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ABSTRACT

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Synaptic plasticity is the process whereby connections between neurons can be altered in an experience dependent manner. For example, drugs of abuse alter plasticity in the ventral tegmental area (VTA) of the midbrain. A large amount of research has been applied to uncovering the mechanism whereby synapses on the reward signaling dopamine cells is altered, however, less is known regarding the VTA inhibitory GABA neurons. Our objective was to examine the ability of GABA neurons to exhibit plasticity, and determine how drugs of abuse could influence it. Here we report a novel type of plasticity of excitatory neurotransmission onto VTA GABA cells. This plasticity is dependent on the metabotropic glutamate receptor 5, to signal for diacylglycerolipase alpha to make the endocannabinoid 2-arachadonoyl glycerol to signal via cannabinoid receptor 1 (CB1). Marijuana and cocaine are drugs of abuse that have been shown to alter the endocannabinoid system. Tetrahydrocannabinol is the active ingredient in marijuana, and is a known agonist of CB1, and cocaine is able to attenuate endocannabinoid signals. We tested the effects of these drugs on VTA GABA plasticity and found that it can be blocked by chronic injections of tetrahydrocannabinol, as well as acute and chronic injections of cocaine. If VTA GABA neurons are depressing excitatory inputs, that could lead to less inhibition onto VTA dopamine cells, and therefore, more reward signaling in the brain. This new type of plasticity could be an additional mechanism whereby cocaine and marijuana exert their rewarding and addictive effects.

Another brain structure known to exhibit use-dependent plasticity is the hippocampus, which is involved in learning and memory. The stratum oriens is a layer of inhibitory interneurons in the hippocampus that is involved in feedback inhibition onto the principle excitatory cells in the stratum pyramidale. Our goal was to determine whether oriens interneurons were capable of producing an endocannabinoid signal, and if so, whether they could influence plasticity. We identified 2 major subtypes of oriens interneurons, oriens lacunosum-moleculare cells, and parvalbumin-positive basket cells, which are capable of receiving and producing an endocannabinoid signal. Furthermore, we demonstrated that one such endocannabinoid, anandamide, is responsible for signaling for synaptic plasticity. This plasticity is also dependent on CB1, and is unique in that there are few examples of CB1 signaling for potentiation rather than depression. Collectively, these experiments demonstrate two mechanisms of endocannabinoid mediated synaptic plasticity, which could influence reward signaling, addiction and memory.

Keywords: GABA, long-term depression, reward, addiction, oriens

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CHAPTER 1: Introduction

Mammalian brains are among the most complex and elaborate structures in nature, which allow animals to rapidly sense, interpret and act in their changing environment. The ability to recruit previous knowledge to future decision making is essential to everything from survival skills such as finding food, to building and maintaining relationships. While most of the gross anatomy is present and intact from birth, a mechanism must exist for which new connections can be made or altered. Novel memories and skills are not encoded in new neurons, but changeable, or plastic synapses that can quickly be modified. Several disease states result from the inability to alter synapses that encode new memories, or retrieve stored ones, including dementia and Alzheimer's disease that according to the Alzheimer's Association currently effects over 5 million Americans. In addition to explicit memories, the same mechanisms can allow a person to remember rewarding events such as eating. While this process can be useful for healthy activities, it can be hijacked by abusable substances to produce implicit memories of rewarding drugs leading to addictive behaviors. Certainly, understanding the mechanism of various types of synaptic plasticity can provide applicable insights to several disease states.

Synaptic Plasticity is a process whereby synapses can be strengthened or weakened based on the activity of the cells around them by either presynaptically altering neurotransmitter release, or postsynaptically modifying the receptor trafficking to and from the membrane. Plasticity is a critical event that allows us to modify brain activity based on the experiences in our lives and is thus experience-dependent brain modification. Two major forms of synaptic plasticity are present in the brain, Long-term potentiation (LTP) and long-term depression (LTD). For post-synaptic LTP to occur, there must be a large calcium ion influx through N-Methyl-D-Aspartate (NMDA) glutamate receptors, which triggers α-Amino-3-hydroxy-5-

methyl-4-isoxazolepropionic acid (AMPA) receptors to be inserted into the membrane. These non-selective cation channels allow the post synaptic cells to respond to further glutamatergic transmission. In contrast, a weaker signal would result in a lower amount of calcium ion influx which results in AMPA receptor internalization, known as LTD [1]. Plasticity has been observed in various brain regions, notably, the hippocampus where it is crucial for memory encoding and consolidation.

The Hippocampus is a highly organized and intricate region of the temporal lobe, and contains a variety of cell types, and is known to be involved in memory consolidation. The hippocampus consists of several layers including stratum radiatum, stratum pyramidale, and stratum oriens, which can be divided in main sub-regions including cornu ammonis 1 (CA1), CA2 and CA3. Distributed in these layers are both excitatory and inhibitory neurons. Excitatory pyramidal cells are located in stratum pyramidale of the CA regions and diverse inhibitory interneurons are present in stratum radium and stratum oriens [2]. Other less well known forms of plasticity in addition to NMDA dependent forms also occur in the brain mediated by various other mechanisms such as induced by lipid messengers known as endocannabinoids.

Endocannabinoids (eCBs) are produced by components of the lipid membrane, a reaction catabolized by various enzymes, for example phospholipase c, and diacylglycerol lipase α, normally following second messenger activation. Usually, upon post-synaptic activation of type 1 metabotropic glutamate receptors, enzymes are recruited to produce eCBs which then can retrogradely travel to the pre-synaptic cell to alter neurotransmission via cannabinoid receptor 1 (CB1) [3]. The two best characterized endocannabinoids are anandamide and 2-arachadonyl glycerol (2-AG). Anandamide, a common eCB, is produced by n-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) and can bind to cannabinoid receptor 1, or other G-protein

coupled receptors throughout the brain. It is then degraded by the enzyme fatty acid amide hydrolase (FAAH). When this enzyme is inhibited, baseline anandamide is increased. [4, 5]. 2-AG is produced in the brain by diacylglycerol lipase alpha (DAGLα) and is also involved in retrograde signaling via CB1 [6] and other receptors. It remains unknown whether some or all of the various subtypes of interneurons utilize eCBs for plasticity.

Hippocampal interneurons are a diverse group, and can be classified into several different subtypes based on their function, projections, and firing pattern. Often interneuron subtypes are characterized into these groups using calcium binding proteins and neuropeptides, including somatostatin, cholecystokinin and parvalbumin (Freund, 1996). The stratum oriens of the CA1 region of the hippocampus houses interneurons of these varying types. Oriens lacunosum-moleculare (O-LM) interneurons are one such group, and can be identified by the expression of cholecystokinin and somatostatin, as well as a regular and fast spiking pattern and sag potential upon injection of hyperpolarizing current [7, 8]. O-LM cells are named for their morphology, with somas residing in stratum oriens and distant projections to stratum lacunosum moleculare. Another subtype is parvalbumin positive basket cells, which project locally and lack a sag potential. These cells are also involved in network oscillations in the hippocampus [9]. While projections and spiking patterns are well defined in these neurons, information on endocannabinoid biosynthetic capability, or eCB-dependent plasticity of these interneurons is lacking. Based on the role interneurons play in feed-forward inhibition and widespread network control and oscillations, a thorough study of the mechanism of their plasticity is timely and relevant.

Other areas of the brain experience endocannabinoid-mediated plasticity as well, such as the ventral tegmental area (VTA). The VTA is critically involved in reward stimuli, including

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behavior components of rewarding and aversive stimuli. The VTA projects to structures such as the nucleus accumbens and prefrontal cortex, where reward is mediated and conscious perception of the reward occur, respectively. The VTA contains both dopaminergic neurons projecting to the nucleus accumbens, and GABAeric neurons that regulate DA neuron firing and DA release. GABA cells are common and heterogeneous throughout the VTA, and can be innervated by multiple sources, including excitatory glutamatergic input from distant prefrontal cortical cells [10]. As the VTA is so important to reward, alterations to its synaptic activity have been implicated in forming addictions.

Drugs of abuse, such as cocaine and marijuana, are known to modulate neuronal activity in the VTA and result in addiction [11, 12]. While increased dopamine release in nucleus accumbens mediates the initial reward, synaptic plasticity of VTA circuits is thought to mediate the addiction [13, 14]. Cocaine produces a reward by blocking the dopamine transporter DAT, resulting in dopamine remaining in the synapse for a longer period of time. Δ9-tetrahydrocannabinol (THC), the active component in marijuana activates CB1 receptors. This can lead to reward by disinhibiting or removal of inhibition to dopamine cells. While both cocaine and THC increase dopamine to mediate reward, they can also produce lasting associations, modified synapses in the VTA that can lead to an addictive state.

For example, eCBs and CB1 mediate plasticity either directly by THC or via cocaine which decreases postsynaptic mGluR activation and therefore, eCB synthesis (Luscher & Malenka, 2011). The link between CB1 and drugs of abuse is demonstrated in a variety of conditions, suggesting an additional role of endocannabinoids in the reward effects via increased dopamine, and addiction processes through synaptic plasticity. Figure 1 summarizes the known targets of drugs of abuse on dopaminergic neurons. The right side highlights the synapse of

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interest, between the glutamatergic cell and the VTA GABA cell where we identify a novel form of CB1-dependent synaptic plasticity.

Specific Aims

The effects of drugs of abuse on ventral tegmental area physiology is a relatively new focus of research for many years with the hope of unraveling the molecular processes of reward and addiction states. While much is known regarding alterations of synaptic plasticity in dopamine cells, the possible influence of GABA plasticity on reward remains unknown. Our aim was to determine the mechanism of synaptic plasticity in VTA GABA cells, and examine the influence of cocaine and THC on this plasticity.

In addition, we studied the endocannabinoid capabilities of hippocampal stratum oriens interneurons. These inhibitory cells are regulators of the hippocampal principle cells. We wanted to determine whether these neurons are capable of producing endocannabinoid signals, and how they influence synaptic plasticity.

- Marijuana
- Benzodiazepenes
- Opioids
- Cocaine

- Nothing known
- Could impact reward
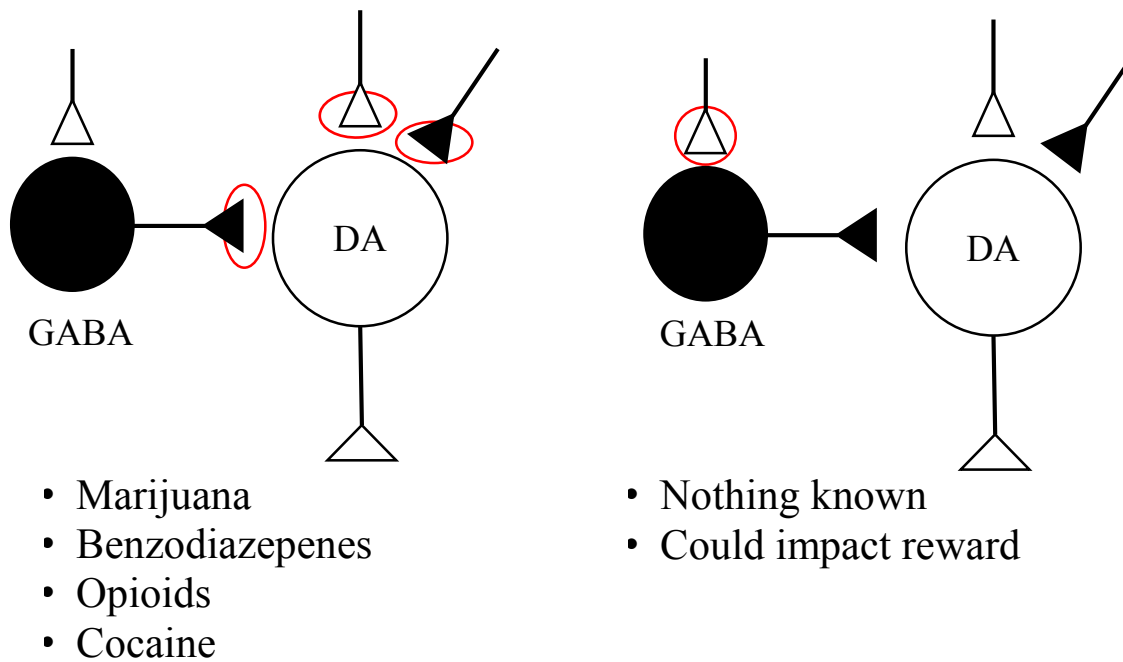
Figure 1.1: VTA Reward Circuitry. Rewarding behaviors can be associated with increases in firing rate of VTA dopamine neurons projecting to the nucleus accumbens. It is known that some drugs of abuse directly target dopamine cells by modulating plasticity at both the inhibitory (solid) and excitatory (open) synapses. However, the modulation of excitatory inputs onto GABA cells remains unknown, but likely could also affect dopaminergic neurons indirectly.

