in mice (van Zessen, Phillips, Budygin, & Stuber, 2012). Optogenetic activation of VTA GABA neurons also facilitates conditioned place aversion (Tan *et al.*, 2012).

The GABA neurons in the VTA are locally connected together via gap junctions (Allison *et al.*, 2006), and regulate activity of both local DA neurons and project to neurons in other areas of the mesocorticolimbic system (van Zessen *et al.*, 2012). Synaptic plasticity in VTA GABA neurons is not well-studied, but inroads are being made to understand the process. It was recently shown that methamphetamine administration in mice causes GABA_B receptors and G protein-gated inward rectifying potassium (GIRK) channels to be internalized in VTA GABA cells, causing the neurons to become more excitable (Padgett *et al.*, 2012). As the study of VTA GABA neurons becomes easier, more mechanisms governing plasticity of these neurons will be uncovered.

Involvement of Endocannabinoids in VTA GABA Neuron Plasticity

Unpublished data collected in this lab show that eCBs play an important part in the synaptic plasticity of VTA GABA neurons. Using mice that were engineered to co-express glutamic acid decarboxylase 67 (GAD67) and green fluorescence protein (GFP) (Tamamaki *et al.*, 2003), it was shown that these GABA neurons exhibit LTD in excitatory currents in response to high frequency stimulus (HFS) in brain slices. This LTD was also elicited by bath application of the endocannabinoid and CB1 agonist R-methanandamide, and this drug occluded further LTD by subsequent HFS. To rule out another possible endocannabinoid receptor, TRPV1, it was demonstrated that the TRPV1 antagonist capsazepine failed to block LTD.

Mechanisms of LTD in VTA GABA Neurons

These findings give a clearer picture of how synaptic plasticity occurs in VTA GABA cells, but more research needs to be done to further characterize the pathways at work in these synapses. The present study shows that CB1 activation with 2-arachidonylglycerol (2-AG) causes LTD in VTA GABA neurons, most likely in a presynaptic manner. However, long-term CB1 activation with 2-AG does not prevent additional HFS-LTD from occurring. We also show that activation of type I mGluRs with DHPG results in LTD of VTA GABA neurons. Although CB1 activation may not be completely responsible for the effects of HFS-LTD, it is necessary for HFS-LTD to occur. Because type I mGluR activation can lead to eCB production such as 2-AG, mGluRs provide a plausible mechanism whereby HFS can lead to CB1 receptor-mediated LTD.

MATERIALS AND METHODS

Slice Preparation

GAD67-GFP mice were anesthetized using isoflurane and decapitated. The brain was quickly removed and horizontal slices 220 μ m thick were obtained using a vibratome. Slices were cut at the level of the anterior commissure in Artificial Cerebrospinal Fluid (ACSF) containing in mM: 220 Sucrose, 3KCl, 1.25 NaH₂PO₄, 25 NaH₂CO₃, 12 MgSO₄, 10 dextrose, 0.2 CaCl₂, saturated with 95% O₂/5% CO₂ (pH 7.3). After sectioning slices were removed and incubated in ACSF containing 119 NaCl, 26 NaHCO₃, 2.5 KCl, 1.0 NaH₂PO₄, 2.5 CaCl₂, 1.3 MgSO₄ and 11 dextrose, saturated with 95% O₂/5% CO₂ (pH 7.4) warmed to 37 °C for at least 30 minutes. Slices were then incubated for at least one hour at room temperature before being transferred to the recording chamber.

Electrophysiology

When transferred to the recording chamber brain slices were continuously perfused with oxygenated ACSF warmed to 32-34 °C. Recording electrodes were obtained using a Model P-1000 Flaming/Brown Micropipette Puller. The resistances of recording pipettes were kept between the values of 2-4Mohms. Recording pipettes were filled with an intracellular solution consisting of in mM: 117 cesium gluconate, 2.8 NaCl, 5 MgCl₂, 20 HEPES, 2 ATP, 0.3 GTP, 1 QX-314, and 0.6 EGTA. In all experiments picrotoxin (100 μ M) was added to the ACSF bath in order to block GABA_A receptors. The VTA was identified as immediately lateral to the fasciculus retroflexus in horizontal slices at the level of the anterior commissure. In EPSC experiments a bipolar stainless steel stimulating electrode was placed in proximity of fluorescent GABA neurons in order to activate local axon terminals. EPSCs were invoked at 0.1Hz with a paired pulse 50ms apart. Fluorescent neurons were patched in the whole cell configuration and

clamped at -65mV. EPSCs were recorded and amplified using an axoclamp 700B amplifier (axon instruments), and low-pass filtered at 4 kHz. Amplified EPSCs were digitally sampled to a PC at 20kHz using pClamp 10.2 Clampex software (Molecular Devices). The input resistance and series resistance were recorded throughout the experiments. If input resistance changed by more than 10% during the experiment it was discarded. GAD67-GFP cells were identified using an Olympus USH-1030S fluorescent light source.

Drugs

AM-251, 2-AG, and DHPG were purchased from Tocris Bioscience. AM-251 and 2-AG were dissolved in DMSO (not more than 0.1% DMSO in final solution), and DHPG was dissolved directly into the ACSF used for experiments. Picrotoxin was purchased from Tocris Bioscience and was present in ACSF at a concentration of 100 μ M in order to isolate excitatory currents in the recorded cells. To confirm isolation of glutamate-gated excitatory currents, APV (Tocris Bioscience) and CNQX (Abcam Inc.) were used in order to block glutamate receptors.

Data Analysis

EPSC amplitudes were measured relative to pre-stimulation baseline currents for each sweep recorded from the cell. The EPSC amplitudes were obtained from raw data using pClamp 10.2 Clampfit software (Molecular Devices). EPSC amplitudes from 6 sweeps were averaged together into 1-minute bins, and were normalized to the amplitudes obtained during the 5 minutes before conditioning. To determine whether the EPSC amplitudes significantly differed between pre-conditioning and post-conditioning epochs, a two sample (unequal variance) two-tailed t-test was performed. The averages of the normalized EPSCs were compared between the last five minutes of the pre-conditioning epoch and minutes 16-20 of the post-conditioning epoch. Statistical significance was determined with an α level of 0.05. 95% Confidence intervals

are reported using standard error as the measure of variability. All calculations were performed using Microsoft Excel and graphs were created using Origin 7.5.

Paired pulse ratio (PPR; $EPSC_2/EPSC_1$) and coefficient of variation (CV) analysis was performed for several sets of experiments. Experiments in which EPSC amplitude was depressed by more than 10% were included in PPR and $1/CV^2$ analysis (Gibson *et al.*, 2008). For each experiment to be analyzed, PPR and CV were calculated within 5-minute epochs of 30 EPSCs each. The first epoch was the 5 min immediately prior to conditioning and the second epoch was between 14-19 minutes. PPR for each epoch was calculated by dividing $EPSC_2$ from the paired pulse by $EPSC_1$. $1/CV^2$ was calculated for each epoch by dividing the squared mean EPSC amplitude by the variance of the EPSC amplitudes. Because the results obtained were not normally distributed, distribution-free nonparametric inferential statistics were used to determine significance. The Wilcoxon matched-pairs signed-ranks test was performed using JMP statistical analysis software, and significance was determined using an α level of 0.05.

Statement of Animal Model

Male CD1 mice (ages: p18-p24) expressing GFP on the GAD67 promoter were used in order to distinguish GABA neurons from other neuron types in the VTA (Tamamaki *et al.*, 2003). The VTA lacks the easily identifiable structure of other well-studied brain areas in plasticity, such as the hippocampus and cerebellum. Using these GAD-GFP mice, we were able to easily tell which cells produced GABA by detecting fluorescence from the GFP that was concurrently produced in GABA cells in this breed of mice. It is possible that affixing GFP to one of the GAD67 alleles may reduce expression of GAD67, resulting in a decreased level of GABA production (unpublished data). Nevertheless, the effects of this possible defect were diminished by the fact that only excitatory (non-GABA_A) currents are recorded in this study.

