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Variable Modulation of Inputs to GABA Cells in the Ventral Tegmental Area and Hippocampus

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Doctor of Philosophy

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ABSTRACT

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The ventral tegmental area (VTA) is an important component of the mesolimbic dopamine circuit and processes reward and motivational behaviors. In response to drug exposure, synaptic connections of this circuit can be rewired via synaptic plasticity—a phenomenon thought to be responsible for the pathology of addiction. While much is known about dopamine neuron plasticity, less is known regarding plasticity exhibited by VTA GABA cells, specifically inhibitory inputs from outside the VTA. Expanding on the work of Bocklisch et al. (2013), we investigated the plasticity of inhibitory inputs to VTA GABA neurons. Using whole cell electrophysiology in GAD67 GFP mice, we observed that these VTA GABA cells can experience either long-term potentiation (LTP) or long-term depression (LTD) in response to a 5 Hz stimulus. While neither the LTP nor LTD appear to be mediated by the cannabinoid-1 receptor (CB1), the nitric oxide synthase (NOS) pathway, or the dopamine-2 (D2) receptor, the LTP is blocked by APV, an NMDA receptor antagonist, and the LTD is blocked by CGP 54626, an antagonist of the $GABA_B$ receptor. Additionally, μ-opioid and adenosine-1 receptors modulated plasticity at this synapse, but chronic morphine administration (10mg/kg) did not block the observed LTP or LTD. Furthermore, we used an optogenetic approach in VGAT-Cre mice to target inhibitory inputs from the lateral hypothalamus (LH) to the VTA. An optical stimulus (5 Hz) caused these inputs to depress, which has not been previously described and may be behaviorally important in reward processing. These novel findings increase our understanding of VTA neural circuitry, ultimately increasing our capacity to better comprehend and treat the pathology of addiction.

Additionally, changes in synaptic strength in hippocampal CA1 pyramidal cells are thought to be responsible for the acquisition and retention of short-term memory. Feedforward stratum radiatum interneurons of many subtypes experience LTD, short-term depression (STD), or lack of plasticity, but it is not known whether plasticity correlates with specific interneuron subtypes. Using whole cell electrophysiology and qPCR, we characterized the plasticity expressed by hippocampal interneurons in correlation with their mRNA expression patterns to determine cell subtype. We also assessed the expression of endocannabinoid (eCB) biosynthetic enzymes as well as metabotropic glutamate receptor subunits known to mediate plasticity. Cells exhibiting LTD tended to express mRNA for at least one of the eCB biosynthetic enzymes and the metabotropic glutamate receptor subunit mGluR5. mGluR5 was not expressed by cells exhibiting STD or no plasticity. Cells that exhibited short-term depression tended to express mRNA for at least one of the eCB biosynthetic enzymes, but not mGluR5. This suggests that stratum radiatum interneuron plasticity can be predicted based on mGluR expression, and that these different types of plasticity may have some importance in hippocampal function.

Keywords: GABA, long-term depression, long-term potentiation, addiction, radiatum, mGluR5

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CHAPTER 1: Introduction

The mammalian brain contains billions of neurons and trillions of synapses—allowing for nearly infinite possibilities in their connectivity. Most neurons in the brain receive input from hundreds of other neurons and send their output to many hundreds of others. Changes to the brain in response to the environmental stimuli and other conditions happen on a molecular level through changes at neural synapses. The brain's ability to remodel synapses in response to environmental cues is termed "use dependence" or neural plasticity.

Laboratory studies of rodent brain slices *in vitro* have revealed the phenomena of long-term potentiation (LTP) and long-term depression (LTD). LTP involves strengthening a synapse and classically occurs in response to higher frequency stimulus (Bliss & Lomo, 1973), whereas LTD weakens a synapse and traditionally occurs in response to lower frequency stimulus (Dudek & Bear, 1992). While LTP and LTD are both laboratory phenomena, many studies show that similar mechanisms occur in vivo and behaviorally modulate learning and memory (Mayford et al., 1996; Tsien et al., 1996; Tang et al., 1999). Hence, in many brain areas including the hippocampus, LTP-like mechanisms are most likely associated with "remembering" or "learning," and LTD-like mechanisms are probably linked to "forgetting" (Malenka & Bear, 2004). Recently, LTD has also been reported as a process of synaptic refinement and memory consolidation (Draguhn, 2018).

Synaptic transmission consists of presynaptic release of neurotransmitter from the axon terminal of one cell combined with postsynaptic activation of receptors by that neurotransmitter on another cell. Strengthening a synapse means that signals passing through that synapse are amplified. This can be accomplished by releasing more vesicles of neurotransmitter from the presynaptic terminal, or by increasing the number of receptors on the postsynaptic terminal.

Many times, both presynaptic and postsynaptic mechanisms in concert contribute to synaptic strengthening or weakening (Malenka & Bear, 2004). Some types of LTP may even involve the formation of new dendritic spines and new synapses between neurons. Thus, on the cellular level, remembering and learning are experience-directed changes in synaptic strength. Learning a new skill or fact results in the modification of synapses at any number of locations in the brain (Malenka & Bear, 2004). Researchers generally accept these molecular-level changes to be the basis for learning and memory in brain areas such as the hippocampus, cortex, and cerebellum. LTP and LTD can also modulate behavior in other brain areas including goal-directed behavior in the ventral tegmental area (VTA).

The VTA is a small, medially-located midbrain structure that houses dopamine neurons that project to the nucleus accumbens (NAc) and release dopamine when activated (Berridge & Robinson, 1998). Dopamine release is involved in associative learning of reward and is important for identifying species-perpetuating activities like eating and mating. Hence, eating and sex are pleasurable activities and involve the release of dopamine in NAc by neurons projecting from the VTA (Fields et al., 2007). The pleasurable "thumbs-up" signal associated with eating, drinking, finding shelter, or mating is important for the perpetuation and survival of the species; thus, the mesolimbic dopamine system is an evolutionarily "old" brain structure that is conserved throughout most vertebrate species (Prakash & Wurst, 2006). Dopamine signaling from the VTA is a key component in reward learning (Tsai et al., 2009).

Recent evidence points to VTA dopamine neurons as a target of illicit drugs such as opiates, methamphetamine, tetrahydrocannabinol (THC), cocaine, nicotine, ethanol, benzodiazepines, and other addictive substances (Lüscher and Ungless, 2006). These substances artificially induce excessive dopamine release during their administration but leave lasting

consequences for their users. Drug users can no longer process reward normally and experience withdrawals, cravings, and deterioration of executive function in both the acute and chronic phases of addiction.

Addiction is now widely recognized as a brain disorder involving the rewiring of the brain regions that process reward. Whether a person struggles with a gambling addiction or a cocaine addiction, satisfying his addiction becomes paramount to all other needs, desires, and responsibilities, in spite of negative consequences. Individuals suffer personal consequences such as strained family relations and employment instability. Society pays billions of dollars every year to help treat addiction and manage its consequences. Indeed, addiction is a severe neuropsychological disorder that requires further investigation. According to Koob and Le Moal, there are three parts of the cycle of addiction: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation (2001). As a person passes through these "stages" of addiction again and again, the hedonic potential of the drug decreases. That is, after every binge/intoxication episode, the "high" that the user receives diminishes somewhat as compared to the previous episode. The subsequent withdrawal episode is even more unpleasant than the one before because basal dopamine firing is considerably below its normal level. The only way that the brain knows to return dopamine levels to their former glory is to use the drug again, thus the preoccupation/anticipation phase also becomes more intense. Overtime, the decision-making prefrontal cortex completely goes offline, and instead hedonic mesolimbic desires drive behavior. The substance abuser is left at the mercy of his addiction—there is no longer executive control of "reward, pain, stress, emotion, habits, and decision-making" (George & Koob, 2010). Hence, addicts seek out their preferred high without regard for basic physical needs, physical pain, emotional pain, or neglected family and work responsibilities.

While much has been said and hypothesized about psychological theories of addiction, the neural mechanisms of addiction in the brain were largely ambiguous until the ground-breaking work of Ungless et al. showed plasticity of dopamine neurons in the VTA following in vivo cocaine administration to mice (2001). After a single exposure to cocaine, the animals' brains showed a marked increase in AMPA/NMDA ratio at the glutamate to dopamine synapse in the VTA (see Figure 1.1), while hippocampal glutamatergic and VTA GABAergic synapses displayed no such plasticity. The cocaine-induced plasticity was sensitive to the NMDA receptor antagonist MK-801, suggesting an NMDA-dependent mechanism. Hence, a neural model for drug abuse and addiction was born.

However, dopamine neurons are not the only players in the neural circuitry of reward and addiction. The VTA houses a heterogeneous population of both dopamine and GABA neurons. Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the brain and will bind to both ionotropic ($GABA_A$) and metabotropic ($GABA_B$) receptors, which are both distributed ubiquitously throughout the mammalian brain (Bowery et al., 1987). Numerous studies have shown that GABA cells synapse onto and modulate dopamine cell activity in the VTA (Grace & Bunney, 1979; Kalivas et al., 1990; Klitenick et al., 1992; Nugent et al., 2007; Nugent et al., 2009; Lüscher and Malenka, 2011; Edwards et al., 2017; see also Figure 1.1). Many of these same studies have clearly demonstrated that drugs of abuse can also modulate plasticity of GABAergic afferents to dopamine neurons. Nugent et al. showed that LTP of GABA neurons was blocked by opioid administration (2007). Others have likewise demonstrated that the "learning" and plasticity involved in addiction and drug abuse affect the firing rate of dopaminergic neurons by altering the GABA neurons that innervate them (Liu et al., 2005). Optical stimulation of GABA neurons can disrupt reward consumption (van Zessen et al.,

2012)—thus amending the dopamine hypothesis that all reward learning and addiction pathology depends solely on only dopamine. VTA GABA neurons can also modulate reward learning in important ways. While dopamine release is critical to motivational learning and encoding reward prediction error (Schultz et al., 1993; Mirenowicz & Schultz, 1994; Schultz, 1998; Fiorillo et al., 2003), actual reward is likely mediated by GABA via a dopamine-independent mechanism (Laviolette & van der Kooy, 2001; Laviolette et al., 2004).