CHAPTER 2: CB1-dependent LTD in Ventral Tegmental Area GABA Neurons:

A Novel Target for Marijuana

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Abstract

Ventral tegmental area neuronal circuits have been implicated in reward processing. The activity of the dopamine cells that signal reward are regulated by neighboring GABA cells that inhibit them. While the physiology and plasticity of dopamine cells has been thoroughly studied, less is known about the plasticity of GABA cells. Here we describe a novel form of excitatory plasticity in VTA GABA neurons, and demonstrate how this plasticity can be modified by Δ9-tetrahydrocannabinol, the active ingredient in marijuana. Using whole cell voltage-clamp electrophysiology in GAD67-GFP mouse VTA slices, excitatory inputs on fluorescent GABA cells were recorded. A CB1-dependent long-term depression can be induced by high-frequency stimulus, which is dependent on type 1 metabotropic glutamate receptors, and cannabinoid 1(CB1) receptors. CB1 agonists r(+)methanandamide, 2-AG, WIN55, and Δ9-tetrahydrocannabinol mimic and occlude LTD. This LTD was absent in CB1 knock-out mice, but preserved in CB1 heterozygous littermates. Chronic injections of Δ9-tetrahydrocannabinol significantly occluded LTD compared to vehicle injections, however, a single 24-hour exposure was insufficient to occlude it. This novel form of plasticity wherein VTA GABA cells can depress excitatory inputs, could lead to disinhibition of nearby dopaminergic cells.

Introduction

Drug addiction represents a significant health and economic issue around the world. The United States spends over $193 billion dollars annually on costs related to illicit drug abuse. While there is variation in the mechanism of each drug of abuse, the common theme among drugs of abuse is that they alter ventral tegmental area (VTA) circuitry and function, usually to enhance dopamine release [15]. However, subsequent to the acute actions of drugs of abuse, changes often occur that continue after the drug has left the body. These synaptic changes in VTA, or its projections, mediate some of the addictive components of drugs of abuse. All addictive psychoactive substances examined to date induce long-term changes of excitatory synapses of VTA DA neurons, while non-addictive psychoactive substances do not [16]. For example, one exposure to cocaine can cause LTP of excitatory input to VTA dopamine cells [17]. While the brain regions relevant to addiction plasticity are primarily the VTA and nucleus accumbens, the mechanisms of plasticity are certainly more numerous. Rewarding drugs increase midbrain dopamine levels through a variety of strategies. Within the VTA, dopaminergic cells are involved in motivation and learned reward. Drugs of abuse enhance DA levels more than normal rewarding behaviors, and cause synaptic modifications leading to addiction. Addiction is the compulsive drive to self-administer drugs even after mitigation of DA enhancements. Endocannabinoids (eCBs) mediate many forms of plasticity, in particular long-term depression (LTD). eCBs can signal for LTD and cause a decrease in synaptic strength is weakened due to decreased neurotransmitter release or endocytosis of post-synaptic receptors. eCBs are more recently characterized lipid-based chemicals that induce plasticity at locations throughout the brain [5], including the VTA [18]. The eCBs include 2-arachidonylglycerol (2-AG) and anandamide that activate receptors such as cannabinoid receptor 1 (CB1) and transient receptor

potential vanilloid 1 (TRPV1) [6, 19]. These receptors have been correlated to reward and addiction in the VTA [20, 21]. The regulation of eCB production share common mechanisms throughout the nervous system [22]. Synthesis of eCBs 2-AG and anandamide often occur in the postsynaptic cell, usually due to activation of type I metabotropic glutamate receptors (mGluRs) [23]. When produced, these hydrophobic molecules escape from the postsynaptic cell and either act as retrograde messenger for presynaptic receptors or receptors on adjacent cells. One such presynaptic receptor is CB1, which is particularly relevant to addiction due to its ability to bind to the psychoactive ingredient in marijuana, Δ9- tetrahydrocannabinol (THC). THC is known to be rewarding and potentially addictive, especially in adolescents. Indeed, an addiction state known as Cannabis Use Disorder is on the rise. The recent legalization of marijuana in some states will only increase the public issues associated with it and thus the demand for studies regarding its effects on reward, addiction and cognition. THC is known to increase midbrain dopamine levels, which is consistent with the current theory of addiction [24].

While illicit drugs cause various synaptic modifications onto DA cells, data regarding drug modification of excitatory synapses onto GABA neurons is conspicuously absent. Therefore, examining VTA GABA neurons is timely as drugs of abuse targeting these neurons, altering their activity and synaptic plasticity, could cause addictive behaviors, as decreasing GABA cell activity reduces inhibition to DA cells, thus enhancing DA release. Our goal was to study the mechanism of plasticity of excitatory inputs onto VTA GABA neurons and determine whether THC can influence this plasticity.

Methods

All experiments were performed in accordance with Institutional Animal Care and Use Committee protocols and followed NIH guidelines for the care and use of laboratory animals. IACUC protocols for all experimental protocols were approved by the Brigham Young University Institutional Animal Care and Use Committee, Animal Welfare Assurance Number A3783-01.

Male CD1 GAD67-GFP knock-in mice (P15-P30) were used so that GABA cells in the VTA could be positively identified using fluorescence [25]. Male CB1 knock-out mice (Dr. Ken Mackie's Lab) were bred to female GAD67-GFP+ mice to obtain knock-out and heterozygous littermates.

Mice were anesthetized with isoflurane and decapitated with a rodent guillotine. Brains were rapidly removed and sectioned horizontally on a vibratome at 300μm using an ice cold, sucrose-based cutting solution composed of 220 mM sucrose, 0.2mM CaCl2, 3 mM KCl, 1.25 mM NaH2PO4, 25 mM NaHCO3, 12 mM MgSO4, and 10 mM glucose. After sectioning the slices were placed in an incubator at 37 degrees Celsius, where they remained until recording. Recordings began at least one hour after cutting while tissue was stored in oxygenated artificial cerebral spinal fluid composed of 119 mM NaCl, 26 mM NaHCO3, 2.5mM KCl, 1 mM NaH2PO4, 2.5mM CaCl2, 1.3mM MgSO4, and 11mM glucose with 100μM picrotoxin.

The ventral tegmental area was visualized using an Olympus Center Valley, BX51W1 microscope with a 40x water immersion objective. Patch pipette resistance was 3.5–5.5 MΩ. Cells were held at −65 mV throughout the recording. Cells were patched with a glass pipette filled with internal solution composed of 117mM cesium gluconate, 2.8 mM NaCl, 20 mM HEPES, 5 mM MgCl2, and QX0314 (Tocris) (pH 7.28, 275–285 mOsm). Slices were

oxygenated with 95% O2, and 5% CO2 in artificial cerebrospinal fluid was pumped into and out

of the recording chamber throughout the experiment. Current traces were recorded using

Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). Signals were filtered at

4 kHz and digitized with an Axon 1440A digitizer (Molecular Devices) connected to a Dell

Personal Computer with pClamp 10.2/10.5 Clampex software (Molecular Devices). Plasticity

was induced using 2 stimulations at 100Hz, 20 seconds apart. Cells used for depolarization

induced suppression of excitation (DSE) experiments were initially held at -65mV, then raised to

0mV for 10 seconds before being returned to -65 mV. The peak amplitude of the induced

excitatory post-synaptic current (EPSC) was calculated using Clampfit 10.5 software (Molecular

Devices) and graphed using Origin 7.5 Within individual experiments, current traces were

averaged by minute (6 sweeps/minute). Wilcoxon matched pairs analysis was used to obtain p-

values for the paired-pulse ratios and coefficient of variance. All other p-values were obtained by

a two-way unequal variance t-test comparing the 5 minutes before conditioning to 15-20 minutes

post conditioning.

Picrotoxin (0.2mM), and Capsazepine (10µL) were purchased from Abcam. APV

(50µL), AM-251 (2µM), 2-AG (50µM), R(+)methanandamide (50µL), GDPβS (500µM), DHPG

(100µL), Win55,212-2 (10µM), and RHC80267 (10µM) were purchased from Tocris. CNQX

(10µM) was purchased from Alomone Labs. Δ9-tetrahydrocannabinol was purchased from

Sigma-Aldrich, and the NIDA Drug Supply Program. THC was administered via intraperitoneal

injection at 10mg/kg.

Results

Critical to the success of this study was to positively identify VTA GABA neurons. This was done by using a GAD67-GFP mouse model where GAD67-expressing GABA cells fluoresce green. This was necessary as electrophysiological methods such as Ih and sag potential that many prior studies used to discriminate the relatively fewer GABA cells are now considered fallible in their ability to discriminate GABA cells from DA cells [10, 26, 27]. Therefore, this study is one of the few unequivocal studies of VTA GABA cell plasticity. To examine the ability of VTA GABA cells to exhibit synaptic plasticity of their excitatory inputs we applied either 5 Hz or 100Hz high frequency stimulus (HFS) to voltage clamped GAD67-GFP positive GABA neurons in the mouse VTA. While 5 Hz stimulation produced long-term depression (LTD) in 30% of cells (data not shown), we observed a more consistent LTD following HFS in 71% of GABA cells (40±3.2% decrease in EPSC amplitude; Figure 2.1A). Therefore, we used HFS stimulus throughout these studies. Control rundown experiments did not exhibit depression, and proved significantly different than HFS-LTD (Figure 2.1B). Next, in order to determine whether this plasticity was pre- or postsynaptic, 1/CV2, paired pulse ratios (PPRs) and failure rates were analyzed (Figure 2.1D-F). These data suggested a presynaptic mechanism for the observed plasticity.

Endocannabinoids are signaling molecules involved in multiple types of presynaptic plasticity. Therefore, we examined the application of a non-hydrolyzable form of anandamide, R(+)methanandamide, to see if it would also induce depression of EPSCs. Indeed similar to HFS, R(+)methanandamide induced significant depression (Figure 2.2A). The usual mediator of anandamide production is type 1 mGluR activation. To confirm this we applied the mGLuR agonist DHPG and once again observed significant depression (Figure 2.2B). In the presence of

the mGluR5 antagonist MPEP, HFS-LTD was blocked, suggesting that mGluR5 are required and sufficient for this plasticity (Figure 2.2C). To further confirm that a postsynaptic G-protein coupled receptor such as mGluR was involved, a GDP analogue, GDPβS, was added to the internal solution to block post-synaptic g-proteins (Figure 2.2D). After waiting 10 minutes for GDPβS to dialyze into the cell HFS was applied. In the presence of GDPβS LTD was inhibited, suggesting that postsynaptic G-protein coupled receptors are necessary to initiate LTD at this synapse.

Among the candidate receptors known to bind eCBs such as anandamide that could induce plasticity are TRPV1 and CB1. The TRPV1 antagonist capsazepine did not block HFS-induced LTD, suggesting that TRPV1 is not involved (Figure 2.3A). However, application of the CB1 antagonist AM-251 effectively blocked LTD (Figure 2.3B), suggesting CB1 is required for HFS-induced LTD.

To verify the presence of CB1 receptors at the synapse, we applied CB1 agonists. Win 55,212-5, an exogenous CB1 agonist, and 2-AG an endogenous agonist, both induced depression of ESPCs (Figures 2.4A and 2.4C). The specificity of Win 55,212-2 for CB1 is noted as AM-251 blocked Win 55,212-2-induced depression (Figure 2.4B). To further confirm the involvement of eCBs and CB1 in HFS-LTD, occlusion experiments were done in which the cells were treated with the CB1 agonist Win55,212-2, then HFS was applied. Further depression by HFS was occluded, suggesting a single, common pathway of CB1 activation and HFS-induced LTD (Figure 2.4A).

To show the necessity of the endogenous eCB 2-AG, and to determine whether its production is post-synaptic, RHC 80267 was applied to the intracellular solution to block the enzyme diacylglycerol lipase α (DAGLα), which produces 2-AG. After whole-cell acquisition,

RHC 80267 was allowed to dialyze into the cell for at least 10 minutes. When post-synaptic DAGLα was blocked, no significant depression was seen following HFS (Figure 2.4D). Blocking HFS-LTD by post-synaptically inhibiting 2-AG synthesis suggests that the 2-AG involved in HFS-LTD is made directly by the GABA cell.

To further confirm that eCBs are being produced post-synaptically in GABA cells, we examined an additional type of plasticity unique to CB1: depolarization-induced suppression of excitation (DSE). We observed a significant depression following a 10 second depolarization to 0mV, which was surprisingly persistent throughout the duration of the recording (Figure 2.4D). AM-251 blocked this depression (Figure 2.4E), suggesting VTA GABA cells can produce the eCBs that can bind to CB1 in this type of plasticity.

To verify CB1 involvement using a genetic method, we utilized CB1 knock-out mice and heterozygous littermates. HFS continued to produce a significant depression in CB1+/- mice (Figure 2.5A); however, LTD was not observed in CB1-/- (Figure 2.5B). Collectively our data suggests CB1 is required for HFS-LTD.