RESULTS

Recorded EPSCs Stay Constant with Time and Are Generated by Glutamate-gated Ion Channels

Recording from cells using the whole cell patch clamp technique could potentially weaken cellular health over time. A rundown effect may be observed in EPSC amplitudes over time as a result. Because we were examining LTD, the rundown effect potentially may have biased our results towards LTD. In addition, many experiments entailed measuring differences in EPSCs in response to switching from a control solution (ACSF with 100 μ M picrotoxin) to a solution with a conditioning drug. Switching between solutions potentially introduces confounding factors (such as bubbles generated from the switching process or from differences in solution temperatures or oxygen saturation) that can also bias the results.

To correct for these potential confounders, a rundown experiment was performed in which slices were bathed in control ACSF and after achieving a 5-minute baseline of recording EPSCs, the bath solution was switched to a different container containing the same ACSF. The experiment continued for as long as the cell opening remained intact. 40 minutes after switching the bath solution, the EPSC amplitudes showed minimal signs of rundown (Figure 1A).

Next, we wanted to confirm that EPSCs were indeed glutamate mediated and not by another neurotransmitter. After obtaining a baseline recording in control ACSF, the AMPAR antagonist CNQX (10 μ M) and NMDAR antagonist APV (50 μ M) were added to the bath for 10 minutes to block ionotropic glutamate receptors. Within two minutes of applying the glutamate receptor antagonists, EPSC amplitudes were extinguished (Figure 1B). After reapplying control ACSF to the bath, EPSC amplitude recovered slowly to above 50%. This showed that the EPSCs obtained in the control ACSF were solely mediated by ionotropic glutamate receptors.

High Frequency Stimulation Causes LTD in VTA GABA Neurons

Synaptic plasticity can be mediated by many different mechanisms, and is often achieved in response to changes in frequencies of neuronal activation. High frequency stimulation (HFS) is a well-known method of inducing both LTP and LTD. In the CA1 region of the hippocampus, HFS has is able to cause LTD in stratum radiatum interneurons and LTP in pyramidal cells (McMahon & Kauer, 1997). In the VTA, HFS (2 pulses of 100 Hz for 1 sec intervals given 20 seconds apart) has been shown to induce LTP in field potentials (Nugent, Hwong, Udaka, & Kauer, 2008). Prior research performed in this lab identified that high frequency stimulation causes LTD in VTA GABA neurons, and subsequent experiments have confirmed this effect (Figure 2A). The data we obtained demonstrate a significant difference in EPSC amplitudes due to HFS ($p=0.0098$; 95% C.I.: $18.1\pm 3.2\%$; $n=17$).

Changes in paired pulse ratio (PPR) and coefficient of variation (CV) concomitant with LTD indicate a presynaptic mechanism for plasticity. In 7 of 9 cells analyzed the PPR increased and in 8 of 9 cells $1/CV^2$ decreased (Figure 2B). This is highly suggestive of a presynaptic mechanism, but the difference is not statistically significant (PPR: $p=0.203$, $n=9$; $1/CV^2$: $p=0.074$, $n=9$).

CB1 Receptor Antagonist AM-251 Blocks HFS-LTD

Our research demonstrates that VTA GABA neurons not only undergo LTD in response to HFS, but also in response to cannabinoids. We found that R-methanandamide (an agonist for CB1, TRPV1 and other putative eCB receptors) caused LTD. To see if this effect was mediated by TRPV1, HFS-LTD was attempted in the presence of the TRPV1 antagonist capsazepine (10 μ M). It was found that LTD still occurred in the presence of capsazepine (Figure 3; $p=0.016$, 95% C.I.: $0.094\pm 0.072\%$, $n=9$). Blockade of CB1 receptors with AM-251 (2 μ M), however,

showed a link between cannabinoids and HFS-LTD (Figure 4). Attempting HFS-LTD failed in the presence of AM-251 ($p=0.81$; 95% C.I.: $2.4\pm 21.5\%$; $n=3$). This suggests that CB1 receptor activation is required for HFS-LTD in VTA GABA neurons.

CB1 Receptor Agonist 2-Arachidonylglycerol Causes LTD, but Does Not Occlude HFS-LTD

While CB1 receptors may be required for HFS-LTD, we wanted to see whether CB1 activation alone was sufficient for LTD. We made sure to choose an agonist that was not only specific to the CB1 receptor, but is also found endogenously in the VTA. RT-PCR analysis in our lab (unpublished data) shows that VTA GABA neurons express DAG lipase, an enzyme that is required for the production of the eCB 2-arachidonylglycerol (2-AG). Because 2-AG is plausibly manufactured by VTA GABA neurons, we attempted to achieve LTD via 2-AG application. We found that applying 2-AG (25 μM) to the bath for 10 minutes elicits moderate but significant LTD (Figure 5A; $p=0.035$; 95% C.I.: $22.0\pm 7.8.0\%$; $n=6$). PPR analysis showed that each of the 4 analyzed cells exhibited an increase in PPR and decrease in $1/\text{CV}^2$ (Figure 5B). Not enough experiments were analyzed, however, to establish statistical significance ($p=0.125$; $n=4$ for both PPR and $1/\text{CV}^2$). While the current evidence suggests a presynaptic mechanism for 2-AG mediated LTD, replication of these results is needed to establish statistical significance.

Because the results of the AM-251 experiments suggest that CB1 activation is required for HFS-LTD, we attempted to see if LTD caused by 2-AG application occludes further HFS-LTD. Acute brain slices were incubated in 50 μM 2-AG to saturate CB1 receptors, then maintained CB1 receptor activation in the recording bath by perfusing the slices with ASCF containing 25 μM 2-AG. Not enough full-length experiments were obtained to perform statistical analysis, but initial results show that 2-AG does not fully occlude HFS-LTD (Figure 6).

Type I mGluR Agonist DHPG Causes LTD

According to our results (especially our AM-251 results), it is plausible that HFS can cause release of 2-AG from VTA GABA neurons to result in presynaptic LTD. Metabotropic glutamate receptors (mGluRs) are viable candidates for transducing this signal because when activated they can stimulate phospholipase C (PLC), resulting in the precursor molecule for 2-AG production (Hashimotodani, Ohno-Shosaku, Watanabe, & Kano, 2007; Rao, Hatcher, & Dempsey, 2000). To test this possibility, we measured the effect of 10 minutes of bath DHPG application (100 μ M) on EPSC amplitude in VTA GABA neurons. Application of DHPG results in quick and robust LTD (Figure 7A; $p=0.0019$; 95% C.I.: $34.7\pm 8.3\%$; $n=5$). This LTD coincided with an increase in PPR in 3 of 4 analyzed cells and a decrease in $1/CV^2$ in all 4 cells (Figure 7B). Although these differences in PPR and $1/CV^2$ were not statistically significant (PPR: $p=0.250$, $n=4$; $1/CV^2$: $p=0.125$, $n=4$), this does not rule out a presynaptic mechanism of LTD. Further experiments may establish statistical significance.

DISCUSSION

Plasticity in VTA GABA Cells

We report that HFS in the VTA causes LTD of excitatory currents in GABA neurons. This provides new and perhaps unprecedented information in the context of previous plasticity research performed in the VTA. For at least 14 years, HFS protocols have been used in the VTA to examine plasticity of DA and GABA neurons. However, only two other studies that we know of have examined synaptic plasticity of excitatory synapses onto GABA cells. In one study a pairing protocol (+10 mV, 200 stimuli at 1 Hz) failed to induce either LTP or LTD (Bonci & Malenka, 1999). We report here that a different protocol, HFS (2 trains of 100 Hz for 1s, each train 20s apart) does in fact cause depression of EPSCs in VTA GABA neurons. This is a novel type of synaptic plasticity never before identified. In another study HFS caused NMDAR-dependent LTP of VTA field potentials and in VTA DA neurons though HFS did not produce either LTP or LTD in VTA GABA neurons (Nugent *et al.*, 2008). This contradicts the principle finding of our investigation. This discrepancy can't be explained by a difference in stimulation protocol because both groups used identical HFS protocols, and this can't be explained by circuit-wide effects of ionotropic receptor antagonists, because picrotoxin (100 μ M) was present in the bath in both sets of experiments. One main difference is that our data were the result of 17 HFS experiments whereas Nugent *et al.* analyzed 4 experiments, and in fact we do not see LTD in every single GABA cell. Another potential difference is that each group could have been recording from different populations of neurons. For our experiments, we defined the VTA as lateral to the fasciculus retroflexus, which could be a different area from where Nugent *et al.* collected their data. Furthermore, we used genetic methods to positively identify VTA GABA neurons, whereas Nugent *et al.* used I_h current to distinguish between DA neurons and non-DA

neurons (that were assumed to be GABAergic), which has since been reported as a poor way to distinguish between GABA and DA neurons (Chieng, Azriel, Mohammadi, & Christie, 2011). Therefore, our data of positively identified GABA neurons we feel confirm that indeed LTD of EPSCs is exhibited by VTA GABA neurons in response to HFS.