The functional role of VTA GABA plasticity in motivational learning, reward, and addiction pathology requires further investigation. While we have some understanding of how local dopamine and GABA neurons interact in the VTA, we understand very little about the excitatory and inhibitory inputs onto VTA GABA cells. Our lab recently showed how excitatory inputs to VTA GABA cells exhibit CB1-dependent LTD that is blocked by chronic THC administration (Friend et al., 2017). Bocklisch et al. used an optogenetic model to demonstrate that D1-expressing GABA projections from the NAc to VTA GABA neurons experience long-term potentiation (LTP) following a high-frequency optical stimulation (2013). This plasticity appeared to be presynaptic and was dependent on L-type calcium channels, the D1 receptor, activation of adenylyl cyclase, and the scaffolding protein Rim1α. The LTP they described was occluded by chronic cocaine administration. However, questions remain about the mechanism of plasticity at this synapse of inhibitory inputs to VTA GABA neurons.

So not only is the GABA-dopamine synapse affected by drug exposure, but the next synapse upstream (the GABA-GABA synapse) is also affected by addictive drug exposure. However, inhibitory inputs to the VTA do not originate solely from the NAc, but also from the pedunculopontine tegmental nucleus, the posterior laterodorsal tegmental nucleus, the ventral pallidum, the rostral medial tegmental nucleus, lateral hypothalamus, and the possibly other

brain areas (Fields et al., 2007). Perhaps these inputs exhibit plasticity that could be altered by illicit drug exposure. To fully comprehend the pathology of addiction, we must first understand the mechanisms of plasticity in a drug-naïve state. Second, we need to understand the effect that addictive drugs have on each synapse of the mesolimbic dopamine system, including the inhibitory inputs to VTA GABA cells.

Currently, molecular addiction research focuses on better understanding the complex circuitry that controls reward learning and processing in the brain. Developing a more complete understanding of the pathology of addiction will allow scientists to eventually discover a cure for addiction that will (hopefully) reverse the plasticity that has occurs in response to drug administration. There is currently no treatment for substance addictions that actually reverses the disorder. Rehabilitation centers focus on removing individuals from people and environments that stimulate drug-seeking and drug-taking behavior. Patients learn techniques of avoiding situations in which they may be tempted to relapse and are taught more healthy methods of dealing with their anxiety and stress as an alternative to drug use. Other methods of addiction treatment are more pharmacological and instead focus on "weaning" addicts off their drug of choice and attenuating withdrawal symptoms. This is the idea behind nicotine patches/Nicorette gum (for nicotine addiction) and prescription drugs like methadone and buprenorphine (for opioid addiction). Buprenophine (brand name Suboxone) is a partial μ-opioid receptor agonist that mimics that effects of opioid administration and reduces cravings to better manage opioid addiction recovery (Ling et al., 2013). While all these treatment options are steps in the right direction, they are still just Band-Aids for a deeper problem—that is, permanently altered synaptic plasticity within the mesolimbic dopamine circuit. A study of the plasticity at the

synapse of inhibitory inputs to VTA GABA neurons is a critical component of understanding mesolimbic dopamine circuit function.

In addition, we are also interested in GABA cell plasticity in the hippocampus. As mentioned previously, the hippocampus is another area of the brain where plasticity occurs and has classically been studied. The hippocampus is an area of the neocortex known to be involved in the formation and consolidation of memory. It is organized into several layers including the stratum radiatum, stratum pyramidale, and stratum oriens, which are in the hippocampus proper, or cornu ammonis, an area that can be subdivided into CA1, CA2, and CA3 (Andersen et al., 1971; Andersen et al., 2000). Each of these layers and sub-regions has specific connectivity within the hippocampus and with its efferents and afferents (Laurberg, 1979). The stratum pyramidale contains excitatory, glutamatergic pyramidal cells. CA1 pyramidal cells receive their input largely from CA3 pyramidal cells and project to the subiculum and entorhinal cortex. In general, pyramidal cells are thought to be a mostly homogenous population of cells. Both the inputs and outputs to CA1 pyramidal cells are heavily modulated by GABAergic interneurons in both the stratum oriens and the stratum radiatum (Ribak et al., 1986). Unlike pyramidal cells, GABAergic interneurons of the hippocampus comprise a wildly heterogeneous population of cells. There are many subtypes of these interneurons including, among others, parvalbumin-containing axo-axonic cells, calretinin-containing interneuron-selective cells, and cholecystokinin/calbindin positive basket cells (Fruend & Buzaki, 1996). In 1997, McMahon & Kauer determined that GABA cells, contrary to the expectations of the time, also exhibit neural plasticity in response to a high frequency stimulus, similar to pyramidal cells. While radiatum interneurons induce long-term depression (LTD), short-term depression (STD), or lack of

plasticity (McMahon & Kauer, 1997), it is not known whether these types of plasticity correlate to any specific interneuron subtype.

Hippocampal interneuron plasticity can be modulated by endocannabinoid activity including activation of CB1 receptors and TRPV1 receptors (Gibson et al., 2008). Other data show that Type 1 mGluRs may also be necessary for the modulation of interneuron plasticity (Le Duigou et al., 2011). Surely the influence of these modulators affects the type of plasticity that a radiatum interneuron will experience. Understanding the character of GABA neurons in the hippocampus, specifically the CA1 stratum radiatum, may be critical to comprehending the larger roles of GABAergic modulatory inhibition in memory formation.

As with my studies in the VTA, in the hippocampus the excitatory neurons are important, but their constitutive activity appears to be heavily modulated by inhibitory interneurons. In both the VTA and hippocampus, we might say that the GABA neurons are truly "running the show," even though historically dopamine cells in the VTA and glutamatergic pyramidal cells in the hippocampus have been more thoroughly studied. Now we seek to better understand the function and purpose of the VTA and hippocampus through studying inhibitory modulation of plasticity in these brain areas.

Specific Aims

Understanding the molecular mechanisms of synaptic plasticity paints a clearer picture of how synapses function in the brain and how differences between synapses change their function and output. Our aim was to investigate the molecular mechanisms of plasticity at the synapse of inhibitory afferents to VTA GABA cells. These cells are part of the mesolimbic dopamine circuit, so increasing our understanding of synaptic plasticity in this circuit enhances our knowledge of addiction as a neurological disease.

In addition, we aim to better understand the correlation of plasticity with interneuron subtype in the CA1 stratum radiatum. This knowledge expands our comprehension of the modulatory role of GABAergic interneurons in hippocampal memory consolidation.

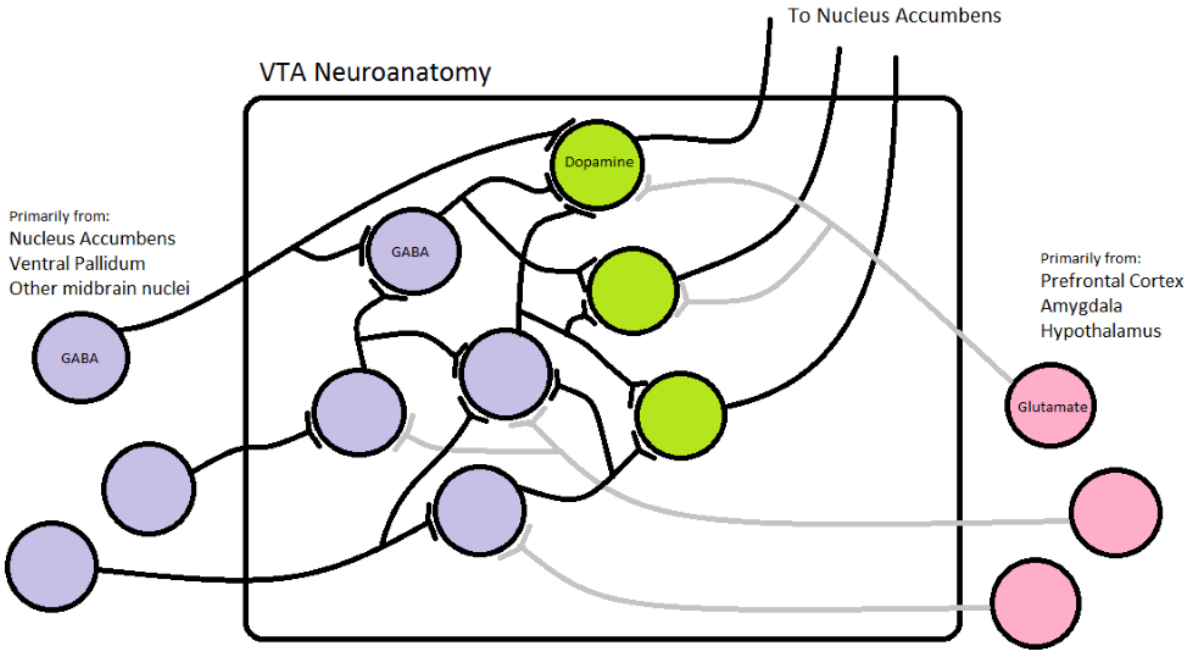

Figure 1.1: Simplified VTA Circuitry. Ungless et al. (2001) described the synapse of glutamatergic inputs to VTA dopamine cells (pink cells to green cells). Nugent et al. (2007) described GABAergic inputs to VTA dopamine cells (purple cells to green cells). Bocklisch et al. described the synapse of GABAergic inputs to VTA GABA cells (purple cells to purple cells).