As CB1 can be activated by THC, this novel form of plasticity we characterized may have relevance to THC-induced reward or addiction-like phenomena such as Cannabis-use disorder. Therefore, we next investigated whether the observed eCB-dependent HFS-LTD could be modified by drug exposure. Extracellular bath application of THC produced a significant depression of VTA GABA EPSCs (Figure 2.6A), and this effect was blocked in the presence of AM-251 (Figure 2.6B), demonstrating THC specificity for CB1. In CB1+/- mice, extracellular THC also produced depression (Figure 2.6C), but not in CB1-/- mice (Figure 2.6D), again confirming specificity. We initially wanted to determine if acute THC bath application could occlude or alter HFS-LTD. Indeed, bath application with THC for 10 minutes occluded HFS-

LTD (Figure 2.6 F). Therefore, THC acutely depresses GABA cell excitability, as well as block LTD induction.

As drugs of abuse cause long-term synaptic modifications may facilitate addiction, we examined the role of acute and chronic THC injections on this LTD. To do this, mice received THC IP injections to study synaptic modification by a single injection, or chronic THC exposure. As THC is less addictive we anticipated that a single injection might not have a long-term effect on plasticity, though chronic injections might, as long-term marijuana users are prone to THC-induced issues. Indeed, single injection failed to occlude LTD (Figure 2.7A). However, chronic injections (7-10 consecutive days) occluded LTD in slices (Figure 2.7B), and in a few cells tended to enhance EPSCs. This suggests that persistent, long-term THC exposure can modify plasticity at the glutamatergic inputs onto VTA GABA cells of juvenile to adolescent age mice that may contribute to some of the long-term consequences of THC use.

Discussion

The present study demonstrates a novel CB1-dependent LTD of excitatory inputs to VTA GABA cells that can be modified by THC, as summarized in Figure 2.8. HFS-LTD is induced by CB1 activation that is mediated by post-synaptically produced 2-AG, formed by PLC and DAGLα via mGluR5 activation and postsynaptic calcium release. THC mimics this plasticity via the CB1 pathway when applied acutely and occludes it when injected chronically. Therefore, this novel form of plasticity has the potential to influence reward and addiction states following THC exposure.

To date no one has carefully studied excitatory plasticity of VTA GABA cells at the cellular level, despite the fact that GABA cells mediate reward consumption [28], associative learning of reward [11], conditioned place preference [29], and conditioned place aversion [30].

As GABA cells are critical to reward, it illustrates the importance of this study, the first to identify synaptic plasticity of excitatory inputs to VTA GABA cells that can modify GABA cell activity by depressing it. While one other study examined plasticity of excitatory inputs to GABA cells, this study employed a pairing protocol (+10 mV, 200 stimuli at 1 Hz) that failed to induce either LTP or LTD [31]. However, this pairing protocol is normally employed to examine NMDA-dependent plasticity. The plasticity we observed is likely mGluR dependent, which accounts for the observed difference. We report that using HFS (2 trains of 100 Hz for 1s, each train 20s apart) protocol does indeed cause depression of EPSCs in VTA GABA neurons, in a CB1-dependent manner. As an important note, there are likely more eCB receptors at this synapse than just CB1 as R-(+)methanandamide and DHPG produce a larger effect than CB1 agonists alone, however CB1 appears to be the only eCB receptor required for this LTD. Finally, while VTA DA cells exhibit DSE of excitatory inputs via presynaptic CB1 receptors [32]. It is important to note that our DSE data indicate that GABAs can modulate their own activity by producing eCBs themselves rather than relying on DA cells for their production.

CB1-dependent forms of LTD exist in many brain regions [33], with the list growing as the eCB system is widely expressed. This plasticity is a form of use-dependent plasticity and the eCB system is now recognized for its importance in synaptic plasticity throughout the brain. The best cellular correlate to compare VTA GABA cells is likely the striatum [34]. Other examples of plasticity can be found that are strikingly similar to the plasticity described here. For example, LTD can be initiated between glutamatergic inputs onto striatal GABA neurons, which is dependent on presynaptic CB1 and post-synaptic activation of mGluRs and eCB synthesis [35, 36].

The importance of GABA cell involvement in reward either directly through axonal projections to brain regions outside the VTA or indirectly by regulating DA cell activity, highlight the critical nature in understanding GABA cell modification by synaptic plasticity, a necessary element in understanding VTA reward mechanisms. Directly, GABA cells in the VTA project to the nucleus accumbens (NAc) where they enhance associative reward learning [11] and to the tegmental pedunculopontine nucleus where they mediate the dopamine-independent reinforcing properties of opioids [37]. Indirectly, decreased GABA cell activity results in increased DA cell activity by disinhibiting DA neurons [29], and alternatively increased GABA cell activity inhibits DA cells activity and DA release [28, 30], which alter reward behavior. Thus, this study fills a critical missing piece of our understanding of reward physiology and suggests this novel form of glutamatergic LTD could modulate reward behavior either directly by its projections or indirectly by modulating DA levels via DA cell disinhibition. Indeed, VTA DA cells are under tonic inhibition from GABAergic cells, including from the recently identified RMTg [38], making any change in GABA function pertinent to DA cell function as only phasic or brief and inhibition or excitation of DA cells mimic negative reward [39] or reward behavioral conditioning [40], respectively.

Regarding reward, acute exposure to THC can directly modify dopaminergic cells by increasing their firing rate. This is thought to occur via presynaptic CB1 receptors on GABA inputs to dopamine cells, thus resulting in disinhibition of DA cells (Szabo- your ref #12 and maybe others). Our current study proposes a novel mechanism that THC could induce reward, namely that THC reduces excitatory input to GABA cells and thus decreasing inhibition to VTA DA cells via disinhibition, which is a similar mechanism but one synapse upstream in the circuit.

Therefore, acutely ingested or bath applied THC could simultaneously alter both synapses that synergistically result in increased DA and reward.

To date all addictive psychoactive substances examined induce long-term changes of excitatory synapses of VTA DA neurons, while non-addictive psychoactive substances do not [16]. This finding portends the importance of understanding forms of glutamatergic VTA GABA plasticity and their potential role in addiction. Drug-evoked or drug-inhibited synaptic plasticity alters VTA circuits and thus can lead to drug addiction by creating drug-adaptive behaviors.

While the true addictive nature of THC is debated, alterations in the mesolimbic pathway following chronic THC use is particularly relevant for adolescents. Indeed, human adolescent use of marijuana correlates to decreased IQ and cognitive impairment [41], cortex development issues, schizophrenia [42], alterations in nucleus accumbens [43], depression and anxiety and increased probability for long-term substance abuse [44, 45]. Therefore, studies examining THC impacts on adolescent brain are extremely important, particularly in the reward center. The mice age range employed here correlate to juvenile/adolescent ages, making this study of potential importance as to THC effects on immature brain, which could have implication in adolescent related increases in substance abuse.

While a single THC injection was sufficient to alter plasticity in the NAc [46], we anticipated and noted that a single injection of THC had no effect on CB1-dependent LTD. However, chronic THC did occlude this LTD, suggesting this occlusion mechanism responds to only chronic repeated usage. As during cannabis withdrawal there is a decrease in mesolimbic dopamine [47], it is possible that THC-occluded LTD prevents GABA cells from reducing their activity, potentially contributing to some of decreased DA levels in withdrawal.

The effects of chronic THC use were recently reviewed by Colizzi et al [48]. The plasticity described here is a similar mechanism to previously reported acute and chronic synaptic modifications, wherein a pre-synaptic CB1 receptor alters glutamate plasticity affecting changes in mesolimbic dopamine, however ours is the first study to identify this plasticity on GABA neurons.

Again, while the addiction potential of THC is not completely apparent, this CB1-dependent glutamatergic synaptic plasticity may also be modified by more addictive drugs making it critical to examine. Cocaine is known to modify glutamate plasticity onto DA cells in nearly every study [14, 29, 49]. In addition, cocaine blocked eCB forms of plasticity such as a CB1-dependent form of LTD in the NAc [49]. Another example where the pharmacological inhibition of the synaptic breakdown of the eCB anandamide enhanced the increase of VTA DA release caused by cocaine administration in mice, which demonstrates that CB1 activation enhances the effects of cocaine in the VTA [50]. Finally, cocaine can stimulate 2-AG to suppress GABA activity onto dopaminergic neurons via CB1 [51].Therefore, cocaine can modify eCB-dependent forms of plasticity and thus this plasticity may have relevance to modification of other drugs of abuse. Indeed, this is a matter of current investigation.

Significance

Marijuana use and legalization has recently been an issue for multiple states. Although marijuana is the most commonly used illicit drug, the implications of legalized, widespread or continued usage are speculative. Additionally, there are misconceptions regarding whether marijuana can be addictive. THC may be more addictive that previously thought, especially for adolescents, and the addiction state Cannabis Use Disorder is on the rise. After smoking and alcohol, marijuana ranks second behind opiate abuse for those entering substance abuse

treatment programs, portending the importance of understanding THC's effect on the brains reward center. If nothing else, state legalization of marijuana will only increase its usage and public issues associated with it as well as the demand for studies regarding its effects on reward, addiction and cognition. Even THC for medical purposes will be important to understand, again particularly when it is prescribed for adolescents. This study in adolescent mice emphasizes vital role VTA GABA cells play in reward circuitry, and the potential synaptic remodeling that can occur after THC use.

Conclusion

The results of the present study shed more light on how the population of VTA GABA neurons, an important cell in the brain's reward circuit, can be affected in the long term by upstream excitatory activity. This study specifically addresses a mechanism whereby marijuana could exert its rewarding or addictive effects. Although marijuana addiction has a high prevalence among illicit drugs [52], it remains difficult to treat partly because its addictive mechanisms are poorly understood [53]. 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. As those attempting to overcome their addiction often relapse, novel targets to treat addiction are essential.

Figure 2.1: Pre-Synaptic Long-Term Depression (LTD) In VTA GABA Cells. A. Glutamate currents recorded from GAD67-GFP positive VTA GABAs. 100Hz high frequency stimulus (HFS) induces long-term depression. Responses were reduced by 40±3.2% compared to baseline (n=14, p<0.001 comparing baseline to post conditioning). B. No treatment controls showed no change in EPSC amplitude, which demonstrates HFS-LTD is not due to washout effect (n=8). Following HFS the coefficient of variance (1/CV2) decreased, paired-pulse ratio increased, and failure rates increased (n=15 for 1/CV2 and PPR, n=6 for failure rates, p>0.05 for each) suggesting a presynaptic mechanism for HFS-LTD. Scale bars 100 pA, 10 msec. Plots, mean with s.e.m. Arrow indicates 100Hz high frequency stimulus (HFS).
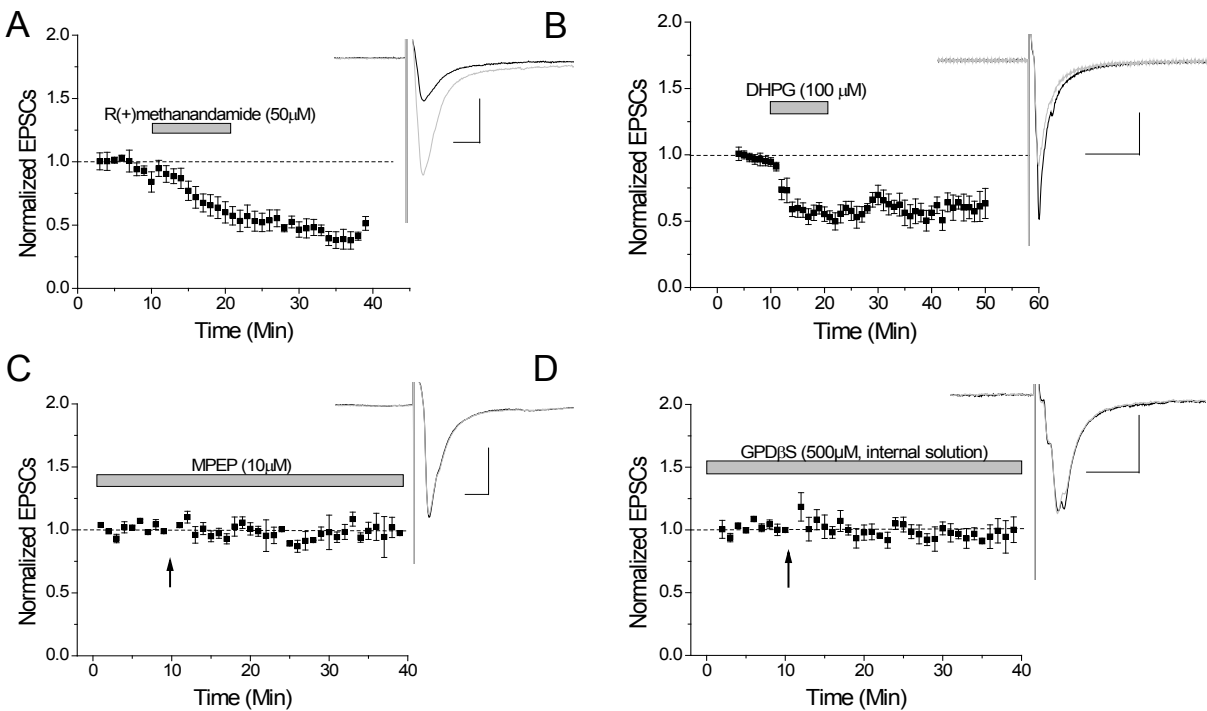
Figure 2.2: Endocannabinoids (eCBs) Are Likely Involved In VTA GABA Cell Plasticity.
eCBs are signaling molecules involved in presynaptic plasticity mechanisms. A. A non-hydrolyzable form of an eCB, R(+)methanandamide, induces depression of glutamatergic neurotransmission (n=6, p<0.001). B-C. Group 1 mGLuRs are normally required to signal for eCB synthesis. The Group 1 mGluR agonist DHPG caused depression similar to HFS-LTD (n=7, p<0.001) and the antagonist MPEP blocked HFS-LTD. Therefore, mGluR5 are required and sufficient for depression. D. To further test for the necessity of a post-synaptic g-protein coupled receptor (such as an mGluR), a GDP analogue GDPbS was added to the internal solution, and indeed blocked HFS-LTD (n=7).