Plasticity of GABAergic Synapses and Disinhibition

We theorize that LTD of VTA GABA neurons would cause reduced GABA release onto VTA DA neurons, which we term as disinhibition. Our examination of excitatory input on VTA GABA neurons fit this inhibition model. Several other studies have examined plasticity of inputs to GABA and DA neurons, which can be compared to our overall finding that GABA cells have depressed excitatory input in response to HFS. For example, Nugent *et al.* reported that HFS (the same protocol used in our study) caused LTP of GABA_A currents, albeit onto DA neurons rather than GABA neurons, in a presynaptic and heterosynaptic fashion, and that this LTP_{GABA} could be blocked by *in vitro* or *in vivo* administration of morphine (Nugent, Penick, & Kauer, 2007).

This finding of Nugent *et al.* (2007) does not necessarily fit with the disinhibition model that we describe is at work in the VTA. It should be noted, however, that in order for Nugent *et al.* to isolate GABA_A currents they bathed slices in DNQX (AMPA antagonist; 10 μ M) and strychnine (glycine receptor antagonist; 1 μ M), whereas we bathed slices in picrotoxin (100 μ M) to isolate excitatory currents. The differences we see in our data compared to Nugent *et al.* could be purely due to circuit-wide effects of bath application of drugs that could have broad effects on the slices. Also, if HFS does cause a decrease in GABA release from VTA GABA neurons (via a heterosynaptic mechanism), this could be a sort of built-in compensatory mechanism for the decrease in excitability that HFS produces in VTA GABA neurons according to our data. This interpretation fits results reported elsewhere, in which HFS failed to induce LTP of field

potentials in rat brain slices devoid of ionotropic receptor antagonists (Liu *et al.*, 2010).

Alternatively, it should be considered that the LTP_{GABA} noted by Nugent *et al.* (2007) is solely synapse specific, causing increased GABA release onto dopamine neurons directly, and not dependent specifically on the activity level of the GABA cells. Therefore, this does not rule out the possibility that depression of excitatory input to GABA cells that we noted could be occurring simultaneously with LTP_{GABA}; where overall depressed GABA neuron activity occurs concomitantly with enhanced GABA release.

Later findings of Nugent *et al.* (2008) actually partially fit with the disinhibition model and our data. However, they describe the HFS-LTP of field potentials as NMDAR-dependent, while we describe HFS-LTD of VTA GABA neurons as CB1 receptor dependent. In truth, both mechanisms may be at work. It was reported earlier that VTA GABA neurons have a basal AMPA/NMDA ratio of 0.68 ± 0.1 , which indicates that NMDARs are abundant at GABA neuron excitatory synapses (Bonci & Malenka, 1999). We did not assess whether AMPA/NMDA ratio changes in VTA GABA neurons in response to HFS; nor did we evaluate whether HFS-LTD occurs in the presence of APV, but doing these experiments will clarify the relative roles of CB1 receptors and NMDARs in HFS-LTD.

One plasticity study of note that affirms the inhibition model came from the group led by Christian Lüscher. They demonstrated that medium spiny neurons (MSNs) originating in the nucleus accumbens formed inhibitory synapses onto VTA GABA neurons, and that optogenetic activation of these MSNs inhibited GABA neuron firing, thereby increasing VTA DA neuron firing. They further showed that selective HFS of the MSNs (4 trains of 100 pulses at 50 Hz, each initiated 20s apart) potentiated this inhibitory input onto VTA GABA neurons, and that this HFS-iLTP was occluded by *in vivo* cocaine administration (Bocklisch *et al.*, 2013). Our study

differs in the inputs and the type of currents that we investigate, but we show another way in which HFS of inputs onto VTA GABA neurons causes a long-term reduction in excitability of the GABA neurons. Furthermore, Bocklisch *et al.* show the relevance that our findings have in relation to VTA DA neurons, namely that depressing VTA GABA neuron activity will enhance VTA DA neuron activity and increase DA release. Our findings show further support for this disinhibition model.

Our study also verifies previous data obtained in this lab and further contextualizes how VTA GABA cells undergo plasticity. The simplest model that could be drawn from the results in this study is summarized in Figure 8. HFS causes high amounts of glutamate to be released from excitatory cells onto VTA GABA neurons. The glutamate then activates group I mGluRs, activating the PLC pathway, resulting in DAG lipase to generate 2-AG as a cleavage product of arachidonic acid. 2-AG then diffuses to the presynaptic terminal and activates CB1, decreasing the amount of glutamate released from the presynaptic terminal. This model fits with well-known cellular pathways (Rao *et al.*, 2000).

The data obtained in this study fit the model proposed above, but the factors that govern plasticity of VTA GABA neurons are likely much more complex. Our results do not definitively prove a causal link between HFS and mGluR activation of these neurons, nor do they demonstrate a causal link between mGluR activation and CB1 activation. Further study can demonstrate whether these associations exist. If a group I mGluR antagonist prevents HFS-LTD from occurring, and if a CB1 antagonist prevents DHPG from inducing LTD, then these links can be established.

Endocannabinoids and CB1 in the VTA

We demonstrated that CB1 activation by 2-AG causes a long-term decrease in EPSC amplitude in VTA GABA neurons, and we hypothesize that this process can aid in disinhibition VTA DA neurons. We acknowledge, however, that the CB1 receptor has many functions within the VTA that can either promote or oppose this paradigm. CB1 receptors are expressed in both glutamatergic and GABAergic terminals in the VTA that directly input to DA neurons (Mátyás, Urbán, & Watanabe, 2008). It has been reported that CB1 activation can depress IPSC amplitude in VTA DA neurons (Szabo, 2002). This supports the disinhibition model, but this mechanism of disinhibition is distinct from the one that we have found as we are examining different synapses. Another study found that CB1R agonists cause presynaptic inhibition of EPSCs in VTA DA neurons in the presence of picrotoxin (100 μ M) (Melis *et al.*, 2004). The findings of Melis *et al.* mainly reveal only how CB1 receptor activation affects the glutamatergic synapses of DA neurons themselves, and not how CB1 receptor activation affects the VTA at large.

Likewise, our study examines how CB1 receptor activation affects excitatory synapses of VTA GABA neurons. More research must be performed to see how CB1 receptor activation affects the VTA more broadly. One recent attempt at this showed that chronic *in vivo* activation of CB1 receptors using the agonist HU210 occluded a form of LFS-LTD of field EPSPS (with no ionotropic receptor antagonists) that was discovered in the VTA (Liu *et al.*, 2010). This suggests that chronic CB1 receptor activation actually depresses VTA DA neuron activity. The combination of our research and the other studies summarized here shows that CB1 receptor activation leads to reduced neurotransmitter release at many synapses in the VTA, and there are many parallel mechanisms that determine how exogenous tampering with eCB signaling can lead to addiction.

The present study shows that CB1 activation is required for HFS-LTD in VTA GABA neurons. Furthermore, the eCB 2-AG results in LTD. However, attempts at occluding HFS-LTD with prior CB1 activation showed mixed results. What factors could explain why HFS-LTD happened after CB1 receptors were activated for more than 10 minutes prior to HFS?

One explanation we considered as to why 2-AG did not consistently occlude HFS-LTD is that the cells may have become sensitized to the drug. It has been observed elsewhere that acute exposure to 2-AG can increase CB1 mRNA levels in the midbrain (Landa, Jurajda, & Sulcova, 2010). However, it is unlikely that the amount of time the slices were exposed to 2-AG was enough for synthesis and trafficking of more CB1 receptors to the glutamatergic terminals. Furthermore, there is considerable evidence in studies with monoacylglycerol lipase (MAGL) $-/-$ mice that overexposure of CB1 receptor-containing synapses to 2-AG actually desensitizes CB1 receptors to 2-AG (Chanda, Gao, Mark, & Btsh, 2010; Pan *et al.*, 2011; Schlosburg *et al.*, 2010).