CHAPTER 2: Inhibitory Inputs to VTA GABA Neurons Exhibit LTP or LTD

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Scott Steffensen, Jeffrey Edwards

Abstract

The ventral tegmental area (VTA) is an essential component of the mesolimbic dopamine circuit and processes reward and motivational behaviors. The VTA contains dopamine cells thought to mediate reward as well as GABAergic inhibitory cells that can regulate dopamine cell activity. In response to drug exposure, synaptic connections of this circuit can be rewired via synaptic plasticity—a phenomenon thought be responsible for the pathology of addiction. While much is known about dopamine neuron plasticity, less is known regarding plasticity exhibited by VTA GABA cells, specifically inhibitory inputs from outside the VTA. Expanding on the work of Bocklisch et al. (2013), we investigated the plasticity of inhibitory inputs to VTA GABA neurons. Using whole cell electrophysiology in GAD67 GFP mice, we observed that these VTA GABA cells can experience either long-term potentiation (LTP) or long-term depression (LTD) in response to a 5 Hz stimulus. Paired pulse ratios suggest a presynaptic mechanism for both types of plasticity. While neither the LTP nor LTD appear to be mediated by the cannabinoid-1 receptor (CB1), the nitric oxide synthase (NOS) pathway, or the dopamine-2 (D2) receptor, the LTP is blocked by APV, an NMDA receptor antagonist, and the LTD is blocked by CGP 54626, an antagonist of the $GABA_B$ receptor. Additionally, we found some cells to be depressed by the acute application of DAMGO, a μ-opioid receptor agonist, while others were slightly potentiated. To further investigate these pathways, we used an optogenetic approach in VGAT-Cre mice to target inhibitory inputs from the lateral hypothalamus (LH) to the VTA. A 5 Hz optical stimulus caused these inputs to depress. LTD at this synapse has not been previously

described and could be an important component of the reward processing pathway. We also found that neither acute or chronic morphine administration (10mg/kg) occluded the observed LTP or LTD. Additionally, these novel findings regarding the molecular mechanisms of synaptic plasticity at this inhibitory synapse onto VTA GABA cells help us better understand VTA neural circuitry, ultimately increasing our capacity to better understand and treat the pathology of addiction.

Introduction

The mesolimbic dopamine circuit contains important, evolutionarily-conserved pathways that normally help animals to identify and seek species-perpetuating behaviors, as well as avoid harmful behaviors (Berridge & Robinson, 1998). Dopamine neurons in the ventral tegmental area (VTA) project to the nucleus accumbens (NAc) where they release dopamine onto medium spiny neurons (MSNs; Fields et al., 2007). Drugs such as opiates, amphetamines, tetrahydrocannabinol (THC), cocaine, nicotine, ethanol, and other addictive substances artificially increase dopamine release and/or increase its duration at the synapse and cause persistent changes at synapses within and connecting to this mesolimbic circuit (Di Chiara & Imperato, 1988; Lüscher and Ungless, 2006). Addiction is a brain disease involving the rewiring of neural circuitry in response to drug exposure (Wise, 2004; Creed & Lüscher, 2013). Drug users can no longer process reward normally and experience withdrawals, cravings, and deterioration of executive function in both the acute and chronic phases of addiction.

Synaptic plasticity following drug exposure was initially observed in excitatory inputs to VTA dopamine cells (Ungless et al., 2001), and drug-dependent plasticity has since been thoroughly described at both excitatory and inhibitory inputs onto dopamine cells (Bonci & Malenka, 1999; Saal et al., 2003; Nugent et al. 2007; Argilli et al., 2008; Stuber et al., 2008; Zweifel et al., 2008; Nugent et al., 2009; Kodangattil et al., 2013). While the dopamine cells involved in addiction have been thoroughly studied, research shows that the inhibitory GABA neurons modulating those dopamine cells are also altered in addiction (Roberts et al., 1996; Liu et al., 2005, Barrett et al., 2005). However, less is understood about the inputs to these GABA neurons and how they change following drug exposure. Inhibitory inputs to VTA GABA neurons come from many areas of the brain (Fields et al., 2007; Morales & Margolis, 2017), but perhaps

the most predominant and well-studied in recent years are from the nucleus accumbens (NAc) and the lateral hypothalamus (LH).

The NAc is a bilaterally located nucleus in the medial forebrain composed mostly of inhibitory medium spiny neurons (MSNs) as well as small populations of glutamatergic and cholinergic interneurons (Yamaguchi et al., 2011; Brown et al., 2012). MSNs project back to the VTA to inhibit both GABA and dopamine cells (Xia et al., 2011; van Zessen et al., 2012; Edwards et al., 2017). Bocklisch et al. used an optogenetic model to demonstrate that D1-expressing GABA projections from the NAc to VTA GABA neurons experience long-term potentiation (LTP) following a high-frequency optical stimulation (2013). This plasticity appeared to be presynaptic and was occluded by chronic cocaine administration. More recently Edwards et al. used a Cre-driven optogenetic mouse model to discover compelling evidence that NAc D1 MSN input to VTA GABA neurons is $GABA_A$ mediated and modulated by the adenosine-1 receptor (2017). These NAc D1 MSN inputs to the VTA showed cocaine, but not morphine, sensitization. We hypothesized that the NAc to VTA pathway could be influenced by other neuromodulators beyond those already studied and aimed to confirm the LTP described by Bocklisch et al. (2013).

The LH also provides inhibitory input to the VTA and contains many cell types including GABA, glutamate, and neuropeptide-releasing cells (Berthoud & Munzberg, 2011). The LH has classically been studied for its role in regulating feeding behaviors—electrical stimulation of the LH produces voracious feeding in sated animals, while ablation of LH neurons leads to aphagia and emaciation (Wise, 1971; Hoebel, 1965). Jennings et al. used an optogenetic model to target specific subpopulations of LH cells and reveal the importance of LH GABA neurons in regulating feeding behavior (2015). Additionally, the LH to VTA pathway facilitates both

feeding and compulsive sucrose-seeking behaviors, and stimulation of LH GABAs inhibits VTA GABAs and increases dopamine release in the NAc (Nieh et al., 2015; Nieh et al., 2016). Stimulating this pathway at higher or lower frequencies appears to facilitate pleasurable reward or drive-like feeding behaviors, respectively (Barbano et al., 2016). We hypothesized that this LH pathway may be involved in drug-induced plasticity.

We used whole cell electrophysiology *ex vivo* to study the modulation of inhibitory inputs to VTA GABA neurons. We not only observed the previously reported LTP at this synapse, but we also describe a novel form of plasticity at this synapse that has not previously been reported—inhibitory inputs onto VTA GABA neurons can exhibit either LTP or LTD. Interestingly, neither form of plasticity is blocked by chronic morphine administration. We investigated the hypothesis that the observed LTP or LTD could be input-dependent, and we found evidence for plasticity at the inhibitory LH to VTA GABA afferent using an optogenetic model.

Methods

Electrophysiology

All experiments were performed in accordance with Institutional Animal Care and Use Committee protocols and NIH guidelines for the care and use of laboratory animals. Male GAD67 GFP (Tamamaki et al., 2003) mice P15-30 days old were anesthetized with isoflurane (1.5-2%) and decapitated. Brains were removed and sectioned transversely on a vibratome at 300 μm using ice cold oxygenated sucrose solution composed of 220 mM sucrose, 3 mM KCl, 1.25 mM NaH_2PO_4, 0.2 mM $CaCl_2$, 12 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM glucose, and 400 μM ascorbic acid. Recordings began at least one hour after cutting while tissue was stored in oxygenated ACSF composed of 119 mM NaCl, 26 mM $NaHCO_3$, 2.5 mM KCl, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 11 mM glucose. Excitatory glutamate currents were blocked using 10 μM CNQX (Alomone Labs) throughout. NMDA antagonist APV was not constitutively applied because cells were constantly held in voltage clamp at -65 mV throughout experiments. An Olympus BX51WI microscope with a 40x water immersion objective was used to visualize the VTA, identified as a nucleus of GFP+ cells surrounding the fasiculus retroflexus. GFP+ cells were patched with a borosilicate glass pipette (3-6 MΩ) filled with internal solution composed of 117mM KCl, 2.8mM NaCl, 20mM HEPES, 5mM $MgCl_2$, and 1 mM QX-314 (Tocris) with pH 7.28 and osmolarity 275–285 mOsm. Recording were made in voltage clamp with cells being held at -65 mV throughout experiments. Plasticity was induced using a 5 Hz stimulation in current clamp mode and delivered using a concentric bipolar electrode (Microprobes for Life Science) 200-400 μm from the patched cell. Currents were recorded using Multiclamp 700B amplifier and digitized with an Axon 1440A digitizer (both from Molecular Devices, Sunnyvale, CA, USA). Signals were filtered at 4 kHz and recorded using Clampex 10.4

(Molecular Devices) on a personal computer. Electrophysiological data was analyzed using Clampfit software (Molecular Devices), Microsoft Excel, and Origin 10.8 (OriginLab Corporations, Northampton, MA, USA). All p-values were obtained by a Student's t-test comparing the 5 minutes before conditioning to 15-25 minutes post conditioning.

Surgery and Optogenetic Manipulation

Male VGAT-Ires-Cre mice (Jackson Labs, stock# 028862) were crossed with female GAD67 GFP+ mice. Male GFP+ cross mice older than 21 days received stereotaxic neurosurgery in a Kopf stereotaxic apparatus (Model 940). Mice were anesthetized using 1-3% isoflurane using a Kent SomnoSuite. Mice received carprofen tablets 24 hours before and for at least 72 hours following surgery, as well as a subcutaneous injection of buprenorphine (0.1 mg/kg) after induction of anesthesia and immediately before surgery. Mice received bilateral injections of AAV2/1-EF1a-DIO-hChR2(H134R)-mCherry (UNC Vector Core) in the Nucleus Accumbens (AP +1.6, ML ±0.6, DV -4.4 to -4.5 DV) or in the Lateral Hypothalamus (AP -1.3, ML ±0.6 to 0.8, DV -5.2 to -5.3). Animal recovery and viral incubation lasted 3-8 weeks post-surgery, after which time animals were sacrificed for experiments via the protocol previously described. Cutting took place in either the previously described sucrose solution or in an NMDG-based solution (92 mM NMDG, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 30 mM $NaHCO_3$, 20 mM HEPES, 25 mM glucose, 2 mM thiourea, 5 mM Na-ascorbate, 3 mM Na-pyruvate, 0.5 mM $CaCl_2 \cdot 4H_2O$ and 10 mM $MgSO_4 \cdot 7H_2O$, titrated to ~ pH 7.4 with HCl) and was proceeded by transcranial perfusion with said solution (Pan et al., 2015). Slices were then maintained in a HEPES holding solution (92 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 30 mM $NaHCO_3$, 20 mM HEPES, 25 mM glucose, 2 mM thiourea, 5 mM Na-ascorbate, 3 mM Na-pyruvate, 2 mM $CaCl_2 \cdot 4H_2O$ and 2 mM $MgSO_4 \cdot 7H_2O$) in an incubator for at least one hour before

electrophysiology was attempted. Blue light for optical activation was generated by a TLED+ from Sutter Instruments with individual light pulses lasting 1-2 ms.