Figure 2.3: CB1 Mediates HFS-LTD. Two candidate receptors that could act as a pre-synaptic target for eCBs include TRPV1 and CB1. A. The TRPV1 antagonist capsazepine failed to block LTD, suggesting TRPV1 is not involved in this HFS-LTD (n=9, p=0.014). B. The CB1 antagonist AM-251 blocked LTD (n=9, p=0.03 compared to HFS control).
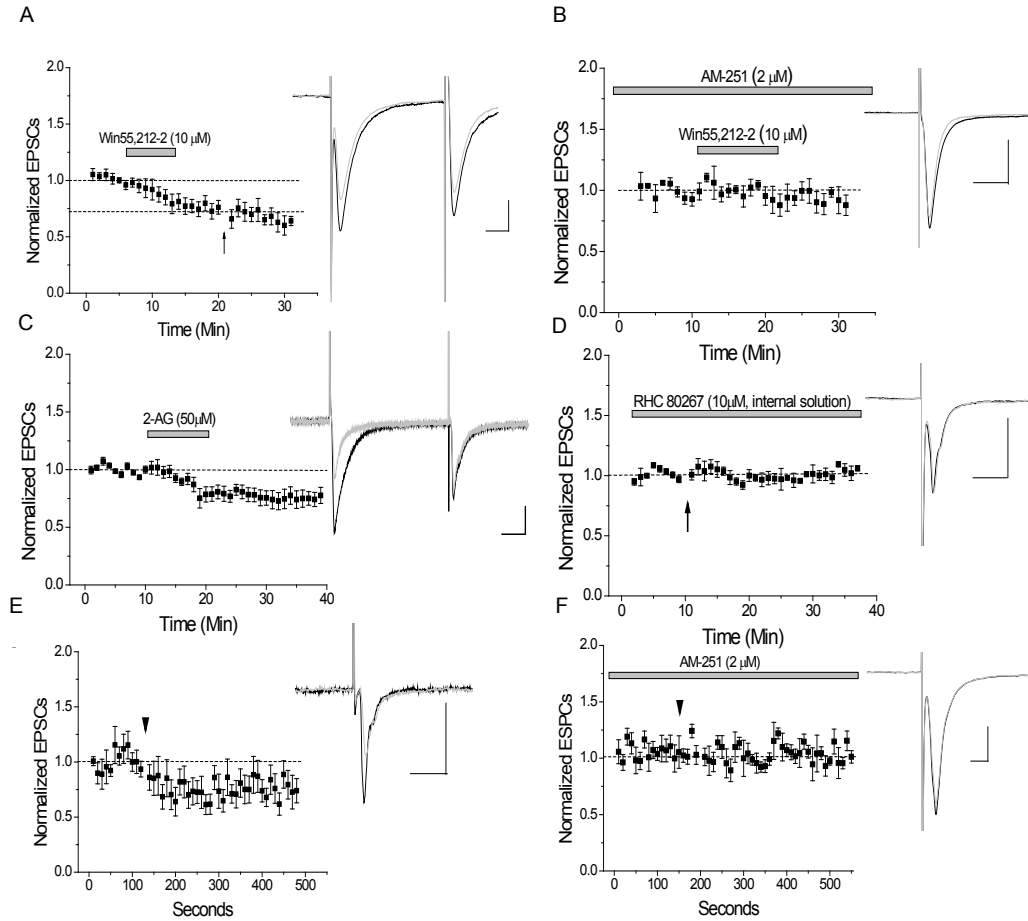
Figure 2.4: Post-Synaptic 2-AG Mediates LTD Of VTA GABA Cells. A. Application of the CB1 agonist Win55,212-2 depresses this synapse (n=6). There is a significant change between baseline and drug application (p=0.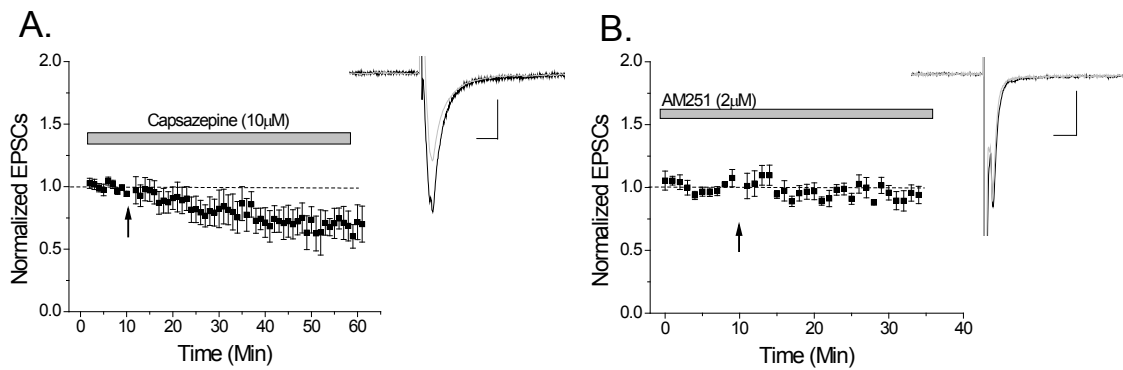002), but further HFS depression was occluded (p=0.57). B. When the CB1 antagonist AM251 was present Win55,212-2 no significant change was observed, further demonstrating the specificity of Win 55,212-2 for CB1 (n=5, p=0.86 compared to Win 55,212-2 only). C. Application of endogenous CB1 ligand 2-AG induces significant depression (n=9, p=0.03). D. When the DAGLα inhibitor RHC 80267 was applied to block postsynaptic production of 2-AG, HFS failed to produce a significant depression, suggesting that 2-AG is produced post-synaptically in the GABA neuron (n=6, p=0.49). E-F. To further test for post-synaptic eCB production, depolarization induced suppression of excitation (DSE) was employed. VTA GABA neurons were depolarized to 0 mV for 10 seconds to induce DSE. We observed a persistent depression following DSE protocol (n=7, p=0.01), which was CB1 dependent, as it was blocked by AM-251 (n=7).
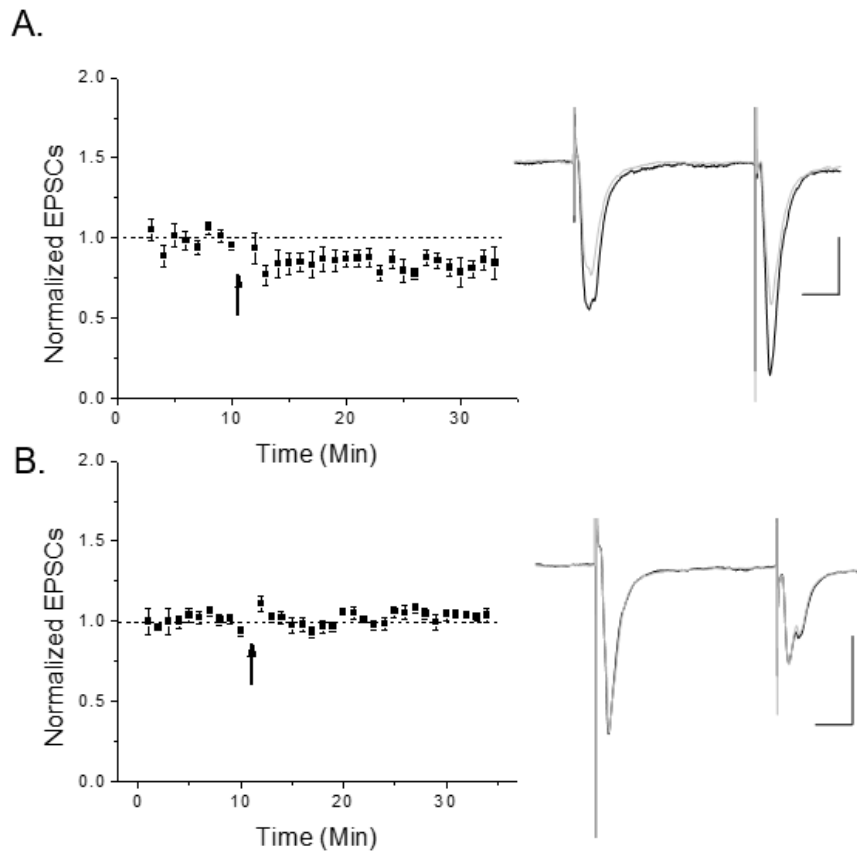
Figure 2.5: HFS-LTD Is Blocked In CB1 KO Mice. A. CB1 knock-out and heterozygous mice were used to confirm CB1 involvement in the VTA. HFS in heterozygous mice caused a significant depression compared to baseline (n=7, p=0.01). B. In CB1 knock-out mice, HFS failed to produce LTD, further demonstrating the importance of CB1 in this plasticity (n=9, p=0.34).