The lack of occlusion we observed might more easily be explained by experimental variation. More replication of this experiment would show how VTA GABA neurons behave to this protocol on average. There is still precedent for us to potentially see occlusion of HFS-LTD by CB1 activation, because unpublished data from our lab shows that the CB1/TRPV1 agonist R-methanandamide occludes HFS-LTD. If CB1 activation causes maximal LTD, then we would expect both R-meth and 2-AG to occlude further LTD via HFS. We acknowledge the possibility that more than one plasticity mechanism may be at work at this synapse. The HFS-LTD we observed may partially be due to NMDAR activity or that of another receptor, notwithstanding that our AM-251 results show that CB1 receptor activation is required for HFS-LTD.

VTA GABA Neurons, CB1 Receptors, and Addiction

VTA GABA neurons play an important role in motivation and reward by disinhibiting VTA dopamine cells, and there are many ways in which VTA GABA neurons can be modulated in both normal activity and addictive processes. It has been reported that VTA GABA neurons disrupt reward consumption (van Zessen *et al.*, 2012) and mediate conditioned place aversion (Tan *et al.*, 2012). The current study focuses on synapses onto VTA GABA neurons themselves specifically, but not the outputs of these neurons. These GABA neurons synapse not only onto local DA neurons, but also onto neurons in the nucleus accumbens where they can alter reward and associative learning (Brown *et al.*, 2012; Johnson & North, 1992). These GABAergic terminals are then subject to further modification in order to fine-tune their firing rates. For example, drugs such as cocaine and nicotine can abolish potentiation of GABAergic inputs onto DA neurons, in turn increasing DA levels in the mesocorticolimbic system (Niehaus, Murali, & Kauer, 2010). Thus VTA GABA neurons play an important role in the reward pathway.

CB1 receptors have been demonstrated to be directly or indirectly involved in reward pathway and addiction as well. CB1 receptors are prevalent throughout the brain, and in the VTA they are present in both excitatory and inhibitory axon terminals (Kortleven *et al.*, 2011; Mátyás *et al.*, 2008; Pan *et al.*, 2008b). Although CB1 receptors are directly activated by THC, other drugs can exert their effects indirectly through CB1 receptors. Conditioned place preference (CPP) studies with rats showed that activation of CB1 in the VTA enhances the ability of morphine to induce CPP in mice, and that blocking CB1 receptors in the VTA diminishes CPP induced by morphine (Rashidy-Pour *et al.*, 2013). While morphine does not directly bind to CB1 receptors, its effects on the neural network of the VTA may result in CB1 activation, which will cause LTD in VTA GABA neurons and disinhibit VTA DA neurons. Additionally,

pharmacological inhibition of the synaptic breakdown of the eCB anandamide will enhance the increase of VTA DA release caused by cocaine administration in mice, which shows that CB1 activation enhances the effects of cocaine in the VTA (Mereu *et al.*, 2013). Therefore, future examination of the CB1-dependent plasticity reported here, in combination with various drugs of abuse, could provide a link between this pathway and addiction.

Significance

The results of the present study shed more light on how the population of VTA GABA neurons, an important hub in the brain's reward circuit, can be affected in the long term by upstream excitatory activity. This study specifically addresses a potential mechanism whereby marijuana could exert its addictive effects. This is important because although marijuana addiction has a high prevalence among illicit drugs (Kandel, Chen, Warner, Kessler, & Grant, 1997), it remains difficult to treat partly because its addictive mechanisms are poorly understood (Elkashef, Vocci, & Huestis, 2008). This study shows how both glutamatergic activity and cannabinoid receptor activation are tied to LTD of VTA GABA neurons. These events can lead to disinhibition of VTA DA neurons. The resulting increase of DA in the VTA and its projections in turn could mediate rewarding and addictive behavior.

FUTURE DIRECTIONS

The data obtained support the hypothesis that CB1 receptors are intimately involved in the plasticity of VTA GABA neurons. However, due to the lipophilic nature of drugs and endogenous molecules that bind to CB1 receptors, the results we have collected may not be definitive. Performing similar experiments on CB1 knockout mice will provide important genetic cross-validation to our current results, and may broaden our understanding of the mechanisms whereby CB1 receptors are normally activated. We have already shown that activation of group I mGluRs results in LTD of VTA GABA neurons, but we haven't directly linked mGluR activation with CB1 activation. CB1 KO mice will help to establish whether or not there is a causal link between these two types of receptors.

The results from this study also set the stage for studying THC, a mild drug of abuse (Haney, Comer, Ward, Foltin, & Fischman, 1997). Because CB1 receptors are involved in VTA GABA neuron plasticity, and these neurons are important in reward circuits, VTA GABA neurons may be important substrates that mediate the addictive effects of THC. The experiments conducted with 2-AG in this study should be replicated using THC to see if the drug can cause LTD via CB1 activation. Another experiment with more physiological importance would be to inject mice with THC and examine how the drug affects plasticity in VTA GABA neurons either 24 hours later or several days after administration. It will eventually be important to know how antagonists or modulators of CB1 receptors can mitigate the effects THC injection may have on plasticity.

FIGURE LEGENDS

Figure 1: Control EPSC Amplitudes Stay Constant over Time and Currents are mediated by Glutamate Receptors. A) EPSC Amplitudes Stay Constant over Time. Bath application of control ACSF (Same as the ACSF that was already in the bath) for 10 minutes does not cause EPSC amplitudes to diminish. EPSC amplitudes may begin to lessen after 45 minutes of establishing cell opening. Black bar indicates time period when second solution of control ACSF was present in bath. Inset: EPSC traces from whole cell recording of VTA GABA cell. Black trace is baseline and red trace is 28-30 minutes after application of control ACSF, scale bar: 100 pA, 10 ms. B) Bath application of APV (NMDAR antagonist, 50 μ M) and CNQX (AMPA antagonist, 10 μ M) completely extinguish EPSCs, and EPSC amplitude partially recovers after resuming control ACSF. This shows that the currents isolated were completely mediated by glutamate-gated ion channels. Note that all solutions of ACSF already contained the GABA_A antagonist picrotoxin (100 μ M). Black bar indicates the time period when solution containing APV and CNQX was applied to bath. Inset: EPSC traces from whole cell recording of VTA GABA cell. Black trace is baseline and red trace is 10-15 minutes after application of conditioning ACSF, scale bar: 100 pA, 10 ms.

Figure 2: High Frequency Stimulation (HFS) Mediates LTD in VTA GABA neurons, most likely in a presynaptic manner. A) VTA GABA neurons undergo LTD after HFS treatment ($p=0.0098$; $n=17$). Arrow indicates time of HFS. Error bars indicate standard error of the mean (SEM). Inset: representative EPSC traces from whole cell recordings of VTA GABA cells. Black trace is baseline and red trace is 28-30 minutes post-HFS, scale bar: 100 pA, 10 ms. B) HFS-LTD increases paired pulse ratio (PPR) (NS: $p=0.203$; $n=9$) and decreases $1/(\text{coefficient of variation})^2$ ($1/CV^2$) (NS: $p=0.074$; $n=9$). PPR increased in 7 of 9 cells and $1/CV^2$ decreased in 8 of 9 cells. Open squares indicate values from individual experiments; closed squares represent average values.

Figure 3: TRPV1 Antagonist Capsazepine Fails to Block HFS-LTD. When slices were preconditioned in ACSF containing capsazepine (10 μ M), LTD still occurred after HFS ($p=0.016$; $n=9$). Black bar indicates time period that capsazepine was present in bath. Arrow indicates time of HFS. Error bars indicate SEM. Inset: representative EPSC traces from whole cell recordings of VTA GABA cells. Black trace is baseline and red trace is 28-30 minutes after HFS, scale bar: 100 pA, 10 ms.

Figure 4: CB1 Receptor Antagonist AM-251 prevents HFS-LTD in VTA GABA neurons. Blockade of CB1 receptors by AM-251 (2 μ M) results in no significant change in EPSC amplitude ($p=0.81$; $n=3$) in response to HFS (arrow). Error bars indicate SEM. Inset: representative EPSC traces from whole cell recordings of VTA GABA cells. Black trace is baseline and red trace is 28-30 minutes after HFS, scale bar: 100 pA, 10 ms.