PCR

Cells from GFP- littermates of GFP+ mice were used for PCR analysis and 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 manufacturer's 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. We performed additional no-template-control tests to ensure that there were no primer dimer or hairpin interactions between primers. The samples were 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. The primer and probe sequences were those used in our previous study (Merrill et al., 2012). 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.

Treatment with morphine

Male GAD67 GFP+ mice (P15-30) were injected with morphine (Sigma-Aldrich) at 10 mg/kg via intraperitoneal injection either acutely (approximately 24 hours before sacrifice and

experimentation) or chronically (one dose each day for 7 days with the last dose approximately 24 hours before sacrifice and experimentation).

Drugs

APV, L-NAME, baclofen, DAMGO, and eticlopride were obtained from Tocris and dissolved in ddH_2O to make stock solutions. AM251, CPG 54626, SNAP, and N^6-CPA were obtained from Tocris and dissolved in DMSO to make stock solutions. CNQX was obtained from Alomone Labs and dissolved in ddH2O. All stock solutions were frozen at –20° C until dilution into ACSF to give the reported concentrations.

Statistical Analysis

Peak amplitude was analyzed using ClampFit software (Molecular Devices) and normalized to the average of the last six minutes of baseline. Individual failures were excluded from minute averages of IPSC amplitude. If a minute was left with fewer than three sweeps, the entire minute was excluded.

One-way ANOVA analysis was performed on every electrophysiology experiment to determine if the plasticity was statistically significant comparing baseline to post-conditioning within every individual experiment. For LTP experiments, baseline was compared to 20-30 minutes postconditioning (as most of the LTP in our studies had a delayed onset). For LTD, baseline was compared to 10-20 minutes postconditioning.

PPRs were calculated using the average of the last 5 minutes of the baseline with 5-10 minutes postconditioning for both the first and second pulses. PPRs were taken from the following sets of data: 5Hz control, AM251, CB1 KO, L-NAME, and APV (LTP only). Statistical significance was calculated using the Wilcoxon ranked sign test, and critical values

were taken from the Ranked Sign Test Table. Statistical significance was determined using Student's T-test in Microsoft Excel.

Results

We used GAD67-GFP mice to target GABA neurons in the VTA (Tamamaki et al., 2003), as both GABA and dopamine neurons can display similar electrophysiological characteristics (Margolis et al., 2006) and GAD65 can co-express with tyrosine hydroxylase (Gonzalez-Hernandez et al., 2001; Olson & Nestler, 2007). The inhibitory synapse onto VTA GABA neurons can experience two types of plasticity—LTP or long-term depression (LTD) (Figure 2.1A). Experiments were individually classified as expressing either LTP or LTD based on single factor ANOVA analysis (data not shown). Both LTP and LTD were induced in response to a 5 Hz stimulus (3 minutes or 900 stimuli), though we note that a high frequency stimulus (2 pulses of 100 Hz) also induced both LTP and LTD (LTP: n = 4, LTD: n = 5; data not shown). We continued to use a 5 Hz stimulus throughout our study as it gave reliable results and was deemed to be more physiological. We confirmed that the currents we recorded were $GABA_A$-mediated, as they were abolished by bath application of the known $GABA_A$ antagonists picrotoxin (100 μM, Figure 2.1B) or bicuculline (30 μM, data not shown). We acknowledge that IPSCs can also be mediated by the neurotransmitter glycine, which is known to facilitate neurotransmission at the GABA to dopamine synapse in the VTA (Ye et al., 2004). We found that the prototypical glycine receptor antagonist strychnine (1 μM) did indeed reduce IPSCs by ~60-70%, but we discontinued this investigation due to the non-specific effects of strychnine (Hernandes & Troncone, 2009; data not shown). We also saw both LTP and LTD in GFP- littermates of GFP+ animals (Figure 2.1C). In the absence of GAD67-GFP fluorescence, we confirmed that these cells were GABA cells using a combination of spiking and PCR. To isolate GABA currents, we eliminated glutamate currents using CNQX (10 μM) in all experiments described in Figures 2.1-4. Application of CNQX to a recording of all afferents to a VTA GABA

cell reduces the current to 78.9% ±2.1 of baseline (Figure 2.1D), suggesting that VTA GABA cells receive most of their input from other GABA cells. Paired pulse ratio (PPR) analysis for LTP and LTD experiments suggests that both types of plasticity may have a presynaptic mechanism (Figures 2.1E and 2.1F). We note anecdotally that some GABA cells had notable failure rates which usually increased following the stimulus protocol, though not all cells experienced a significant number of failures.

To determine the molecular mechanism of both the LTP and LTD observed at this synapse, we examined several classic presynaptic mediators of plasticity including the nitric oxide synthase (NOS) pathway and cannabinoid receptor-1 pathways. While NOS plays an important role in the mechanism of local GABA to DA plasticity within the VTA (Nugent et al., 2007; Nugent et al., 2009), we found that it did not alter the induction of either LTP or LTD at the inhibitory synapse onto VTA GABA neurons as the NOS antagonist L-NAME (200 μM) did not block either form of plasticity (Figure 2.2A). Similarly, application of the NO donor SNAP failed to induce plasticity (Figure 2.2B). Furthermore, in the presence of the CB1 antagonist AM251 (2 μM; Figure 2.2C) or in GFP+ CB1 knock-out animals (Figure 2.2D) significant LTP and LTD were still exhibited. Chelating calcium with intracellular BAPTA (20 mM) yielded mixed results and did not definitively block either form of plasticity (n = 5; data not shown). Similarly, bath application of the selective CB1 agonist WIN55 (10 μM) yielded inconclusive results (n = 3, data not shown), leading us to rule out CB1 as an important modulator of the plasticity we observed. Continuing our investigation, both the LTP and LTD were also independent of D2 dopamine receptor activation, as the D2 antagonist eticlopride (10 μM) did not block either response (Figure 2.2E). However, surprisingly this LTP appears to be NMDA-dependent, as it was blocked by the NMDA receptor antagonist APV (50 μM) when applied

during baseline and conditioning (Figure 2.2F). This finding is like that of Nugent et al., who described NMDA-dependent plasticity of GABAergic afferents to VTA dopamine neurons (2007). Collectively, these data expand our understanding of inhibitory plasticity onto VTA GABA neurons and suggest a presynaptic mechanism of LTP yet to be fully determined, but production of a retrograde factor produced from excitatory synaptic activation is likely involved. However, the previously observed LTD persists in the presence of APV (see Figure 2.2F).

While investigating possible presynaptic mechanisms of plasticity at inhibitory inputs to VTA GABA cells, we examined the potential role of $GABA_B$ receptors. $GABA_B$ receptors are G-protein coupled metabotropic receptors that classically function as autoreceptors (Scanziani, 2000) and could be important molecular targets in pro-cognitive medicine (Serrats et al., 2017). Edwards et al. identified NAc D1 MSN input to VTA dopamine neurons that was $GABA_B$ mediated (2017), so we used the $GABA_B$ agonist baclofen to assess the presence of $GABA_B$ at the synapse of inhibitory inputs to VTA GABA cells. Baclofen (50 μM) strongly depressed recorded IPSCs (Figure 2.3A). We wondered in $GABA_B$ might mediate plasticity at this synapse, and we found that application of the $GABA_B$ antagonist CGP 54626 (2 μM) blocked the previously observed LTD, while not affecting LTP (Figure 2.3B). These data reveal a previously unknown synapse at which $GABA_B$ mediates plasticity. We attempted to occlude this $GABA_B$-mediated plasticity by applying a lower concentration of baclofen (1 μM, closer to EC_{50}; Cruz et al., 2004) throughout the experiment in which a 5 Hz stimulus was used to induce plasticity. Contrary to our hypothesis, we were unable to occlude plasticity, but instead saw a depression following 5Hz stimulus in the presence of 1 μM baclofen (Figure 2.3C). We believe that perhaps constitutive activation of local $GABA_B$ receptors by a low concentration of baclofen may

predispose GABA neurons to experience LTD, similar to the depression evoked in Figure 2.3A with 50 μM baclofen, only less extreme.

We also investigated the presence of known presynaptic modulators of plasticity at the inhibitory synapse onto VTA GABA neurons, namely the μ-opioid receptor and the adenosine-1 receptor (A_1R). μ-opioid receptor activation is known to depress neurotransmission at GABAergic synapses though negative coupling with adenylyl cyclase and activation of GO (Johnson & North, 1992; Williams et al., 2001). Upon acute application of DAMGO (1 μM), a highly selective and potent μ-opioid receptor agonist, we saw some cells depress (Figure 2.4A), while another group of cells did not depress and may have slightly potentiated in response to DAMGO (Figure 2.4A). To obtain these data, DAMGO was applied at the end of another experiment where 5 Hz stimulus was used to induce plasticity. However, we found each cell's response to DAMGO to be independent of both plasticity expression and other drugs used in the experiment. We also tested for the presence and involvement of A_1R at the inhibitory synapse onto VTA GABA neurons and found that all cells tested depressed in response to bath application of N^6-CPA (1 μM), a non-hydrolysable analog of adenosine (Figure 2.4B). These results are consistent with the findings of Edwards et al., who found that D1 MSN afferents to VTA GABA and dopamine cells experience depression with N^6-CPA application, while local VTA GABA to dopamine synapses do not (2017). Together these findings highlight roles of μ-opioid receptors and A_1Rs at the inhibitory synapse onto VTA GABA neurons.

We went on to test the effects of chronic morphine administration on the LTP and LTD we observed at the synapse of inhibitory inputs to VTA GABA neurons. Interestingly, chronic morphine administration (10 mg/kg via IP injection) for 7 to 9 consecutive days did not block neither the previously observed LTP nor the LTD (Figure 2.4C). Morphine and other opioids are

classic presynaptic modulators of neurotransmission (Williams et al., 2001), so understanding which synapses morphine alters (and which synapses it does not) within the mesolimbic dopamine circuit may help researchers and clinicians better understand the key neural changes involved in opiate addiction.