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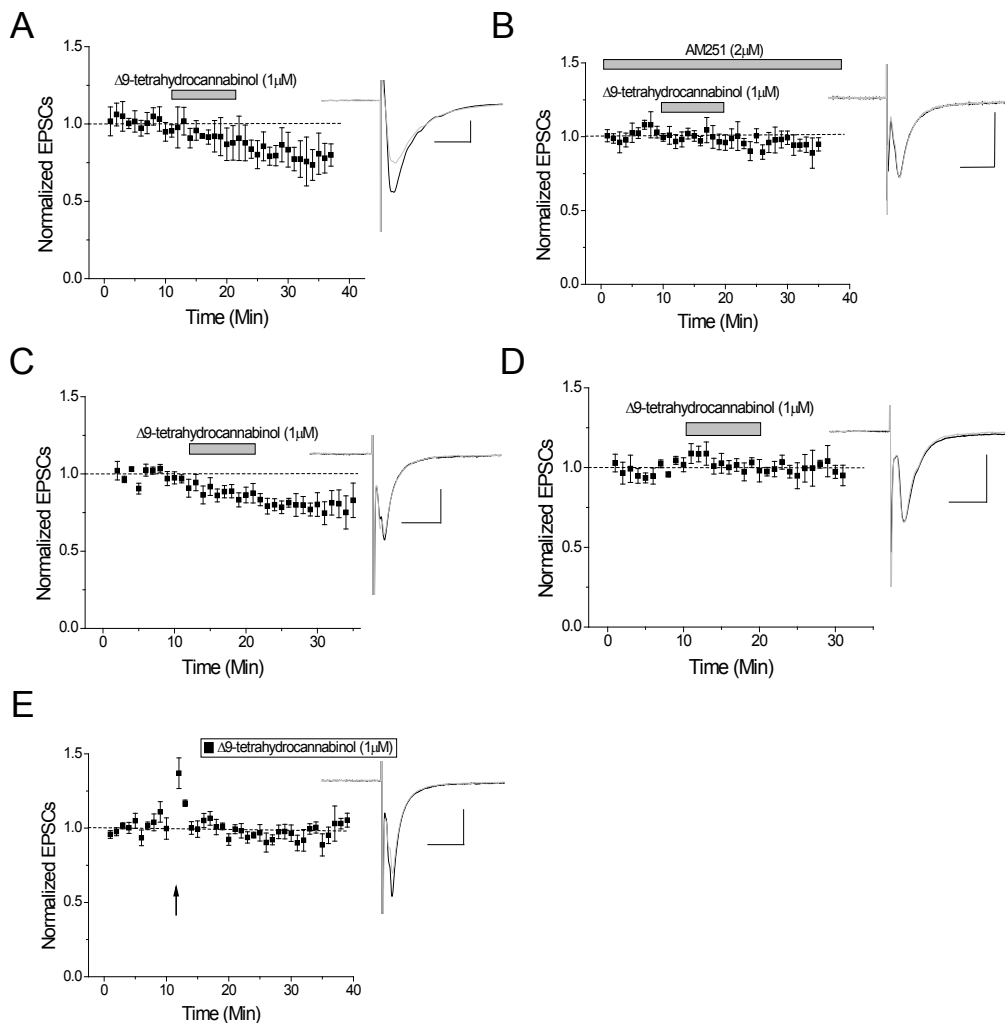
Figure 2.6: THC Induces A CB1-Dependent Depression. A. THC is the psychoactive component in marijuana, and a known CB1 agonist. When THC is applied to extracellular bath it produced a lasting depression (n=6, p<0.001). B. Extracellular exposure to THC failed to produce depression in the presence of AM251 (n=6, p=0.013, compared to THC without AM251). CB1 knock-out and heterozygous mice were used to confirm CB1 involvement. C. THC induced depression in heterozygous mice (n=7, p=0.02). D. In CB1 KO mice, THC failed to produce significant depression (n=9, p=.03). E. THC occluded HFS-LTD, suggesting THC and HFS-LTD use the same pathway (p=0.003).
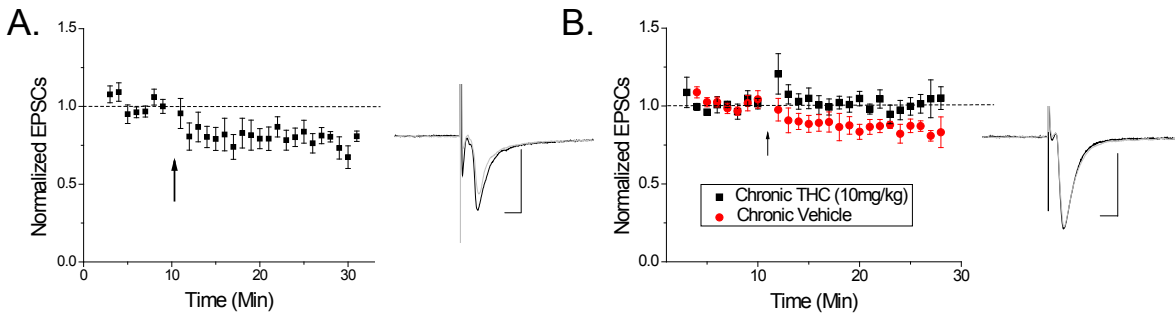
Figure 2.7: Chronic THC Occludes HFS-LTD. A. We examined the effects of administering THC via intraperitoneal injections. Chronic injections (7-10 consecutive days) of THC occluded further depression following HFS (n=6) B. A single THC injection failed to occlude LTD (n=7, p=0.019 compared to vehicle only controls). This suggests chronic THC is inducing synaptic modifications that maybe comparable to drug-induced plasticity changes that alter addiction behavior.
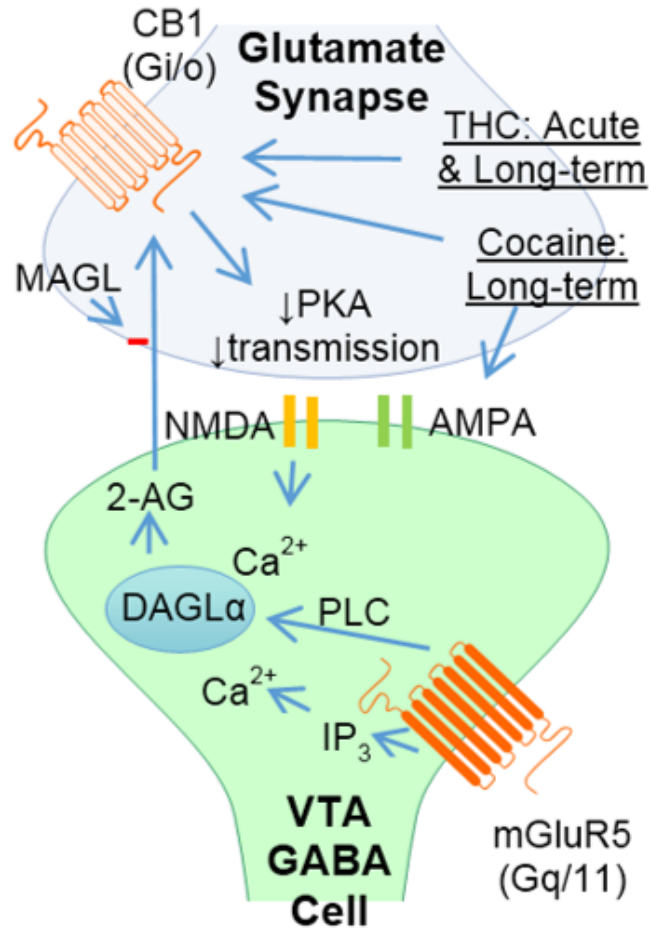
Figure 2.8: Current Working Model. HFS-LTD is induced by CB1 activation that is mediated by postsynaptically induced 2-AG, which is formed by PLC and DAGLα via mGluR5 activation and postsynaptic calcium release. THC modulates this synapse via the CB1 pathway and/or AMPA receptor expression.

CHAPTER 3: Acute and Chronic Cocaine Exposure Occludes LTD

in Ventral Tegmental Area GABA Neurons

Lindsey N. Friend, Jeffrey G. Edwards

Abstract

The ventral tegmental area in the midbrain modulates reward. Drugs of abuse will increase midbrain dopamine activity, and can alter ventral tegmental area glutamate plasticity leading to addiction. While dopamine cells are the principal mediator of reward, they are inhibited by nearby GABA cells. Our lab has demonstrated a form of pre-synaptic CB1 dependent long-term depression of glutamatergic inputs onto VTA GABA neurons. This plasticity is dependent on mGluR5 and post-synaptic diacylglycerol lipase alpha. Δ9-tetrahydrocannabinol, the active ingredient in marijuana can initiate this long-term depression at this synapse by acute slice application, and occlude this plasticity following chronic injections. Our aim was to determine whether cocaine can influence this plasticity. Using whole cell voltage-clamp electrophysiology in GAD67-GFP mouse VTA slices, excitatory inputs on GABA cells were isolated and recorded. Acute and chronic injections of cocaine were both sufficient to occlude long-term depression. Furthermore, chronic cocaine decreased AMPA/NMDA ratios, compared to vehicle only injections. The plasticity can be reversed to the naïve state, as long-term depression was again observed following 7 days of abstinence. This novel form of cocaine-evoked plasticity wherein VTA GABA cells can depress excitatory inputs, which could lead to disinhibition of nearby dopaminergic cells.

Introduction

Drug use leads to addiction and is a costly and debilitating disorder. Within the midbrain is the ventral tegmental area (VTA) which mediates motivation and reward. The VTA is

predominantly composed of dopamine cells that signal reward, and neighboring GABA cells that inhibit them [14]. These dopamine cells are the target of drugs of abuse. All addictive psychoactive substances examined to date induce long-term changes of excitatory synapses of VTA DA neurons, while non-addictive psychoactive substances do not [16].

Cocaine has a robust and profound effect on modifying dopaminergic plasticity, indeed cocaine modifies glutamatergic input to DA cells in every study examining this circuit in the VTA to date [54]. Synaptic changes mediated by cocaine in DA cells include inhibition of long-term plasticity [55], increases in AMPA/NMDA ratios [17], decreases in AMPA rectification indices [56], synaptic AMPA receptor subtype expression [57, 58], as well as indirectly altering endocannabinoid physiology [50, 51, 59]. These synaptic alterations may be able to revert to the naïve state following 1 week or more of abstinence [14].

Cocaine has been shown to influence CB1 plasticity, by influencing endocannabinoid release [50], occluding eCB mediated LTD in the nucleus accumbens [49], which makes it an ideal candidate drug to modify endocannabinoid plasticity. These effects have been studied at synapses onto VTA dopamine cells, however, the effect of acute or chronic cocaine on glutamatergic plasticity on GABA cells is unknown.

We have previously identified a novel form of plasticity on VTA GABA cells, that is initiated by post-synaptic mGluR5, which leads to produce 2-AG synthesis that acts on presynaptic cannabinoid 1 receptors. Δ9- tetrahydrocannabinol (THC), the active ingredient in marijuana, can mimic this plasticity following slice application, and occlude it following chronic intraperitoneal injections. Our goal was to examine the effect of cocaine on glutamate plasticity in VTA GABA cells.

Methods

All experiments were performed in accordance with Institutional Animal Care and Use Committee protocols and followed NIH guidelines for the care and use of laboratory animals. IACUC protocols for all experimental protocols were approved by the Brigham Young University Institutional Animal Care and Use Committee, Animal Welfare Assurance Number A3783-01.

Male Cd1 GAD67-GFP knock-in mice were used to identify GABA cells in the VTA. 15-30 day old mice were anesthetized with isoflurane and decapitated with a rodent guillotine. Brains were rapidly removed and sectioned horizontally on a vibratome at 300 μm. Recordings began at least one hour after cutting while tissue was stored in oxygenated cerebral spinal fluid composed of 119 mM NaCl, 26 mM NaHCO3, 2.5mM KCl, 1 mM NaH2PO4, 2.5mM CaCl2, 1.3mM MgSO4, and 11mM glucose. 4mM picrotoxin (Abcam) was added to the recording solution to block GABA currents. Spermine (0.1 mM) will be included in the patch electrode to avoid polyamine dialyzation to maintain its block of GluR2-lacking AMPA receptors at depolarized potentials, and is used to determine the subunit composition of synaptic AMPA receptors via I-V plots.

The ventral tegmental area was visualized using an Olympus, BX51W1 microscope with a 40x water immersion objective. Patch pipette resistance was 2.5–4.5 MΩ. Cells were held at −65 mV prior to recording. Cells were patched with a glass pipette filled with internal solution composed of 117mM potassium gluconate, 2.8mM NaCl, 20mM HEPES, 5mM MgCl2, and QX-314 (Tocris) (pH 7.28, 275–285 mOsm). Cells were recorded under voltage clamp mode at -65 mV. Traces were recorded using Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). Signals were filtered at 4 kHz and digitized with an Axon 1440A digitizer

(Molecular Devices) connected to a Dell personal computer with pClamp 10.2 Clampfit software (Molecular Devices).

Plasticity was induced using 2 stimulations at 100Hz, 2 times, 20 seconds apart. AMPA/NMDA ratios were obtained by patching cells at -70mV, allowing at least 10 minutes for spermine to dialyze into the cell. The holding was raised to 40mV and APV was applied to isolate the AMPA current. For IV plots, cells were stepped down from 40mV to -70mV, in 10mV increments. All other p-values were obtained by a students t-test comparing the 5 minutes before conditioning to 15-20 minutes post conditioning.
Abcam supplied Picrotoxin (0.2mM). Tocris supplied APV (50µL). Sigma provided spermine (0.1mM in internal pipette solution) and cocaine HCl which was administered via intra-peritoneal injections at 15 mg/kg.

Results

Ventral tegmental area GABA neurons were positively identified using a GAD67-GFP knock-in mouse line. These cells were patched using whole-cell voltage clamp electrophysiology to record glutamate EPSCs. To induce GABA cells glutamatergic plasticity we used a 100Hz high frequency stimulus (HFS) protocol. Following HFS there was a 40% reduction in EPSC amplitude following HFS (Figure 3.1A). As many forms of glutamatergic plasticity require NMDA, we examined the potential involvement of NMDA receptors. We observed HFS-LTD was blocked in the presence of the NMDA receptor antagonist APV.

As cocaine is known to modify glutamatergic synapses to dopamine cells that are thought to correlate to the creation of addiction and drug-addictive behavioral changes, we examined the effect of cocaine on this novel plasticity in GABA cells.

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In order to investigate the possible role of cocaine in modulating this LTD, mice were exposed to cocaine *in vivo* via intraperitoneal injections. Following chronic cocaine injections, HFS failed to induce LTD (Figure 3.2A), which was significantly different than the chronic saline vehicle injections (Figure 3.1A). Similarly, a single injection 24 hours prior to recording also blocked LTD (Figure 3.2B). Cocaine-evoked plasticity can be reversed to the naïve state following a period of abstinence. In order to determine whether LTD can be recovered, mice were injected with a single dose of cocaine, followed by 7 days of abstinence. HFS induced LTD similar to naïve mice (Figure 3.2C), suggesting synaptic changes following cocaine exposure do not persist less than 7 days. These studies suggest cocaine can influence plasticity of VTA GABA neurons, following a single injection.

An additional indication of drug evoked synaptic plasticity is alterations in AMPA to NMDA ratios. VTA GABA neurons were patched and initially held at -70mV with spermine in the intracellular solution. After waiting for 10 minutes for the spermine to dialyze into the cell, the holding potential was raised to 40mV. Glutamate currents were recorded before and after the application of the NMDA antagonist APV, and the ratios of AMPA to NMDA currents were calculated. The AMPA/NMDA ratio from control mice exposed to chronic saline averaged 1.15, while mice treated with chronic cocaine had a significantly decreased AMPA/NDMA ratio, averaging 0.75 (Figure 3.2A).