Figure 5: 2-AG Mediates LTD of VTA GABA Neurons Via a Presynaptic Mechanism. A) Application of CB1 agonist 2-AG (25 μ M, 0.1% DMSO) for 10 minutes induces a significant depression of EPSC amplitude ($p=0.035$; $n=6$) that is long-term. Black bar indicates the period of time that 2-AG was applied to the bath. Error bars indicate SEM.

Inset: representative EPSC traces from whole cell recordings of VTA GABA cells. Black trace is baseline and red trace is post-HFS, scale bar: 100 pA, 10 ms. B) LTD induced by 2-AG coincides with an increased PPR (NS: $p=0.125$; $n=4$) and a decreased $1/CV^2$ (NS: $p=0.125$; $n=4$). Though not statistically significant, the trend observed so far indicates a presynaptic mechanism for LTD. PPR increased in 4 of 4 cells and $1/CV^2$ decreased in 4 of 4 cells. Open squares indicate values from individual experiments; closed squares represent average values.

Figure 6: LTD Mediated by 2-AG Does Not Fully Occlude Further LTD by HFS. HFS in slices pre-conditioned with CB1 receptor agonist 2-AG (25 μ M, 0.1% DMSO) resulted in LTD. Not enough full-length experiments were collected to perform statistical analysis. Only 1 of 3 experiments showed no change in response to HFS. Black bar indicates time period during which 2-AG was present in the bath. Arrow indicates time of HFS. Error bars indicate SEM. Inset: representative EPSC traces from whole cell recordings of VTA GABA cells. Black trace is baseline and red trace is 10-15 minutes after HFS, scale bar: 100 pA, 10 ms.

Figure 7: DHPG Causes LTD in VTA GABA Neurons. A) Robust LTD occurs ($p=0.0019$; $n=5$) soon after application of DHPG (100 μ M) for 10 minutes. Black bar indicates time period that DHPG was applied to the bath. Error bars indicate SEM. Inset: representative EPSC traces from whole cell recordings of VTA GABA cells. Black trace is baseline and red trace is post-HFS, scale bar: 100 pA, 10 ms. B) LTD caused by DHPG coincides with an increased PPR (NS: $p=0.250$; $n=4$) and a decreased $1/CV^2$ (NS: $p=0.125$; $n=4$). Though not statistically significant, the trend observed so far indicates a presynaptic mechanism for LTD. PPR increased in 3 of 4 cells and $1/CV^2$ decreased in 4 of 4 cells. Open squares indicate values from individual experiments; closed squares represent average values.

Figure 8: Proposed Model for HFS-mediated LTD in VTA GABA Neurons. High frequency stimulation allows glutamate to accumulate in the synapse until activation of perisynaptic type I mGluRs occurs. Intracellular signaling downstream of mGluR activation results in synthesis of 2-AG within the VTA GABA neuron. 2-AG diffuses across the synapse to the terminal of glutamatergic neurons and activate CB1 receptors. This activation triggers intracellular signaling within the terminal that results in diminished glutamate release. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience (Kauer & Malenka), copyright 2007.

FIGURES

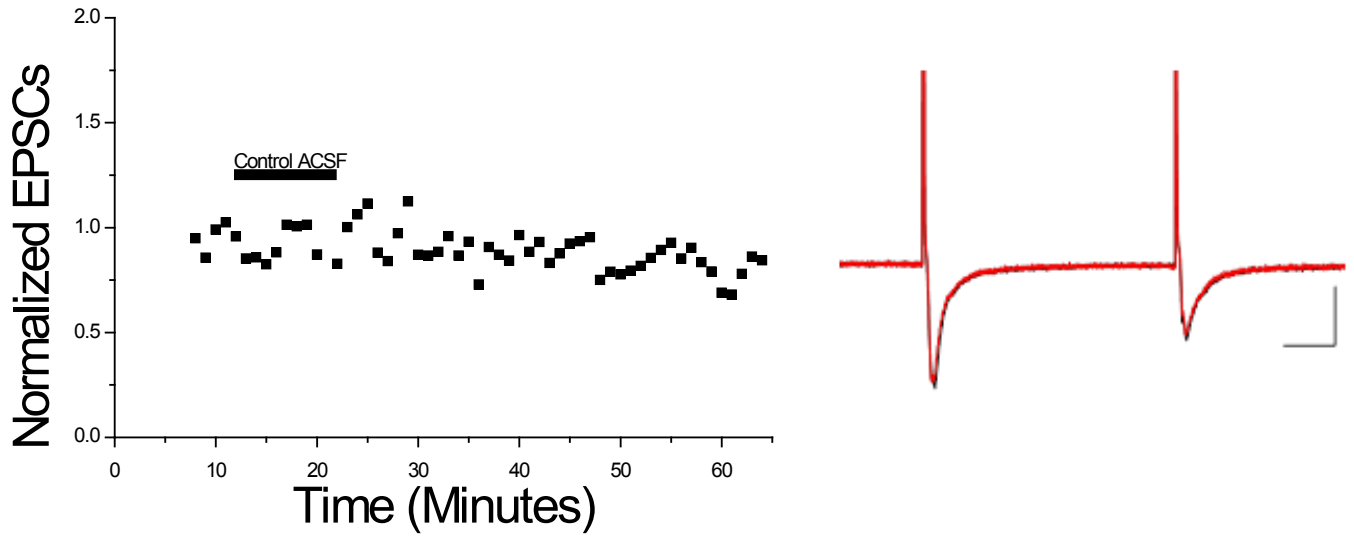


Figure 1A: EPSC Amplitudes Stay Constant Over Time.

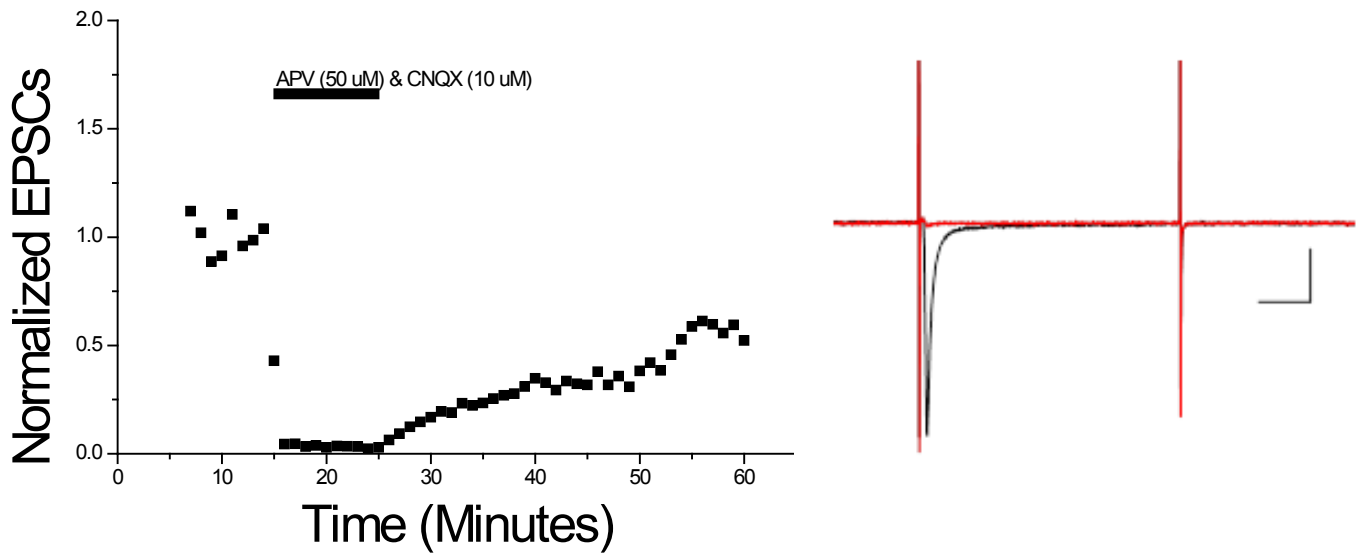


Figure 1B: EPSCs are Fully Attenuated by Antagonists APV and CNQX.