Beyond identifying the mediators of the observed LTP and LTD, another important question is why we see two distinct types of plasticity at the same synapse. Though different responses to the same stimulus have been reported before, they often appear to be gradations of depression that are dependent on the degree of activation or expression levels of necessary factors like biosynthetic enzymes (McMahon & Kauer, 1997; Péterfi et al., 2012). Conversely, here we demonstrate two different types of plasticity present at the same synapse. In response to a 5 Hz stimulus, we observe either LTP or LTD, but have yet to observe (under control conditions) a situation where LTP and LTD appear to occur simultaneously and result in no net change at the synapse. We hypothesized that the LTP and LTD we observed arose from different inhibitory inputs to VTA GABA neurons (Lammel et al., 2012). As previously discussed, two important sources of GABAergic inputs to the VTA come from the NAc and the LH, and Bocklisch et al. previously reported LTP of the D1-MSN feedback to VTA GABA cells (2013). To test the plasticity of LH to VTA inhibitory afferents, we crossed VGAT-IRES-Cre mice to our GAD67-GFP line and injected AAV2/1-EF1a-DIO-hChR2-mCherry bilaterally into the LH of GFP+ cross mice to achieve GABA-specific expression of ChR2. We confirmed the injection site by imaging using a confocal microscope to visualize mCherry infection of cells in the LH (Figure 2.5A) as well as mCherry+ afferents to GFP+ GABA cells in the VTA (Figure 2.5B). We recorded optically evoked currents in the VTA and observed only LTD in response to an optical 5 Hz stimulus (Figure 2.5C). This response was likely $GABA_A$-mediated as it was blocked by

bath application of picrotoxin (100 μM; Figure 2.5D). These results reveal a previously unknown neural pathway that could be important in processing reward, and consequently could play a role in the pathology of addiction. Beyond the previously studied roles that this inhibitory LH to VTA pathway plays in feeding behaviors and compulsive sucrose seeking (Jennings et al., 2015; Nieh et al., 2015), we highlight a novel form of plasticity and suggest a neural mechanism by which this LTD may be mediated.

Another potential explanation for the two distinct types of plasticity observed at the synapse of inhibitory afferents to VTA GABA cells is that there exist subtypes of GABA cells within the VTA that experience different types of plasticity. Though this hypothesis seemed less likely in the light our PPR analysis pointing to a presynaptic mechanism for both LTP and LTD, we investigated this hypothesis by studying the distribution of the GABA synthesis enzymes GAD67 and GAD65 in VTA GABA neurons and the different plasticity they displayed. GAD67-GFP mice were crossed with GAD65-mCherry mice (Jackson Labs) to visualize the co-expression of GAD67 and GAD65 in the VTA. We also stained for tyrosine hydroxylase (TH). We found co-expression of GAD67 and GAD65, particularly in more dorsal transverse slices of VTA (Figure 2.6A). We also saw co-expression of GAD65 and TH in select transverse slices containing more ventral VTA (Figure 2.6B). While we rarely observed GAD67-only cells, we note a prominent population of GAD65 only cells located medial to traditional VTA (Figure 2.6C). We observed no co-expression of GAD67 and TH (Figure 2.6D). In total we observed TH+/GAD65+ cells and GAD67+/GAD65+ cells, as well as cells exclusively expressing TH+ and GAD65+. Clearly there is a significant amount of heterogeneity among both dopamine and GABA populations within the VTA (see also Morales & Margolis, 2017). We used these GAD67/GAD65 cross mice for whole cell electrophysiology experiments and targeted GAD65

only cells. We saw a similar pattern of inhibitory plasticity onto VTA GAD65 cells as we saw with GAD67 cells—inhibitory inputs to these cells express both LTP and LTD (LTP n = 4, LTD n = 4, data not shown). This suggests that the expression of GAD65 or GAD67 is likely not predictive of how inhibitory inputs to those GABA cells will experience plasticity. These results also confirm that GAD65 alone is not a reliable marker of GABA cells, as it can frequently co-express with TH. This subpopulation of GAD65/TH neurons requires further study.

Discussion

We herein present data describing mechanisms of plasticity at the inhibitory synapse onto VTA GABA neurons. Two types of plasticity can be elicited at this synapse in response to a 5 Hz stimulus: LTP or LTD. While these two forms of plasticity appear to be independent of the CB1 signaling pathway, the NOS system, and D2 receptors, the LTP is dependent on NMDA signaling, while the LTD requires $GABA_B$ activation. We also saw depression in response to the application of DAMGO and N^6-CPA. Chronic morphine administration blocked neither LTP nor LTD. Finally, we used an optogenetic model to target the LH to VTA GABAergic afferent and discovered that this synapse experiences LTD in response to an optical stimulus. We are the first to report LTD at an inhibitory synapse to VTA GABA neurons, and specifically the first to report LTD at the LH to VTA GABAergic afferent.

As discussed previously, this work follows that of Bocklisch et al. studying inhibitory inputs to VTA GABA neurons (2013). Their work utilized an optogenetic approach to target projections from the NAc to the VTA, and they discovered LTP at the inhibitory synapse of D1-MSNs to VTA GABA neurons in response to high frequency optical stimulation (trains of 50 Hz). This LTP appeared to be presynaptic, occluded by chronic cocaine, and was dependent on L-type calcium channels, the D1 receptor, activation of adenylyl cyclase, and the scaffolding protein Rim1α. We found the LTP in our study to be dependent on NMDA receptor activation, which could be because we used an electrical stimulus which activated all surrounding neurons and afferents, as opposed to an optical stimulus which would only activate afferents from the NAc to the VTA. NMDA receptor activation can modulate plasticity at GABAergic synapses (Nugent et al., 2007), as well plasticity at nearby excitatory synapses onto both dopamine and GABA neurons (Bonci & Malenka, 1999; Overton, 1999). Nugent & Kauer suggest that this

trigger from activity at an excitatory synapse to induce plasticity at a proximal inhibitory synapse may serve to balance excitation and inhibition within the mesolimbic dopamine circuit (2008). Further work is necessary to understand if the LTP that we observed in response to a 5 Hz electrical stimulus is the same LTP observed by Bocklisch et al. in response to an optical stimulus (2013).

We found the inhibitory LTD onto VTA GABA neurons to be $GABA_B$-mediated. Edwards et al. found extensive evidence that NAc D1-MSN input to VTA GABA neurons is $GABA_A$-mediated, while NAc D1-MSN to VTA dopamine neurons is mediated by postsynaptic $GABA_B$ receptors, and that both inhibitory NAc afferents are modulated by the A_1R (2017). This may suggest that $GABA_B$-mediated LTD that we observed may be from another source besides the NAc, and we did observe optically-induced LTD at the synapse of LH inhibitory inputs to VTA GABA cells. Metabotropic $GABA_B$ receptors have a long history of influencing both excitatory and inhibitory hippocampal plasticity in many ways (Heaney & Kinney, 2016; Jappy et al., 2016). There is also a significant body of research demonstrating the $GABA_B$ agonists, such as baclofen, may attenuate certain drug-seeking behaviors in animals (Roberts et al., 1996; Brebner et al., 2000; Fadda et al., 2003; Cruz et at., 2004; Heaney & Kinney, 2016). The present study is the first report of $GABA_B$ receptor involvement in plasticity of inhibitory inputs to VTA GABA neurons, which may help to in part explain the results of previous behavioral studies. $GABA_B$ LTD could disinhibit VTA GABA cells and thus increase dopamine cell activity. Further studies are required to understand if the LTD that we observed via electrical stimulus is the same LTD that we evoked upon optical stimulation of the LH to VTA inhibitory pathway.

The occurrence of LTP or LTD at the same synapse raises questions about why two types of plasticity can occur and why we have yet to see cells seemingly without plasticity where both LTP and LTD have occurred simultaneously to give a net null effect. Our two hypotheses to explain this phenomenon are that (1) inhibitory inputs from different areas of the brain lead to distinct plasticity outcomes, or that (2) distinct GABA cell subtypes within the VTA experience inhibitory plasticity differently. We believe that our current data support the first hypothesis more than the second, though the variety of GAD, VGAT, and TH expression among "GABA" cells within the VTA should certainly not be overlooked (Morales & Margolis, 2017). We previously demonstrated the variety of expression in calcium-binding proteins and mGluRs among VTA GABA and DA neurons (Merrill et al., 2015). Unlike in cortical areas, identification of calcium-binding proteins and neuropeptides was not very effective at differentiating GABA cells into specific subtypes. Input/output circuitry may lead end up being the best method of classifying VTA GABA cells. In addition, our lab has shown mGluR and CB1 dependent plasticity of excitatory inputs to VTA GABA neurons (Friend et al., 2017). Our current data present a different story—inhibitory plasticity to VTA GABA cells that is likely CB1-independent. Additionally, we herein offer evidence of GABAB-mediated LTD that likely occurs specifically at inputs from the LH.

Interestingly, our experiments testing μ-opioid receptor modulation using DAMGO did not point to LTP or LTD cells having inhibitory afferents exclusively expressing the μ-opioid receptor. Some cells expressing LTP depressed following acute DAMGO exposure (n = 3), while others also expressing LTP did not (n = 3). Similarly, some cells expressing LTD depressed following acute DAMGO exposure (n = 3), while others also expressing LTD did not (n = 3). However, in a recent study by Matsui et al., they found that different inhibitory inputs to VTA

DA cells (including the RMTg, NAc, and VTA proper) have different sensitivities to opiate exposure (2014), so we feel it possible that inhibitory inputs to VTA GABA neurons may also have different responses to acute opiate exposure that vary between inputs. VTA GABA cells may serve as "switch points" for driving reward and aversion, integrating signals from various inputs and modulate dopamine activity differentially.

Another important consideration when recording from VTA neurons is the location of these cells. The VTA is fairly small midbrain nucleus that wraps around the foramen retroflexus (FR) in transverse slices of mouse brain (Franklin & Paxinos, 2008). More ventrally, the VTA is located anterior to the FR (though not an anterior as the LH which contains a larger, readily identifiable nucleus of GABA neurons), and in more dorsal slices is located medial and posterior to the FR (though not as posterior at the pons, which also contains many GABA cells that are smaller and more densely packed than VTA GABAs). We recorded from cells that were more medially located, but still GAD67-GFP+. Our work with GAD65/GAD67 animals suggests that GAD expression in GABA cells changes moving medial to lateral—more medial cells express GAD65 only, more laterally GABA cells strongly co-express GAD65 and GAD67, and the most lateral cells express GAD67 only, though these appear to be rare. We believe that the large majority of GAD67 GPF+ cells used in Figures 2.1-2.4 of this study were also GAD65-expressing. If cells co-expressed GAD65 and TH, they were located more medially and ventrally. Morales and Margolis report similar patterns of enzyme expression in dopamine neurons, with the most "classic" dopamine neurons (TH+, VMAT2+, DAT+, and D2+) located more laterally in the VTA and closer to the substantia nigra pars compacta (SNc), while dopamine cells of a more transient character (TH+ but VMAT2-, TH+ but DAT-, TH+ but D2-, TH+ and VGLUT2+) located more medially. A recent study showed circuit specificity between

the SNc and the dorsal striatum with the medial SNc preferentially receiving afferents from and projecting to the dorsomedial striatum and the lateral SNc preferentially receiving afferents from and projecting to the dorsolateral striatum (Lerner et al., 2015). We believe that this type of location-specific circuitry could also be present between the VTA and NAc and could explain potential variations between our data and that described by Bocklisch et al. (2013).