In order to determine whether decreased AMPA/NMDA ratio was accompanied with alterations in AMPA subunit expression, we analyzed IV plots to look for changes in rectification that are indicative of GluR2-lacking AMPA subunits. AMPA currents were measured from 40mV to -70mV, in 10mV steps. There was no significant difference in the rectification of the AMPA currents, suggesting the change in AMPA/NMDA ratio was not

accompanied by a change in AMPA subunit expression, but overall reduction in AMPA receptor numbers (Figure 3.2 B).

Discussion

The results of the present study demonstrate the role VTA GABA neurons have in reward physiology, and show an additional mechanism through which cocaine mediates synaptic plasticity. Long-term depression can be initiated by 100Hz high frequency stimulus, and this plasticity is blocked by both acute and chronic intraperitoneal injections of cocaine. One injection of cocaine, followed by 7 days of abstinence allows the synapse to revert to a more naïve form, wherein LTD is still elicited.

Previous studies have shown that increased GABA cell activity inhibits DA cell activity and DA release [28, 30], therefore a depression of excitatory inputs onto GABA cells could disinhibit dopamine release and increase reward. Thus, this depression could be an additional means whereby the rewarding effects of cocaine are mediated.

Cocaine has been shown to influence excitatory plasticity in VTA dopamine cells [56], and in medium spiny neurons projecting to the VTA [29]. Therefore, it is likely that cocaine could have an impact influencing LTD at excitatory synapses to GABA cells, although to date there has not been a study examining this effect. Cocaine has been implicated in modulating plasticity through CB1 receptors. For example, cocaine can alter plasticity of GABA inputs onto VTA DA cells [60]. This is particularly relevant as we have previously shown LTD at this synapse is dependent on endocannabinoid signals and CB1 receptors.

An additional measure of drug-evoked plasticity is alterations in the ratio of AMPA to NMDA receptors, as well as AMPA subunit expression. Changes in the AMPA receptor subunit composition have been implicated in cocaine cravings and relapse [61]. Cocaine specifically can

signal for the insertion of GluR2-lacking AMPA receptors, which are unique in their ability to conduct calcium [62]. The plasticity described here does not appear to signal for GluR2 lacking AMPA receptors, as the IV plots for the cocaine-treated animals are not strongly inwardly rectifying. However, calcium could still be conducted through NMDA receptors and used as a second messenger. NMDA receptors are required for the LTD described here and also for other types of cocaine-evoked plasticity [63], as well as conditioned place preference [64].

Significance

Here we report a novel plasticity through which cocaine can modulate reward physiology. This change can occur following a single exposure to cocaine. If GABA neurons undergo long-term depression following cocaine use, this could result in disinhibition of the neighboring dopamine neurons that signal reward.

Acknowledgements
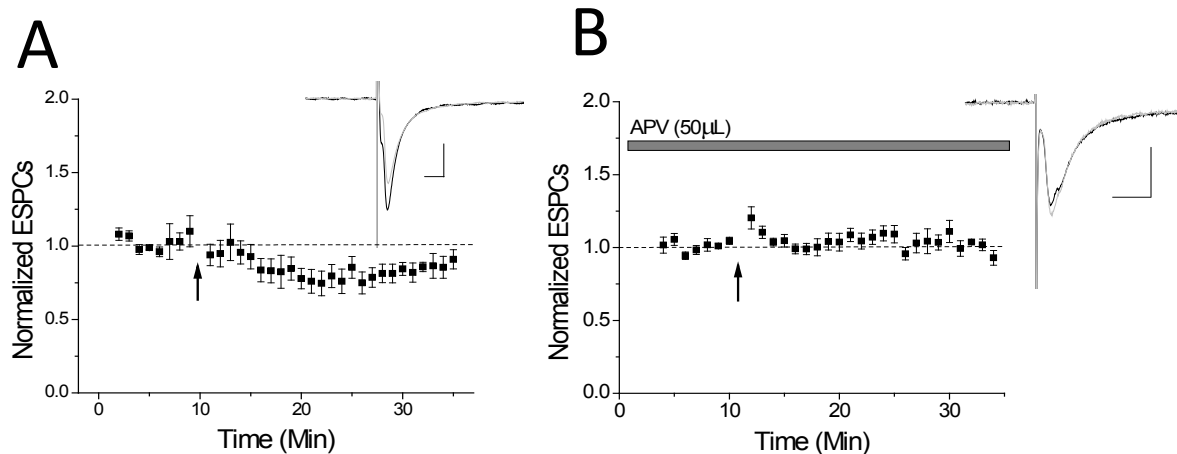
Figure 3.1: Long-Term Depression (LTD) In VTA GABA Cells Is NMDA-Dependent. A. Glutamate currents recorded from GAD67-GFP positive VTA GABA cells from mice chronically injected with saline. 100Hz high frequency stimulus (HFS) induces long-term depression (n=6, p>0.05). B. In the presence of the NMDA receptor antagonist APV blocked HFS-induced LTD (n=9), demonstrating dependence on NMDA receptors. Plots, mean with s.e.m. Arrow indicates 100Hz high frequency stimulus (HFS). Scale bars 100 pA, 10 msec. Plots, mean with s.e.m. Arrow indicates 100Hz high frequency stimulus (HFS).
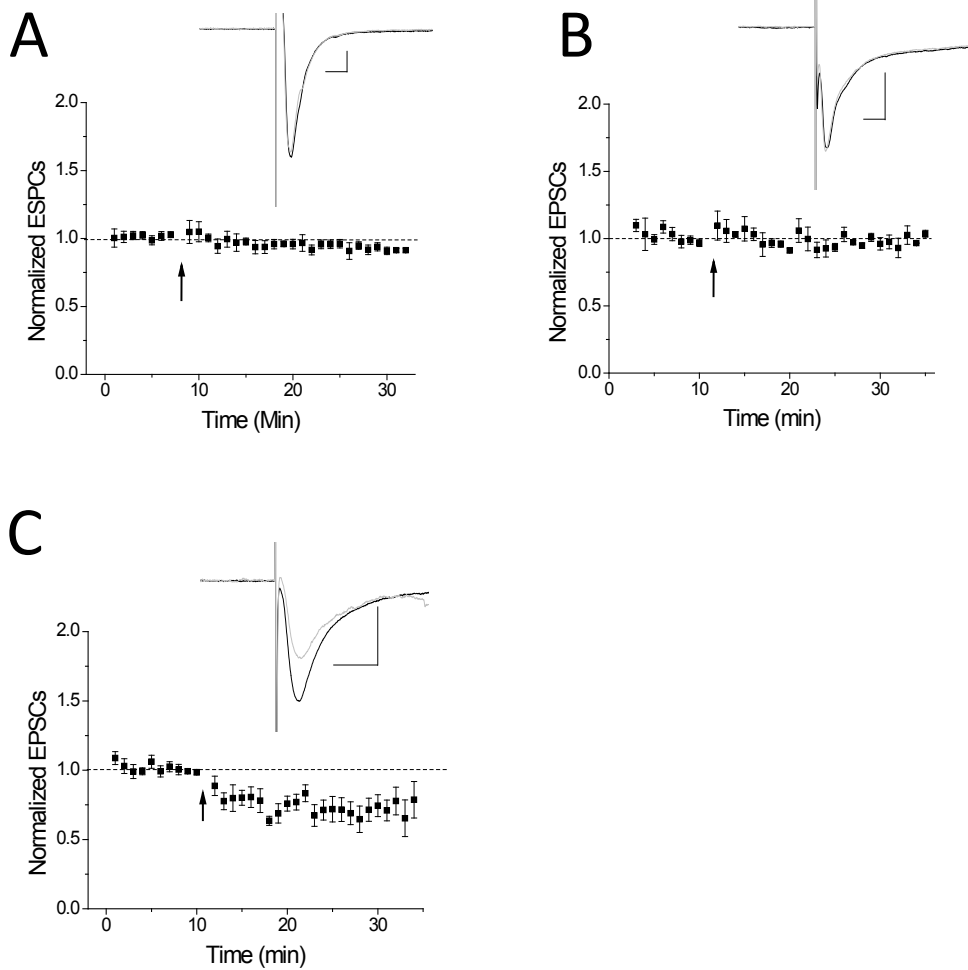
Figure 3.2: Chronic And Acute Cocaine Injections Block HFS-LTD. A. Chronic (7-10 consecutive days of intraperitoneal injections blocks HFS-induced LTD (n=6, p>0.05). B. Similarly, 1 injection of cocaine is sufficient to block LTD (n=7, p>0.05) C. To determine whether this plasticity can revert to the naïve state, mice were given a single injection of cocaine, and plasticity was measured 7 days later. We again observed significant depression following 7 days of abstinence, suggesting the synapse can revert to a native state (n=7, p>0.05).

A

B

C
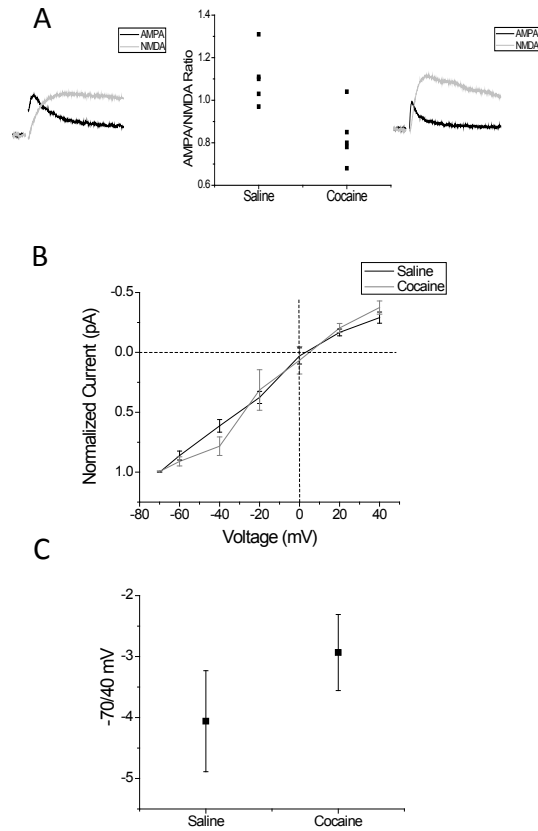
Figure 3.3: Cocaine Decreases AMPA/NMDA Ratios. A. Alterations in the ratio of AMPA/NMDA current provides additional evidence for plasticity. Chronic cocaine injections significantly decreases AMPA/NMDA ratios (n=5, p<0.05). B. IV plots for chronic cocaine and saline treated mice. There was no significant difference in rectification indices between the experimental groups (n=6). C. Measuring current at -70mV and at -40mV is another way to determine if there are changes in rectification. No significant change was observed (n=6).

CHAPTER 4: Hippocampal Stratum Oriens Interneurons undergo a CB1-dependent Potentiation

Lindsey Friend, Ryan Williamson, Collin Merrill, Scott Newton,
Michael Christensen, Jeffrey G. Edwards

Abstract

The hippocampus is thought to mediate learning and memory by altering synaptic

strength within its circuitry. In many cases, synaptic plasticity can be induced by signaling

molecules such as lipid-based endocannabinoids like anandamide. Endocannabinoids modulate

synaptic plasticity of hippocampal pyramidal cells and stratum radiatum interneurons; however,

the role of endocannabinoids in mediating synaptic plasticity among interneurons in the stratum

oriens is still unclear. These interneurons are feedforward inhibitory cells that have unique

synaptic plasticity compared to those in the radiatum as they exhibit presynaptic LTP, rather than

LTD. This plasticity is TRPV1 and nNOS independent. We therefore examined whether oriens

interneurons can produce endocannabinoids and whether these might be involved in presynaptic

LTP. Using patch-clamp electrodes to extract single cells, we analyzed the expression of

endocannabinoid biosynthetic enzyme mRNA using real-time, reverse transcription PCR. The

cellular expression of several calcium-binding proteins and neuropeptides were used to identify

interneuron subtypes. We also analyzed cellular expression of several endocannabinoid

biosynthetic enzymes. qPCR results demonstrate that sratum oriens interneurons do express

mRNA for both biosynthetic enzymes and the type I mGluRs necessary for their production. To

test the role of endocannabinoids in synaptic plasticity, we performed whole-cell experiments

and measured glutamate currents in the presence of a fatty acid amide hydrolase inhibitor

(URB597; 1μM), which increases endogenous anandamide. Interestingly, URB597 potentiated

stratum oriens interneurons in a CB1-dependent manner as it was blocked by AM-251 (2 μM).

Furthermore, high frequency stimulus can induce long-term potentiation in somatostatin-positive cells. This plasticity was also blocked by AM-251, further demonstrating CB1 dependence. Collectively, this suggests oriens interneurons express the cellular machinery needed for endocannabinoid production, and can alter their plasticity in response to anandamide signal.