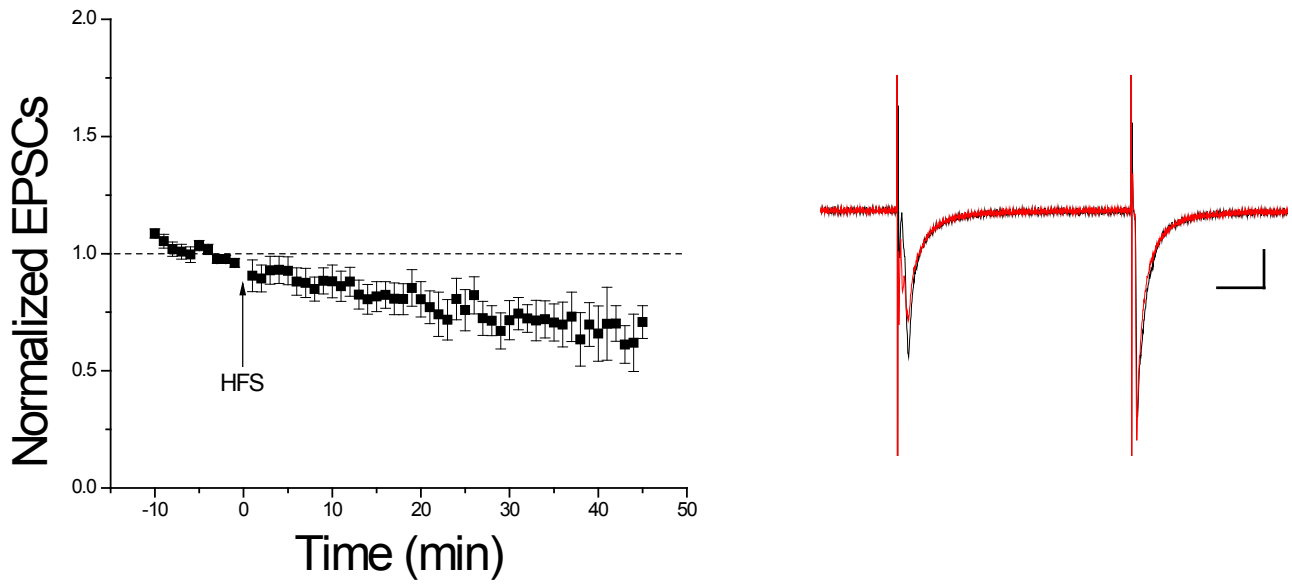


Figure 2A: High Frequency Stimulation Elicits LTD.

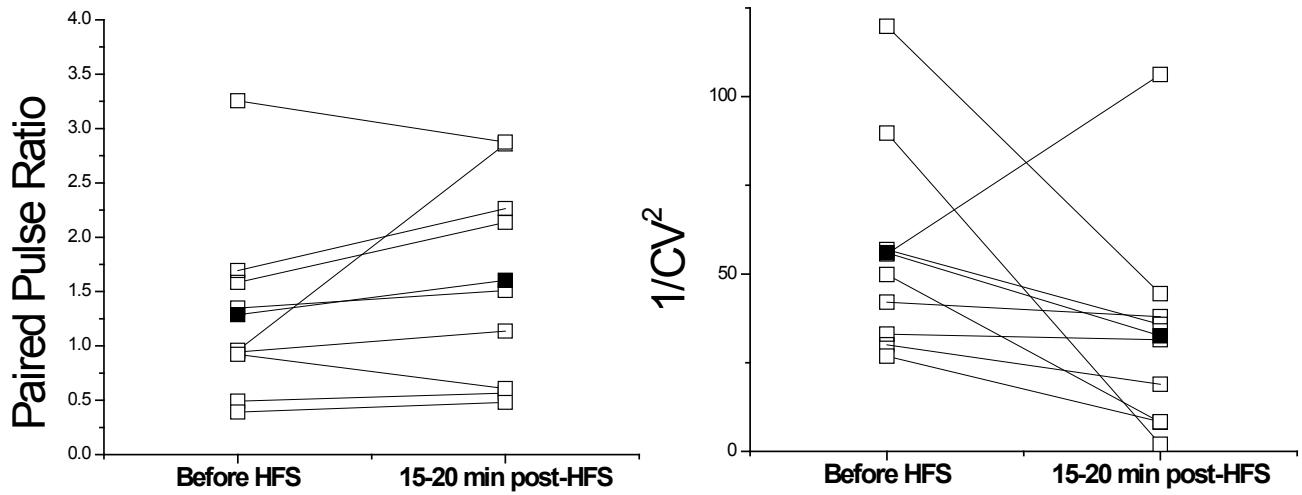


Figure 2B: HFS-LTD Increase Paired Pulse Ratio and Decrease $1/CV^2$.

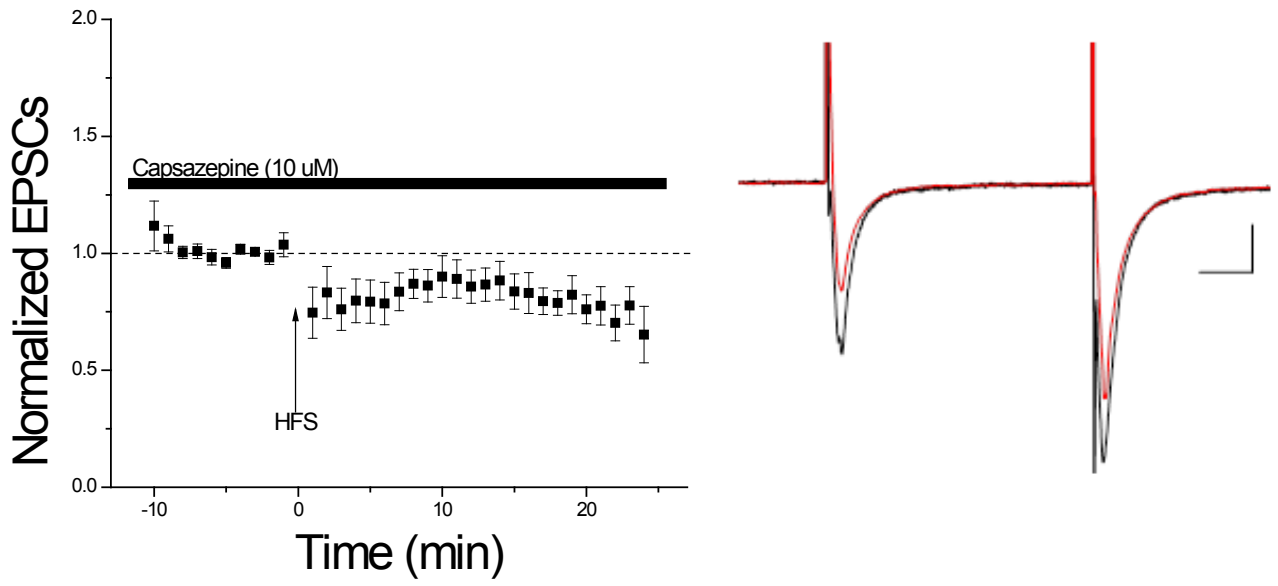


Figure 3: TRPV1 Antagonist Capsazepine Fails to Block HFS-LTD.

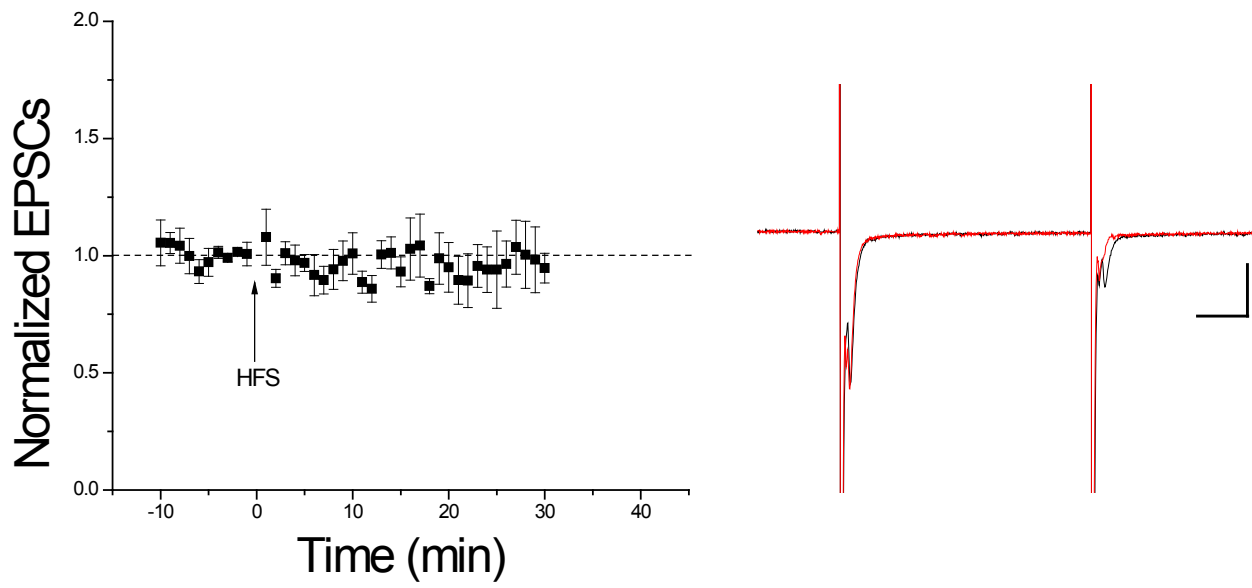


Figure 4: CB1 Receptor Antagonist AM-251 Prevents HFS-LTD.