While many drugs of abuse act directly on dopamine neurons to increase their firing or block reuptake, opiates achieve increases in dopamine largely through the mechanism of disinhibition (Williams et al., 2001). Nugent et al. clearly showed that acute morphine exposure occludes plasticity of GABAergic inputs to VTA dopamine neurons (2007). Conversely, we found that chronic morphine altered neither form of plasticity that we observed at the synapse of inhibitory afferent to VTA GABA cells. Our results more closely align with Edwards et al., who found that D1 MSNs projecting to the VTA experience cocaine sensitization, but not morphine sensitization (2017). Morphine has different effects on mesolimbic synapses acutely vs. chronically, which may be due to phenomena such as tolerance (Bonci & Williams, 1997; Fyfe et al., 2010). Moreover, we note that morphine is not an exclusive μ-opioid receptor agonist—at higher concentrations, morphine can also agonize κ- and δ-opioid receptors, but at concentrations greater than 100 times the dose required to activate μ-opioid receptors (Williams et al., 2001). Additionally, the κ-opioid receptor-specific agonist U69653 did not weaken IPSCs to VTA dopamine neurons, unlike the μ-opioid receptor-specific agonist DAMGO which reduced the amplitude of IPSCs significantly (Matsui et al., 2014). We believe that inputs to VTA GABAs likely show similar sensitivity to μ- and κ-opioid receptor agonists based on our data showing the response of these inhibitory inputs to DAMGO.

The ultimate goal of studying synaptic plasticity in the mesolimbic dopamine circuit is to further our understanding of the molecular mechanisms responsible for experience-dependent synaptic plasticity which mediates behavioral responses to reward and aversion. In the future, we hope to apply this knowledge clinically to aid in the discovery of safe and effective treatments for those struggling with drug abuse disorder and addiction (Luscher et al., 2015). Opioid abuse and addiction are serious problems in the United States and around the world (Imtiaz et al., 2014; Degenhardt et al., 2014). Current treatments are usually only marginally effective and often do little to decrease the growing problem of opioid addiction, sometimes even exacerbating it (Schuckit, 2016; Soyka, 2017). A greater understanding of inhibitory plasticity to VTA GABA neurons is a step toward the development of technology and therapeutic interventions for opioid addiction.

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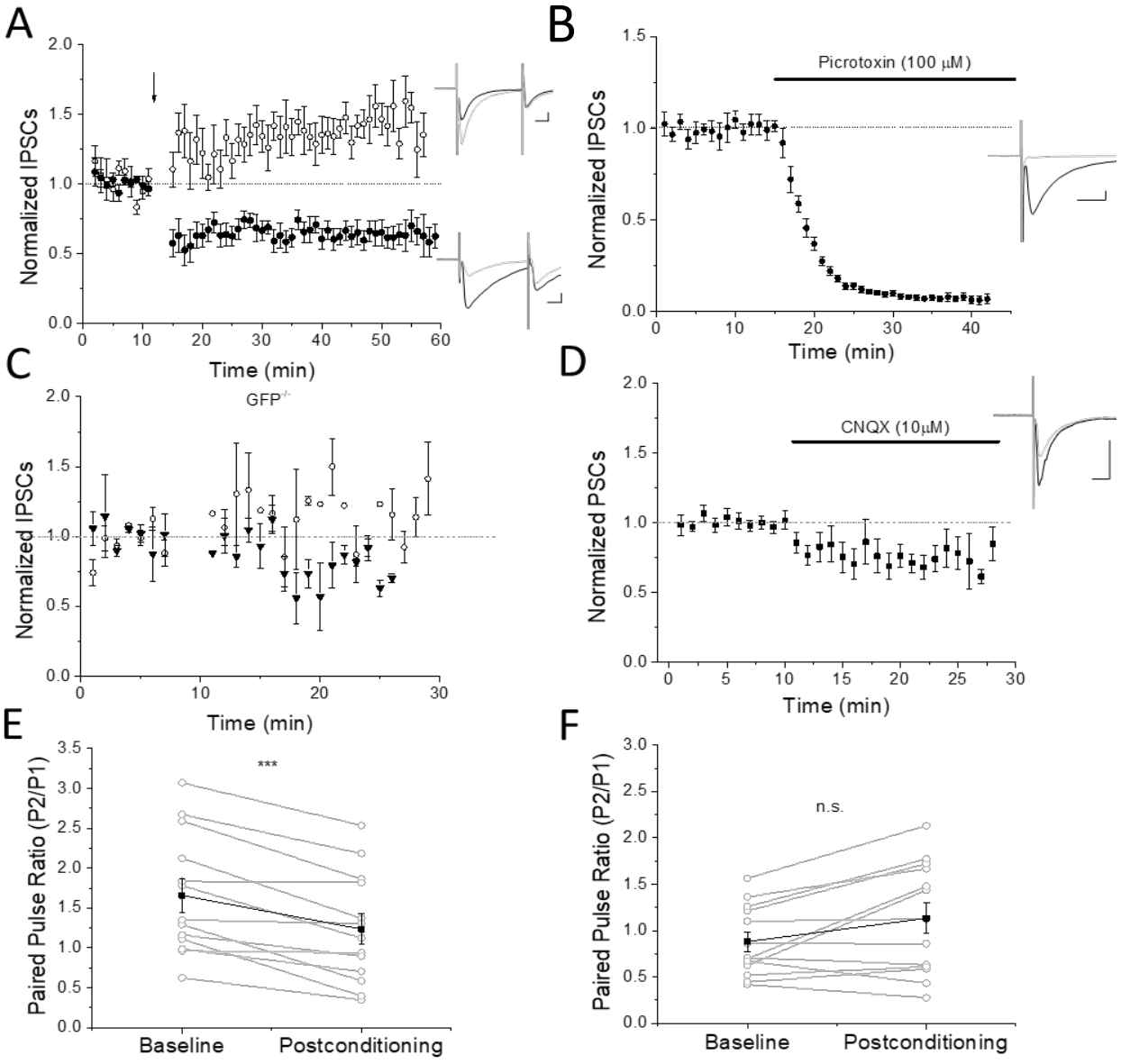

Figure 2.1: Inhibitory Afferent to GAD67+ VTA GABA Cells Exhibit Presynaptic LTP or LTD in Response to 5Hz Stimulus. A) LTP or LTD exhibited at inhibitory inputs to VTA GABA cells in response to 5Hz stimulus (LTP: n = 7, $p<0.001$ comparing 20-30 minutes post-conditioning to baseline using Student's T-Test; LTD: n = 8, $p<0.001$ comparing 20-30 minutes post-conditioning to baseline using Student's T-Test). Scale bars in trace insets represent 100 pA and 10 ms. B) $GABA_A$ antagonist picrotoxin eliminates IPSCs (n = 11). C) GFP- littermates of GAD67 GFP+ animals also exhibit either LTP or LTD in response to 5 Hz stimulus (LTP: n = 2; LTD: n = 2). D) AMPA receptor antagonist CNQX (10 μM) reduces current from all afferent to VTA GABAs to 78.9% ±2.1 of baseline (n = 9, $p<0.001$ comparing baseline to 5-10 minutes after CNQX exposure using Student's T-Test). E) Paired pulse ratio analysis suggests a presynaptic mechanism for the observed LTP (baseline, 1.20 ± 0.19; after stimulation, 0.79 ± 0.14; n = 13, $p<0.001$ using Wilcoxon Ranked Sign Test). F) Paired pulse ratio analysis suggests a presynaptic mechanism for the observed LTD (n = 13, $p>0.05$ using Wilcoxon Ranked Sign Test).