Introduction

Synaptic Plasticity is a process whereby synapses can be strengthened or weakened based on the activity of the cells around them by either presynaptically altering neurotransmitter release, or postsynaptically modifying the receptor trafficking to and from the membrane. Plasticity is a critical event that allows for modification of brain in an experience-dependent fashion. Two major forms of synaptic plasticity are present in the brain, Long-term potentiation (LTP) and long-term depression (LTD). For post-synaptic LTP to occur, there must be a large calcium ion influx through N-Methyl-D-Aspartate (NMDA) glutamate receptors, which triggers α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors to be inserted into the membrane [65]. Plasticity has been observed in various brain regions, notably, the hippocampus where it is crucial for memory encoding and consolidation. Other less well known forms of plasticity in addition to NMDA-dependent forms also occur in the brain that are mediated by various other mechanisms such as lipid messengers known as endocannabinoids.

Endocannabinoids (eCBs) are produced by components of the lipid membrane, a reaction catabolized by various enzymes, for example phospholipase D, diacylglycerol lipase alpha (DAGLα), 12, lipoxygenase, normally following second messenger activation [4, 6, 23, 33, 66]. Usually, upon post-synaptic activation of type 1 metabotropic glutamate receptors, enzymes are recruited to produce eCBs which then retrogradely act on presynaptic receptors to alter neurotransmission via g-protein coupled channels such as cannabinoid receptor 1 (CB1) [3]. The two best characterized endocannabinoids are anandamide and 2-arachadonyl glycerol (2-AG). Anandamide, a common eCB, is produced by n-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) and can bind to CB1, TRPV1 and other GPRs throughout the brain. It is then degraded by the enzyme fatty acid amide hydrolase (FAAH) [4, 5]. 2-AG is produced in the

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brain by diacylglycerol lipase alpha (DAGLα) and is also involved in retrograde signaling via CB1 [6] and other receptors. It remains unknown whether some or all of the various subtypes of oriens interneurons utilize this form of plasticity.

Distributed in hippocampal layers are both excitatory and inhibitory neurons. Excitatory pyramidal cells are located in stratum pyramidale and diverse inhibitory interneurons are present in stratum radiatum and stratum oriens [2]. Hippocampal interneurons are a diverse group, and can be classified into several different subtypes based on their function, projections and firing pattern. Often interneurons are characterized into groups using calcium binding proteins and neuropeptides, including somatostatin, cholecystokinin and parvalbumin. The stratum oriens of the CA1 region of the hippocampus houses interneurons of varying types. Oriens lacunosum-moleculare (O-LM) interneurons are one such group, and can be identified by the expression of somatostatin, as well as a regular and fast spiking pattern and sag potential upon injection of hyperpolarizing current [7, 8]. O-LM cells are named for their morphology, with somas residing in stratum oriens and distant projections to stratum lacunosum moleculare. Another subtype is parvalbumin positive basket cells, which project locally and lack a sag potential. These cells are also involved in network oscillations in the hippocampus [9]. While projections and spiking patterns are well defined in these neurons, information regarding the mechanism of eCB-dependent plasticity of these interneurons is scarce but promising.

Stratum oriens interneurons exhibit plasticity, but markedly unique compared to neighboring interneuron layers such as the stratum radiatum. For example, the group 1 mGluR agonist DHPG will depress radiatum interneurons [67], but it will potentiate oriens interneurons [68]. There is an anti-hebbian form of pre-synaptic potentiation that can be induced in putative O-LM cells by 100 Hz high frequency stimulus (HFS), which is independent of nitric oxide and

TRPV1 [8], but dependent on mGluRs, post-synaptic calcium and M1 muscarinic receptors [69]. our goal was to determine whether this plasticity is dependent on eCBs.

Based on the role interneurons play in feed-forward inhibition of CA1 pyramidal cells and widespread network control and oscillations, and given they also undergo plasticity, it is important to characterize this novel form of LTP. Here we report stratum oriens interneurons have the capability to produce eCB biosynthetic enzymes, and can induce LTP in a CB1-dependent manner.

Methods

All experiments were performed in accordance with Institutional Animal Care and Use Committee protocols and followed the NIH guidelines for the care and use of laboratory animals. Male Sprague-Dawley rats age 15-30 days old were used for all electrophysiological experiments. Rats were anesthetized with isoflurane and decapitated. Brains were removed and sectioned coronally on a vibratome at 400 μm. Recordings began at least one hour after cutting while tissue was stored in oxygenated cerebral spinal fluid composed of 119 mM NaCl, 26 mM NaHCO3, 2.5mM KCl, 1 mM NaH2PO4, 2.5mM CaCl2, 1.3mM MgSO4, and 11mM glucose. 4mM picrotoxin (Abcam) was added to the recording solution to block GABA currents.

Hippocampal oriens CA1 cells were visualized using an Olympus, BX51WI microscope with a 40x water immersion objective. Cells were patched with a glass pipette filled with internal solution composed of 117mM potassium gluconate, 2.8mM NaCl, 20mM HEPES, 5mM MgCl2, and QX-314 (Tocris) (pH 7.28, 275–285 mOsm). Cells were recorded under voltage clamp mode at -65 mV. Plasticity was induced using 2 stimulations at 100Hz, 20 seconds apart, while the cell was in current clamp, then recording resumed in voltage clamp mode. Traces were recorded using Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). Signals were

filtered at 4 kHz and digitized with an Axon 1440A digitizer (Molecular Devices) connected to a Dell personal computer with pClamp 10.2 Clampfit software (Molecular Devices).

Cells used for PCR analysis were extracted using gentle suction and placed into chilled reverse transcriptase reagents (BioRad) within 2 hours. One control sample of artificial cerebral spinal fluid was obtained for each slice and used to identify contamination from extracellular mRNA. Using iScript cDNA Synthesis kit (BioRad), extracted cells were reverse transcribed to cDNA under the manufacturers protocol and cycled in a C1000 Thermocycler (BioRad) at 25°C for 8 min, 42°C for 60 min, and 70°C for 15 min. Following reverse transcription, each cell was divided into three 5μL aliquots which each received a different group of 10-fold diluted primers, iQ Supermix (BioRad), and ddH20. Additional no template control tests were done to ensure there were no primer dimer or hairpin interactions. The samples were then cycled in a C1000 Thermocycler (BioRad) starting at 95°C for 3 minutes, then 15 cycles of 95°C for 15 minutes, 57°C for 20 seconds, and 72°C for 25 seconds. All other p-values were obtained by a students t-test comparing the 5 minutes before conditioning to 15-20 minutes post conditioning.

Most primer and probe sequences were used in our previous study [70]. Primers were designed for somatostatin using the same parameters, efficiency and melting temperature using the Vector NTI software. The somatostatin forward, reverse and probe sequences used were ACCCCAGACTCCGTCAGTTTC, GTTGGGCTCAGACAGCAGTTCT, and ACCGGGAAACAGGAACTGGCCAAGT respectively.

Each pre-amplified cell was run for every target individually and in triplicate. Each cell was run in a CFX96 qPCR machine (BioRad) with a 95°C hot start for 3 minutes, followed by 60 cycles of 95°C for 15 seconds, 57°C for 25 seconds, and 72°C for 25 seconds. Cycle threshold (Ct) values were determined by the BioRad software.

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Results

The goal of our study was to investigate the role of endocannabinoid signaling in mediating LTP in stratum oriens interneurons. We hypothesized that stratum oriens inhibitory neurons possess the machinery necessary for endocannabinoid synthesis and that this machinery was involved in an interneuron mediated LTP. To test this, we extracted putative inhibitory neurons from stratum oriens using patch clamp electrophysiology and then performed RT-qPCR to examine components involved in endocannabinoid biosynthesis. Classification of cells as interneurons was done based on GAD65 and GAD67 mRNA expression. In addition, we further classified putative inhibitory neurons into three subtypes: somatostatin and cholecystokinin expression (SOM+/CCK+), parvalbumin expressing (PV+), and calretinin or calbindin expressing (CR+/CB+). Our results are summarized in Table 1. SOM+/CCK+ neurons were the most abundant (n=14) and expressed mRNA for DAGLα, NAPE-PLD, 12-Lipoxygenase and type 1 mGluRs. PV+ cells were the second largest group, and expressed mRNA for NAPE-PLD, 12-Lipoxygenase and type 1 mGluRs. CR+/CB+ cells were the minority, and only one such cell expressed mRNA for any of the eCB enzymes.

We next used patch clamp electrophysiology to examine the physiological role of eCB production in previously identified stratum oriens potentiation. To do this, we first recorded glutamate EPSCs from oriens interneurons before and after a fatty-acid amide hydrolase (FAAH) inhibitor was applied to the extracellular solution. FAAH inhibition prevents anandamide hydrolysis and increases synaptic levels of anandamide. We found FAAH inhibition produced a significant potentiation compared to baseline (Figure 4.1A). This potentiation was blocked by the CB1 antagonist AM-251 (Figure 4.1B) demonstrating CB1 dependence.

To determine whether the plasticity seen with the FAAH inhibitor is related to the plasticity previously observed, 100Hz high frequency stimulation (HFS), we applied HFS to oriens interneurons (Figure 4.2A) and saw LTP in 63% of interneurons. LTP was blocked the observed plasticity with AM251 (Figure 4.2B). These experiments further confirm the involvement of CB1. When the mRNA was examined, we observed that somatostatin positive cells would potentiate (Figure 4.2C), while somatostatin negative cells would not (Figure 4.2D). Furthermore, LTP of somatostatin-positive neurons was blocked in the presence of AM-251 (Figure 4.2E). This confirms LTP occurs only in O-LM cells, and that this LTP is dependent on CB1.

As the PCR data suggested that oriens interneurons could produce the proteins necessary to produce the anandamide involved in this plasticity, we performed a test of eCB production known as depolarization-induced suppression of excitation (DSE). In this protocol, cells were initially held at -65mV, then raised to 0mV for 10 seconds, after which the cell remained at -65mV for the duration of the experiment. DSE experiments in oriens interneurons showed no change glutamate current amplitude, suggesting that the eCBs involved in plasticity are likely made elsewhere (Figure 4.3).

Discussion

We found that parvalbumin and somatostatin positive cells express mRNA required to receive and produce an eCB signal, and that the eCB anandamide can be used to potentiate oriens interneurons in a CB1 dependent manner. Single-cell PCR revealed that somataostatin-positive cells will respond to HFS. This is the first demonstration of eCB biosynthetic enzyme expression in a subtype-specific manner, and CB1 dependence in oriens LTP.

Hippocampal levels of type 1 mGluRs have been previously studied, and appeared higher than those reported here [68, 71]. Single cell reverse-transcriptase polymerase chain reaction (RT-PCR) has been employed extensively to quantitatively test levels of mRNA, however, this method can slightly underestimate the actual levels. This discrepancy could have resulted from alterations in primer efficiency or be an effect of multiplexing. It is likely that the actual proportion of oriens interneurons that express type1 mGluRs is equal to or greater than the proportion of neurons that exhibited anandamide dependent LTP, as mGluRs are required to signal PLD, and therefore anandamide production.

Several subtypes of oriens interneurons have been identified based on their morphology, protein content, and plasticity. The relative ratios of oriens cell types shown here is similar to what others have seen in the hippocampus [2]. For example, the majority cell type observed was somatostatin-positive O-LM cells. Somatostatin-positive neurons have been previously shown to undergo LTP [72], which is consistent with our current findings. Additionally, calretinin/calbindin positive cells were the minority. Calretinin-positive cells have been implicated in regulating the activity of other interneurons, and tend not to possess eCB machinery [73].

Endocannabinoid signaling in the hippocampus has been previously studied by multiple groups [74], and has particular importance for GABA cells [75]. Therefore, it was likely that oriens interneurons also participate in this type of signaling. The radiatum and pyramidal cell layers express eCB mRNA [70], however, this is the first study that examined the eCB synthetic capability of oriens interneurons.

The anandamide-dependent plasticity observed here signals through CB1 receptors. Typically CB1 activation results in long-term depression [76]. However, a presynaptic CB1-

mediated potentiation is consistent with Kullmann et al observations. The mechanism through which CB1 signals for potentiation is unknown, although there are several possibilities. For example, CB1 could be coupling to different g-proteins intracellularly or signaling for increases in terminal size. Alternatively, the increased anandamide levels could be causing a decrease in 2-AG levels, as observed in the striatum [77], thus, disinhibiting oriens interneurons. Indeed, this is a matter of current investigation.

Significance

The results of the present study demonstrate hippocampal stratum oriens interneurons have the potential to make eCB synthetic proteins and receptors. Further, oriens cells can use anandamide to potentiate glutamatergic inputs in a CB1 dependent manner. This is also the first demonstration of a CB1-dependent long-term potentiation in the hippocampus, suggesting a broader and more variable role of CB1 and other endocannabinoids. The diverse subtypes of oriens interneurons have projections throughout the hippocampus, and understanding the plasticity of these cells is a step to understanding hippocampal circuitry overall.

Acknowledgements

Table 4.1: Summary Of RT-PCR Data. Individual stratum oriens interneurons were patched and extracted for mRNA examination. Cells were sorted by subtype and by endocannabinoid synthetic enzyme expression. Somatostatin-positive cells were the most abundant, followed by parvalbumin positive cells, and finally calretinin/calbindin interneurons. Somatostatin and parvalbumin positive cells had the highest expression of endocannabinoid and mGluR related mRNA.