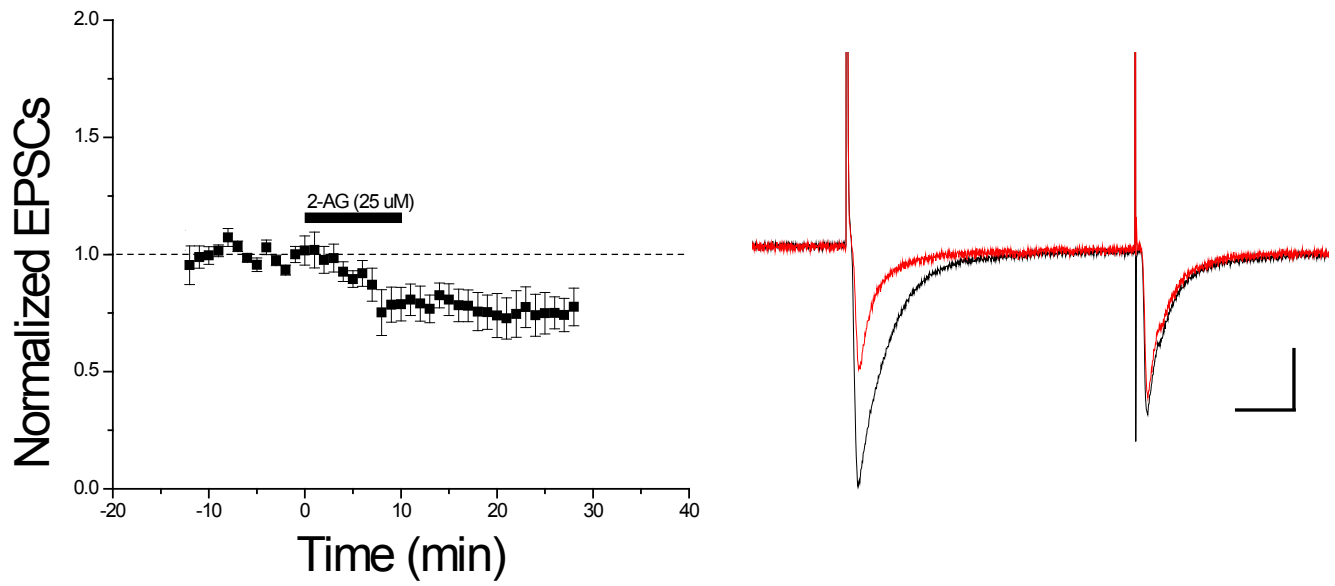


Figure 5A: CB1 Receptor Agonist 2-AG Causes LTD.

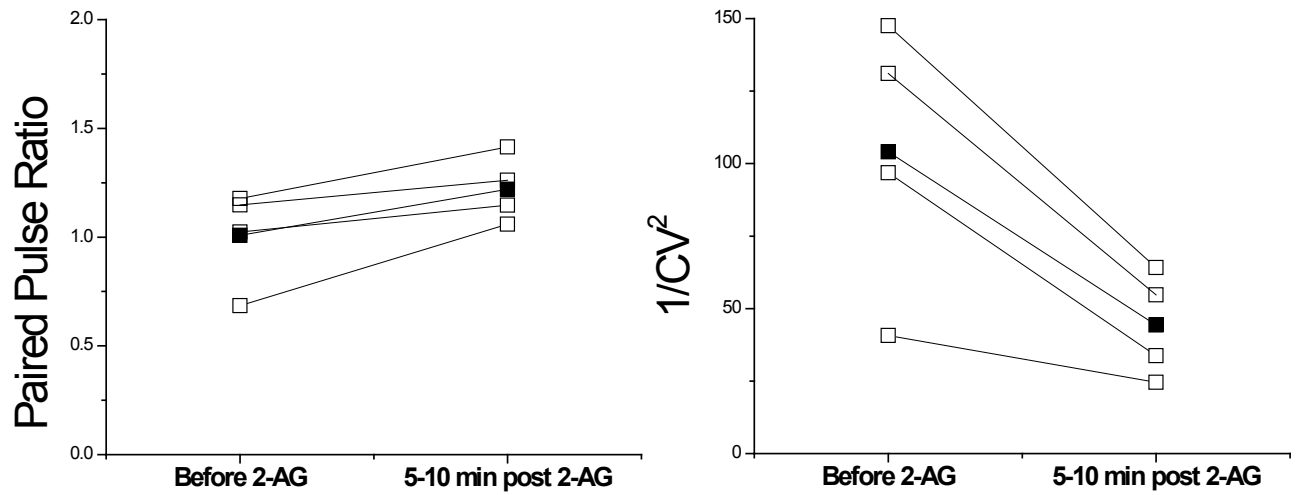


Figure 5B: 2-AG May Increase Paired Pulse Ratio and Decrease $1/CV^2$.

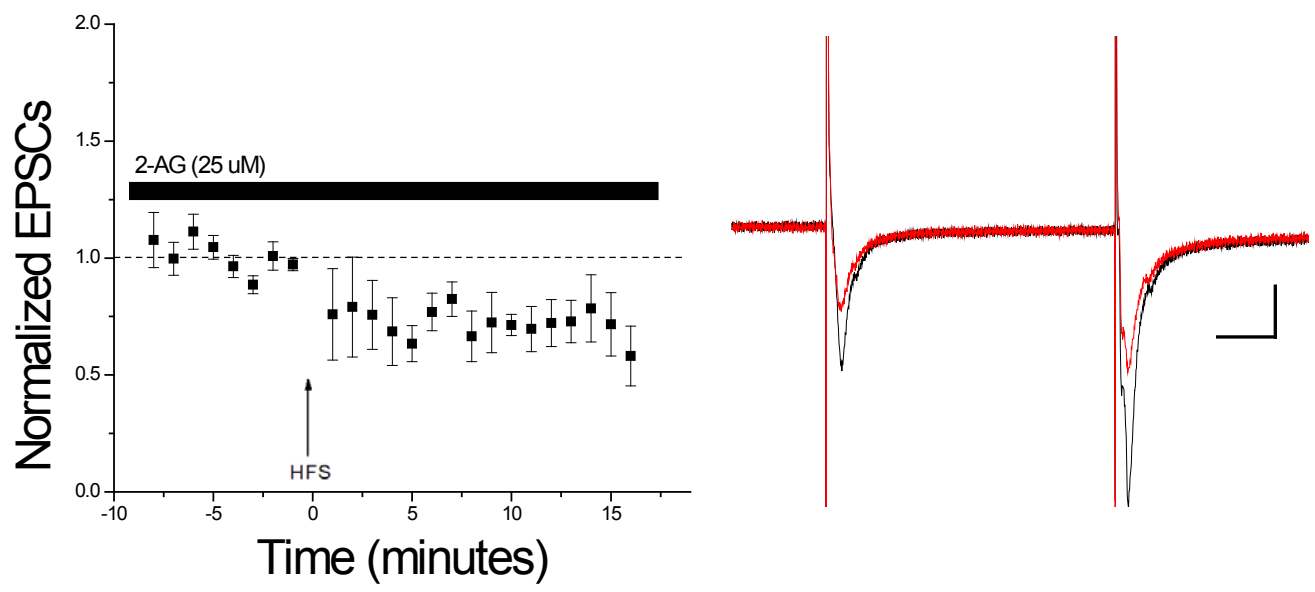


Figure 6: 2-AG Does Not Fully Occlude HFS-LTD.