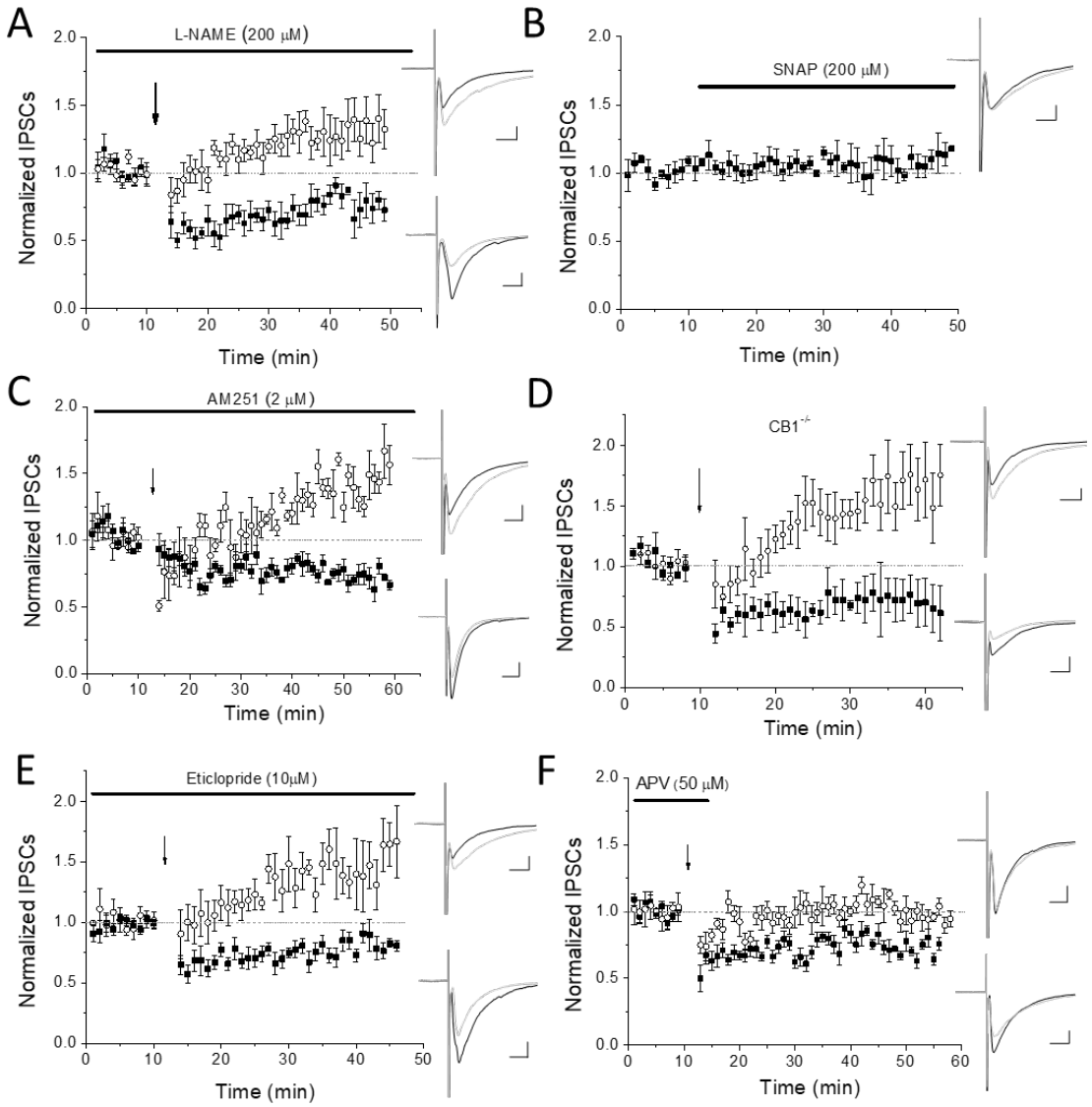

Figure 2.2: LTP and LTD Are Independent of the NOS System, the CB1 Signaling Pathway, and D2 Receptors, while the LTP Is Dependent on NMDA Signaling. A) Both LTP and LTD are still exhibited in the presence of NOS antagonist L-NAME (200 μM; LTP: n = 4, $p<0.001$ comparing 20-30 minutes post-conditioning to baseline using Student's T-Test; LTD: n = 6, $p<0.001$ comparing 20-30 minutes post-conditioning to baseline using Student's T-Test). B) The NO donor SNAP failed to induce plasticity at this synapse (n = 5, $p>0.05$ comparing 20-30 minutes post-conditioning to baseline). C) Bath application of CB1 antagonist AM251 (2 μM) failed to block plasticity (LTP n=4, $p<0.001$ comparing 20-30 minutes post-conditioning to baseline using Student's T-Test; LTD: n = 7, $p<0.001$ comparing 20-30 minutes post-conditioning to baseline using Student's T-Test). D) Both types of plasticity are also still present in GFP+ CB1-/- animals (LTP: n = 6, $p<0.001$ comparing 20-30 minutes post-conditioning to baseline using Student's T-Test; LTD: n = 3, $p<0.001$ comparing 20-30 minutes post-conditioning to baseline using Student's T-Test). E) Blockade of D2 receptors using eticlopride (10 μM) also failed alter plasticity (LTP: n = 5, $p<0.001$ comparing 20-30 minutes post-conditioning to baseline using Student's T-Test; LTD: n = 8, $p<0.001$ comparing 20-30 minutes post-conditioning to baseline using Student's T-Test). F) The NMDA receptor antagonist APV (50 μM) blocked the previously observed LTP (no plasticity group n = 4, $p=0.983$ comparing minutes 20-30 post-conditioning to baseline using Student's T-Test; $p<0.001$ compared to control LTP in Figure 1A post-conditioning using Student's T-Test; for LTD: n = 7, $p<0.001$ comparing baseline to 20-30 minutes post-conditioning using Student's T-Test). Scale bars in trace insets represent 100 pA and 10 ms.

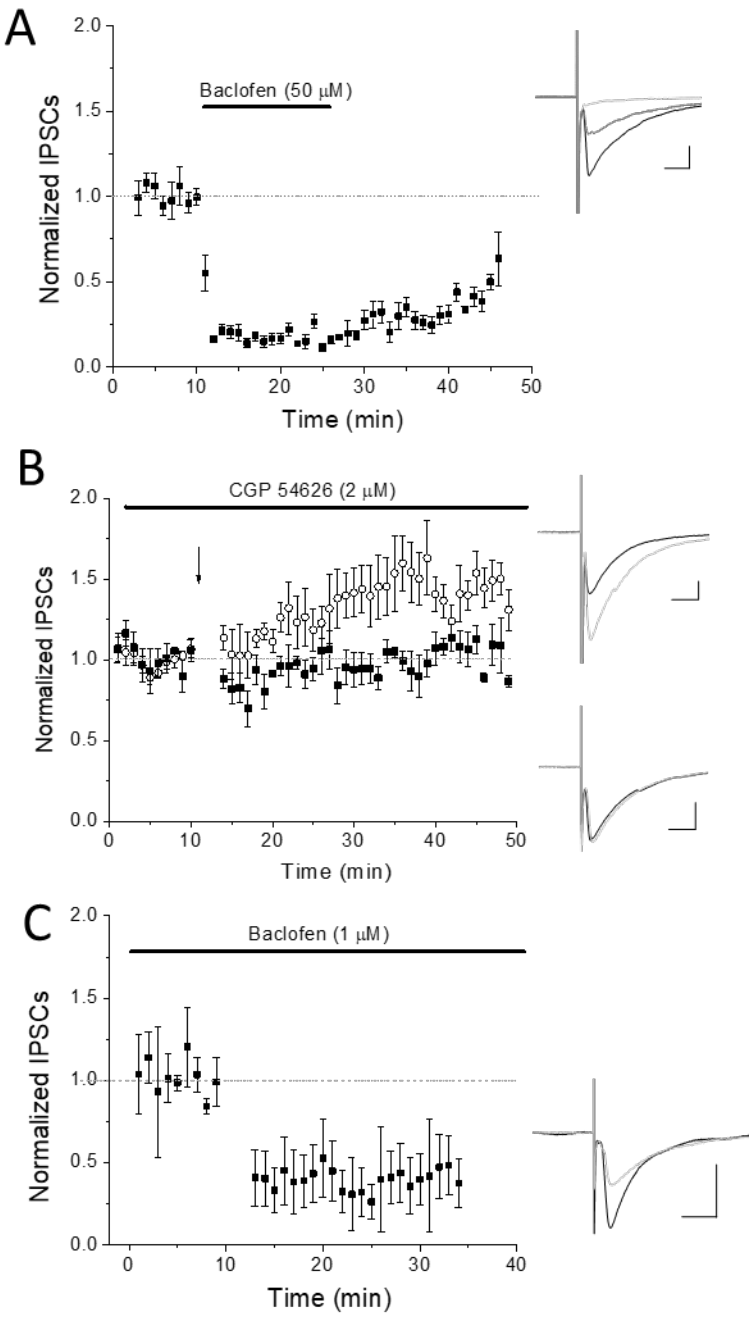

Figure 2.3: $GABA_B$ Receptors Modulate Neurotransmission and Specifically LTD at the Inhibitory Synapse onto VTA GABA neurons. A) Baclofen (50 μM) strongly depresses IPSCs onto VTA GABAs (n = 5). Trace inset shows baseline (black), baclofen conditioning (light gray), and washout (dark gray dot-dash). B) $GABA_B$ antagonist CGP 54626 (2 μM) blocks the previously observed LTD (no plasticity: n = 5; p=0.2570 comparing 20-30 minutes post-conditioning to baseline using Student's T-Test, p<0.001 compared to control LTD in Figure 1A post-conditioning using Student's T-Test; for LTP: n = 7, p<0.05 comparing baseline to 20-30 minutes post-conditioning using Student's T-Test). C) A lower concentration of baclofen (1 μM, closer to EC_{50}) does not produce occlusion, but may promote expression of LTD (n = 3, p<0.05 comparing baseline to 20-30 minutes post-conditioning using Student's T-Test). Scale bars in trace insets represent 100 pA and 10 ms.