Table 1. eCB mRNA expression in Oriens Interneurons				
	DAGLα	NAPE-PLD	12-Lipo	Type 1 mGluRs
SOM/CCK n=14	6	4	1	3
PV n=10	1	5	1	5
CB or CR n=5		1	1	
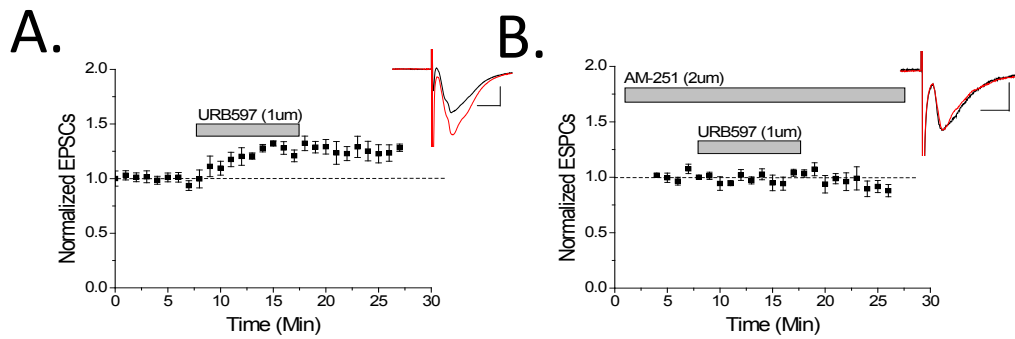
Figure 4.1: FAAH Inhibition And HFS Potentiate Oriens Interneurons In A CB1 Dependent Manner. Stratum oriens interneurons were patched (n=8) in whole cell mode and recorded while the enzyme fatty acid amide hydrolase (FAAH) was inhibited by URB-597. A. A significant (p>.05) potentiating effect was observed suggesting a role of anandamide in oriens synaptic plasticity. B. FAAH induced potentiation is blocked in the presence of the CB1 antagonist AM-251 (n=7). Scale bars 100 pA, 10 msec. Plots, mean with s.e.m. Arrow indicates 100Hz high frequency stimulus (HFS).
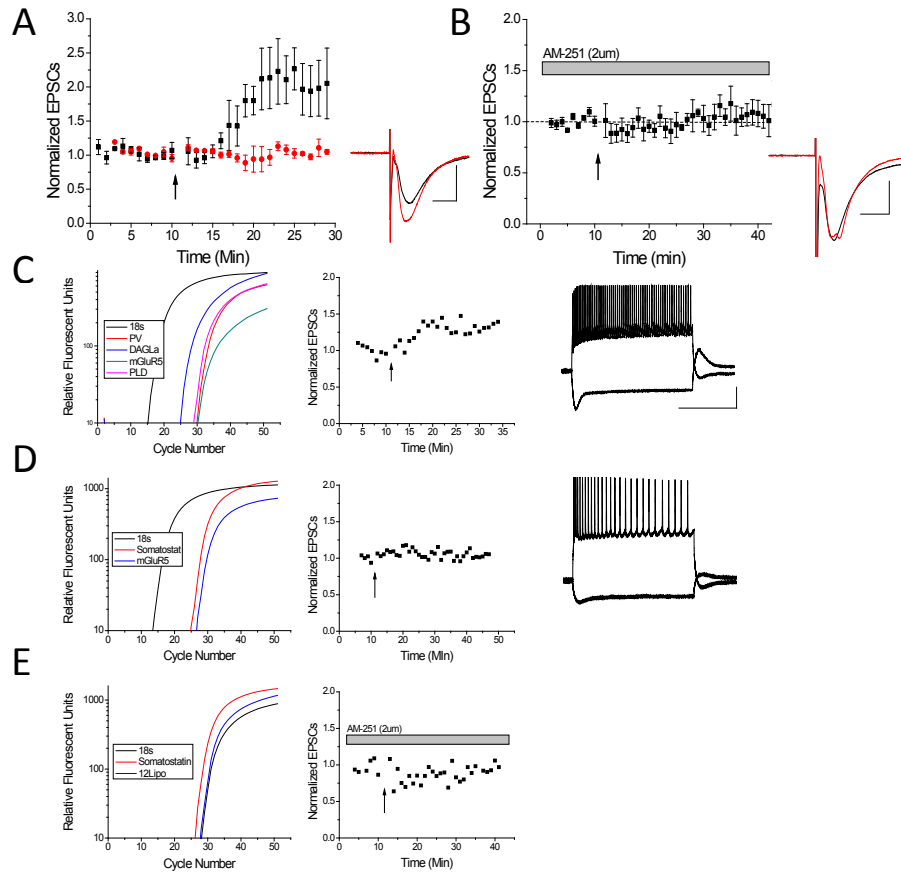
Figure 4.2: HFS-LTP Only Occurs In Somatostatin-Positive Interneurons, And Is CB1 Dependent. A) Oriens interneurons were patched and exposed to 100 Hz HFS (Figure 2.A) A significant potentiation followed (n=8, p<0.05), which was blocked by AM-251 (Figure 2.B), suggesting it is also dependent on CB1 C. An example cell that was somatostatin-positive, and exhibited HFS-LTD, along with an O-LM spiking profile. D. Parvalbumin-positive (somatostatin negative) neuron that did not potentiate following HFS, with spiking figure typical for PV-positive interneurons. E. A somatostatin-positive neuron, that likely could have potentiated. This cell did not exhibit plasticity in the presence of the CB1 antagonist AM251. Collectively, these experiments suggest that parvalbumin positive cells will not potentiate, somatostatin positive (putative O-LM cells) can exhibit LTD following HFS, and that this plasticity is dependent on CB1 receptors.
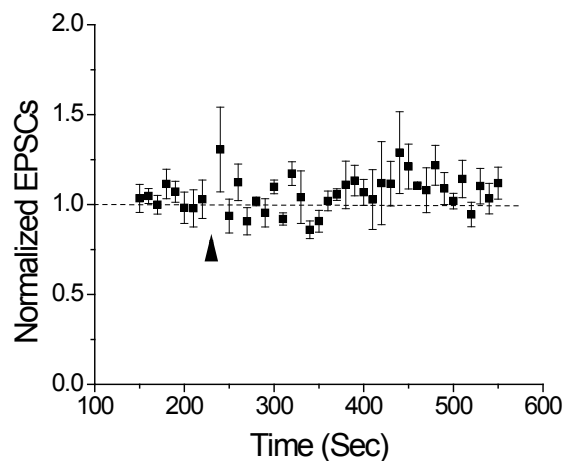
Figure 4.3: No Change In DSE Suggests eCBs Involved In LTP Are Not Made In The Oreins. Depolarization-induced suppression of excitation experiments to determine whether the eCBs involved in the CB1-dependent plasticity were produced in the oriens interneurons. Interneurons were depolarized to 0mV for 10 seconds, then returned to -65mV. There was no significant change following 10 second depolarization to 0mV, suggesting eCBs involved in this plasticity are not made in the oriens (n=6).

CHAPTER 5: Conclusion

The purpose of this work was to determine the mechanism of plasticity in VTA GABA neurons, and in stratum oriens interneurons. In the VTA, we described a novel form of pre-synaptic plasticity dependent on CB1. This plasticity can be occluded by THC and cocaine, demonstrating a link between drugs of abuse and the LTD here observed. In the oriens, we showed eCB biosynthetic capability, as well as a unique type of CB1-dependent LTP.

Future studies will examine the location of the glutamatergic inputs onto VTA GABA cells that participate in the LTD described here. Once they can be isolated and selectively activated, dopamine cells will be recorded before and after LTD induction to confirm disinhibition of dopamine cells. For the LTP in oriens interneurons, future work can be done to elucidate the unique signaling cascades that allow CB1 to initiate potentiation rather than depression.

Collectively, these experiments illustrate the importance of eCBs in modifying synaptic plasticity in the brain. eCBs can be use variably to promote LTD or LTP. Furthermore, eCBs can participate in modifying plasticity involved in reward and memory circuits, demonstrating the diverse impact eCBs have. Understanding the native condition of the eCB system and details of synaptic plasticity is a step toward uncovering the mechanism through which pathologies such as drug addiction and Alzheimer's disease are developed.

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Ventral tegmental area dopamine and GABA neurons: Physiological properties and expression of mRNA for endocannabinoid biosynthetic elements. Collin B. Merrill, Lindsey N. Friend, Scott T. Newton, Zachary H. Hopkins, Jeffrey G. Edwards Sci Rep 2015, 5, 16176. doi:10.1038/srep16176

$\alpha6$ Subunit-containing nicotinic receptors on GABA terminals mediate low-dose ethanol effects on ventral tegmental area GABA neurons and ethanol reward. Scott C. Steffensen, Samuel I. Shin, Ashley C. Nelson, Taylor J. Woodward, Hyun Jung Park, Lindsey Friend, Devin H. Taylor, Ming Gao, Fenfei Gao, M. Foster Oliver, Jeffrey G. Edwards, Sterling Sudweeks, Lori Buhlman, J. Michael McIntosh, and Jie Wu. Submitted.

The nuclear variant of BMP2, nBMP2, affects hippocampal function in a mouse model. Ryan D. Cordner, Lindsey N. Friend, Jaime L. Mayo, Corinne Badgley, Andrew Wallmann, Julia S. Ventura, Brandalyn A. Chidsey, Aubrey C. Rogers, Jeffrey G. Edwards, and Laura C. Bridgewater. Submitted.

The Putative Cannabinoid Receptor GPR55 Modulates Hippocampal Synaptic Plasticity. Katrina Hurst, Corinne Badgley, Jacob Welch, Spencer Bell, Lindsey Friend, Deson Haynie, Brad Prince, Ryan Williamson, Chris Lyon, Jarrod Call and Jeffrey G. Edwards. Submitted.

CB1 dependent LTD in ventral tegmental area GABA neurons, a novel target for marijuana. L. Friend, J. Weed, Phil Sandoval, J. Edwards. In preparation

Hippocampal stratum oriens interneurons express endocannabinoid biosynthetic enzymes and undergo anandamide- dependent potentiation.
L. N. Friend, R. C. Williamson, C. B. Merrill, S. T. Newton, M. T. Christensen, J. G. Edwards. In preparation.

Hippocampal stratum radiatum interneuron plasticity type corresponds with cell subtype and mGluR5 expression.
T. M. Nufer, C. B. Merrill, L. N. Friend, Z. H. Hopkins, J. G. Edwards. In preparation

ABSTRACTS/CONFERENCE PROCEEDINGS

Society for Neuroscience 2015, 2016
Expression of Endocannabinoid Biosynthesizing Enzyme mRNA and protein
in Hippocampal Stratum Oriens Neurons. L. N. Friend, R. C. Williamson,
C. B. Merrill, S. T. Newton, J. G. Edwards

Society for Neuroscience 2016
The Putative Cannabinoid Receptor GPR55 Modulates Hippocampal Synaptic Plasticity. Katrina Hurst, Corinne Badgley, Jacob Welch, Spencer Bell, Lindsey Friend, Deson Haynie, Brad Prince, Ryan Williamson, Chris Lyon, Jarrod Call and Jeffrey G. Edwards.

Society for Neuroscience 2015, 2016
Hippocampal stratum radiatum interneuron plasticity type corresponds with
cell subtype and mGluR5 expression. T. M. Nufer, C. B. Merrill, L. N. Friend,
Z. H. Hopkins, J. G. Edwards

Society for Neuroscience 2014
Expression of endocannabinoid biosynthetic enzyme mRNA within ventral
tegmental area neurons. C. Merrill, L. Friend, S. Newton, Z. Hopkins, J. G. Edwards.

Research Society on Alcoholism Meeting 2014
Rapid adaptation of central and peripheral D2 dopamine receptor responses following acute ethanol and eticlopride R. Folsom, E. Jang, N. Schilaty, J. Linzey, L. Friend, J. Edwards, S. Burnett, G. Burton, and S. Steffensen.

TEACHING EXPERIENCE

Adjunct Professor for Biology 1090
Salt Lake Community College   Fall 2016
Taught Human Biology with Physiology lecture course

Teaching assistant – Advanced Physiology PDBio 362   Winter 2016
Brigham Young University
Taught 15 hours of lecture

Laboratory Instructor – Neuroscience 481   Fall 2015
Brigham Young University
Aided in teaching an advanced neuroscience class for undergraduates

Laboratory Instructor PDBio 305
Brigham Young University   2013
Taught undergraduate physiology lab and helped conduct experiments

Undergraduate Mentor
Brigham Young University   2013-Present
Taught and mentored undergraduate students in electrophysiology and PCR

Teaching Assistant for Advanced Neuro 480
Brigham Young University – Idaho   2011-2012
Lead groups of students through research projects

GRANTS AND AWARDS

Graduate Fellowship Award   2014-2015
    Brigham Young University
    Research grant for $15,000

Travel Award Winter 2015
    Brigham Young University
    Travel grant for $400

Research Assistant Tuition Scholarship   2013 - 2016