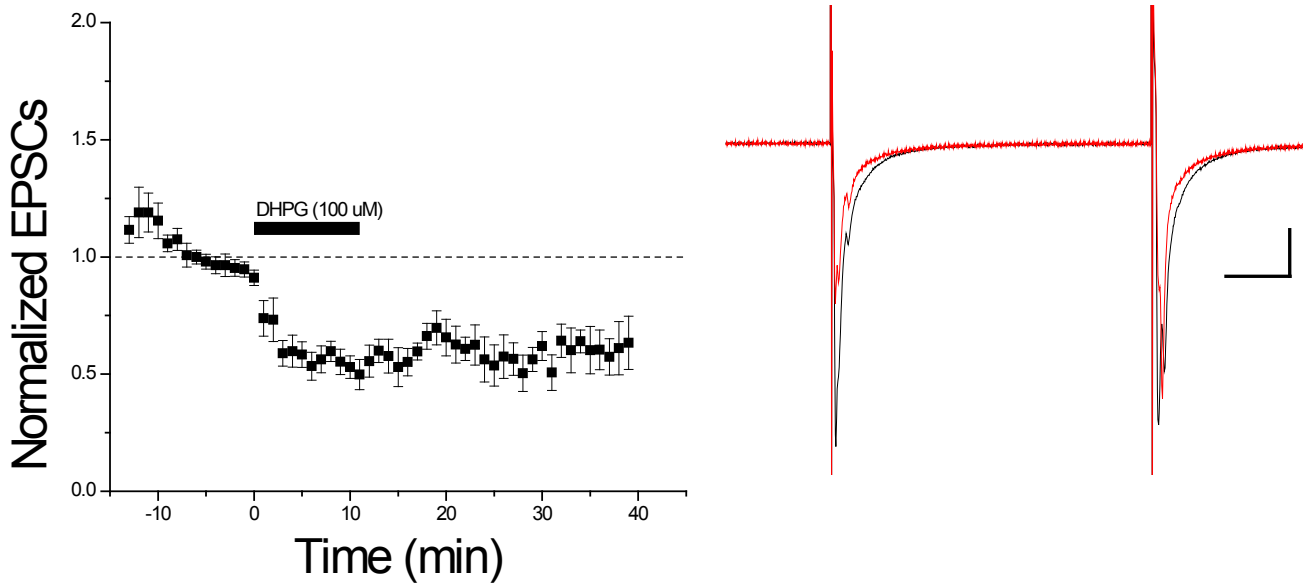


Figure 7A: DHPG Causes LTD in VTA GABA Neurons.

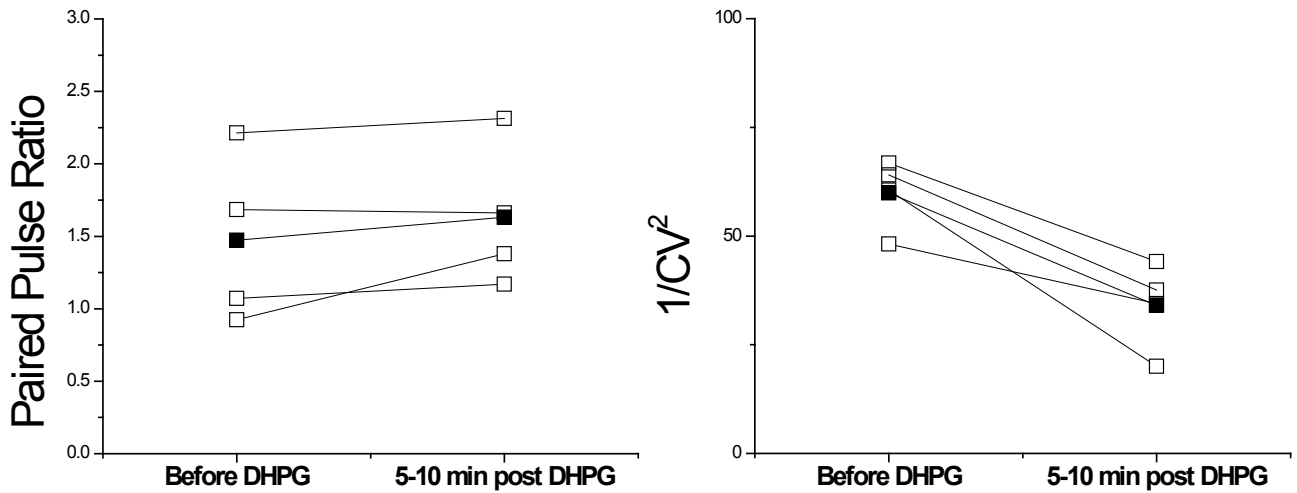


Figure 7B: DHPG May Increase Paired Pulse Ratio and Decrease $1/CV^2$.

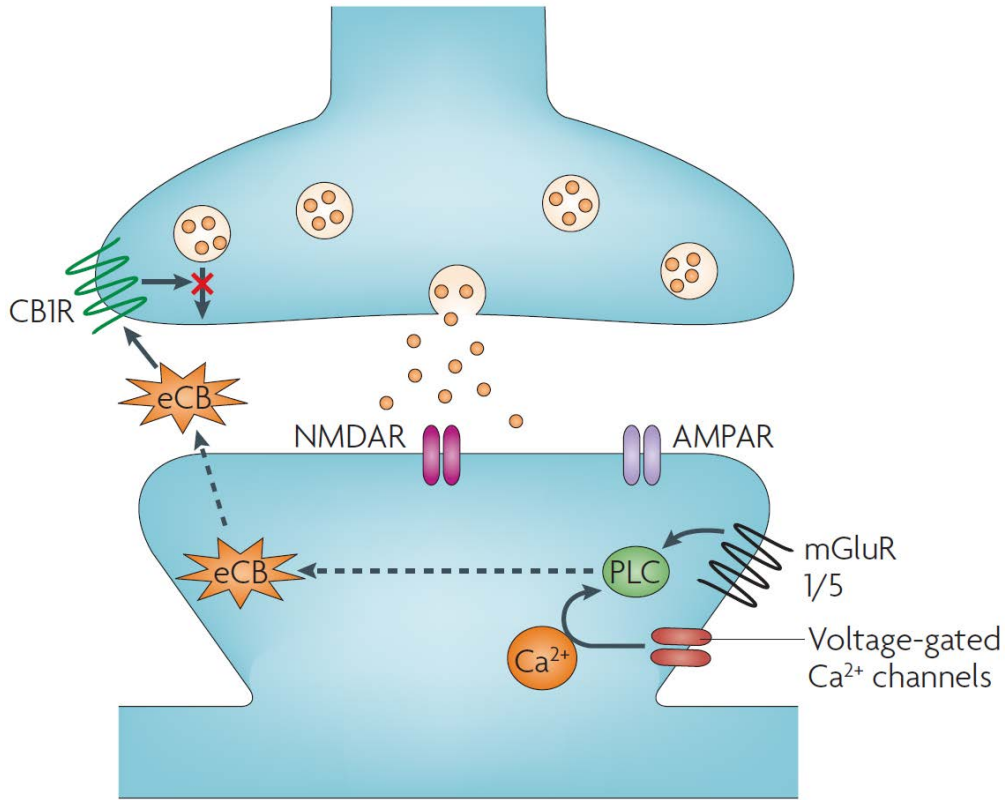


Figure 8: Proposed Model for HFS-mediated LTD in VTA GABA Neurons.

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EDUCATION

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MS, Neuroscience

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BS, Neuroscience. Magna Cum Laude, Phi Kappa Phi

2011

AWARDS

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Department of Graduate Studies, Brigham Young University (\$15,000)

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2011

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Teacher's Assistantship (2)

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Studied mechanisms of synaptic plasticity in mouse ventral tegmental area and rat hippocampus

- Managed breeding of colony of GAD67-GFP knock-in mice.
- Dissected out whole mouse brains and prepared acute slices using vibratome.
- Observed effects of drugs on excitatory postsynaptic currents of VTA GABA neurons using whole-cell patch clamp technique.
- Investigated effects of drugs on field potentials of the CA1 region of rat hippocampus
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