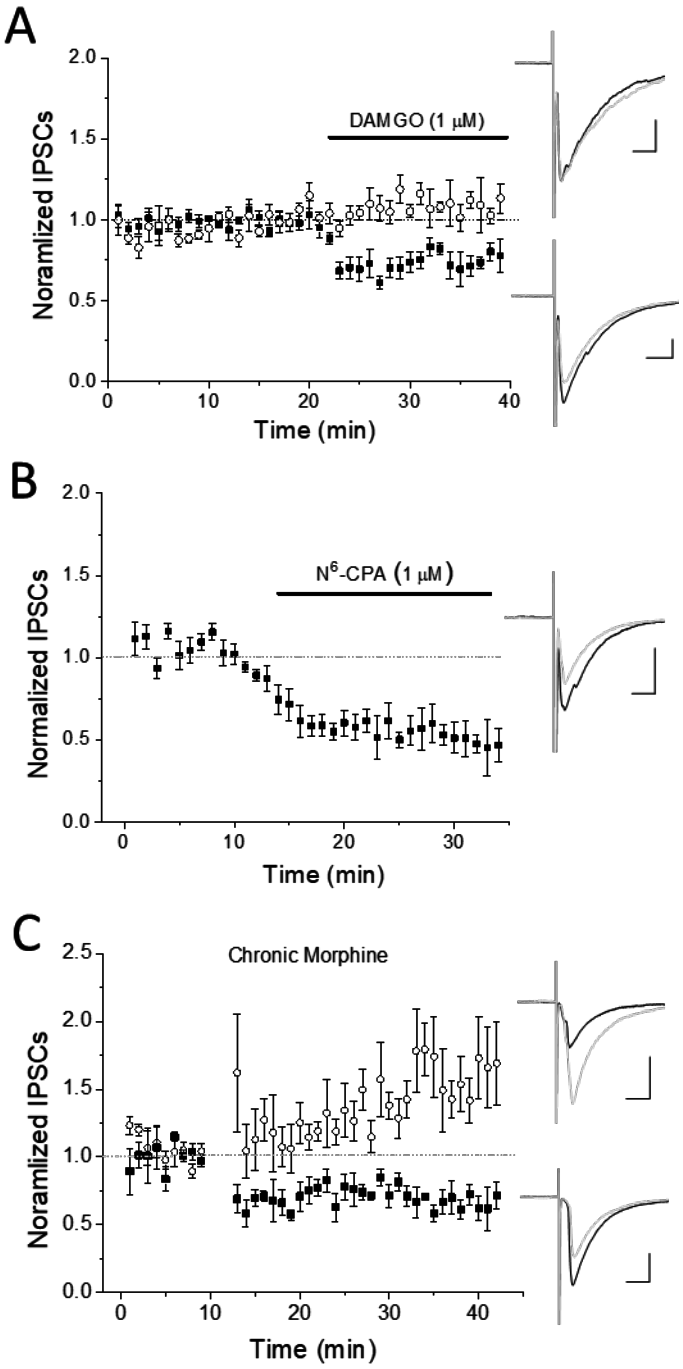

Figure 2.4: μ-opioid Receptor Activation Produces Depression in Some Cells, A_1R Activation Produces Depression in all Cells tested, and Chronic Morphine Administration Does Not Block either Form of Previously Observed Plasticity. A) Acute bath application of the selective μ-opioid receptor agonist DAMGO depresses 50% of synapses tested (depression: n = 6, $p<0.05$ comparing baseline to 5-10 minutes following bath application of drug; no depression: n = 6 $p<0.05$ comparing baseline to 5-10 minutes following bath application of drug). B) Activation of A_1R by N^6-CPA application causes all cells tested to depress significantly (n = 7, $p<0.05$ comparing baseline to 5-10 minutes following bath application of drug). C) Chronic administration of morphine (10mg/kg via daily IP injection) did not alter either form of previously observed plasticity (LTP: n = 5, $p<0.05$ comparing 10-20 minutes post-conditioning to baseline; LTD: n = 4; $p<0.05$ comparing 10-20 minutes post-conditioning to baseline).

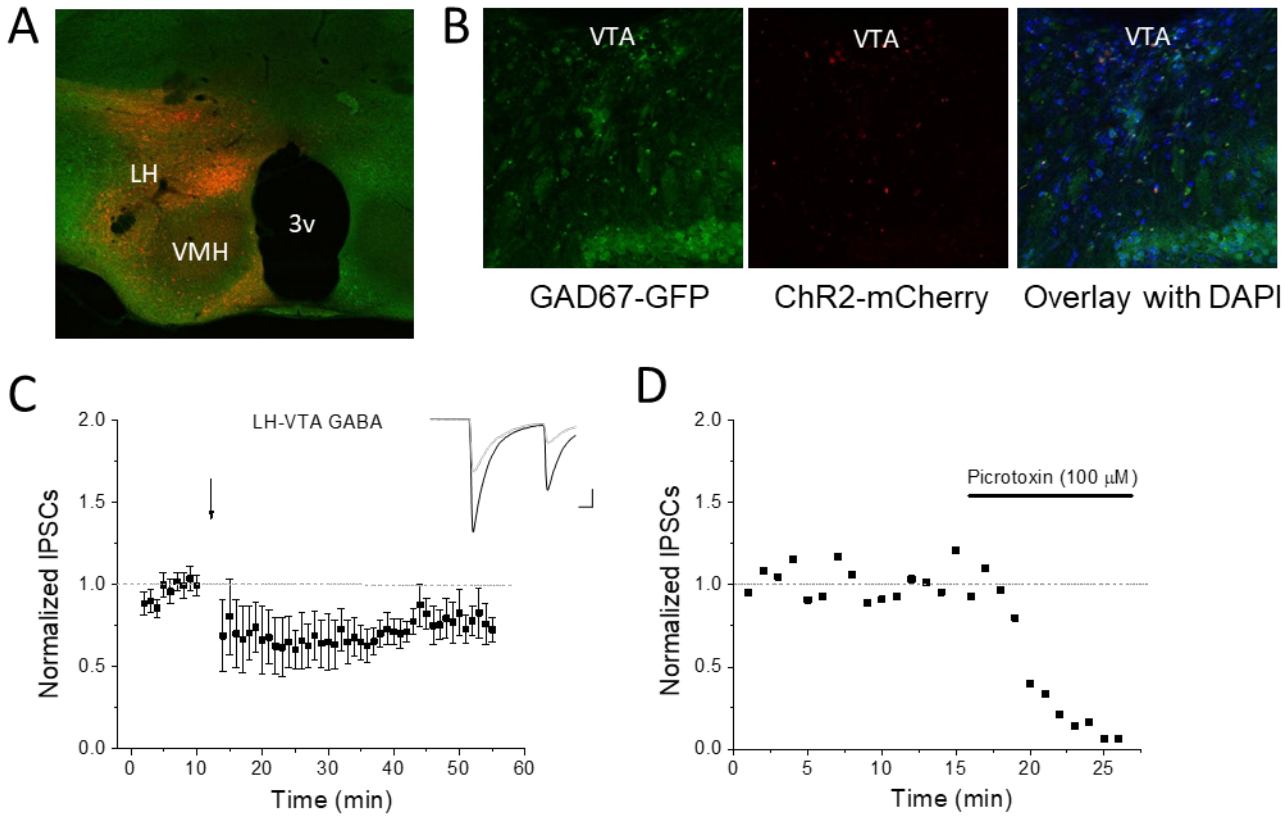

Figure 2.5: The Inhibitory (VGAT+) Projection from the LH to VTA GABA Cells Exhibits LTD in Response to a 5 Hz Optical Stimulus. A) We imaged GAD67-GFP expression and ChR2-mCherry expression in the injection site of surgery mice. B) We were also able to visualize ChR2-mCherry+ axon terminals synapsing onto GAD67-GFP+ cells in the VTA. C) In response to a 5 Hz optical stimulus, the VGAT+ input from the LH to VTA GABAs depressed significantly ($n = 4$, $p<0.05$ comparing baseline to 15-25 minutes post-conditioning). D) This response was indeed GABAA mediated as bath application of picrotoxin eliminated the current. LH=Lateral Hypothalamus, 3v=3rd ventricle, VMH=Ventromedial hypothalamus. Scale bars in trace inset represent 100 pA and 10 ms.

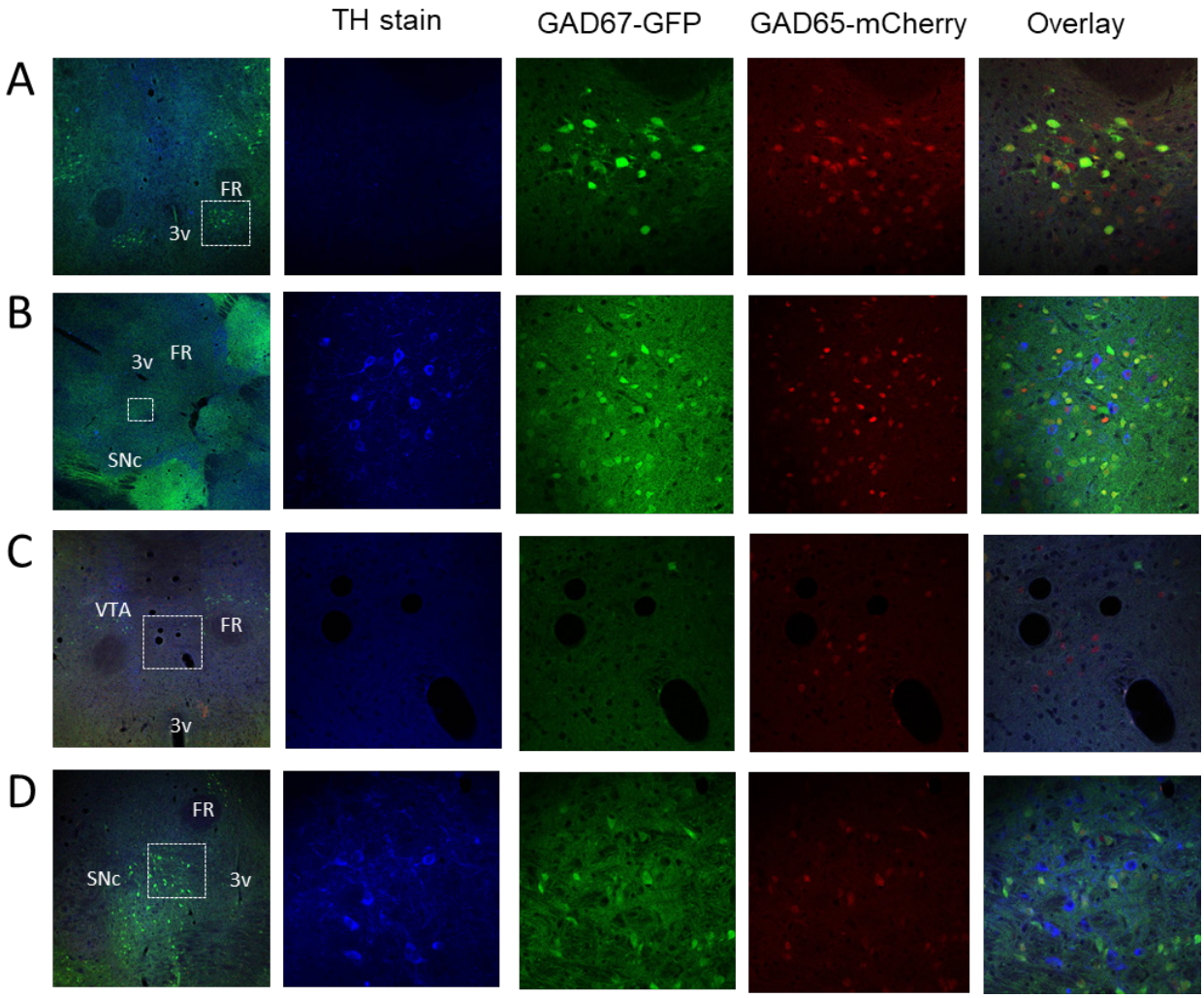

Figure 2.6: Heterogeneity of GAD65, GAD67, and TH Expression among VTA GABA and Dopamine Cells. A) GAD65 (mCherry) and GAD67 (GFP) often co-express, especially in dorsal VTA. B) GAD65 (mCherry) and TH (blue stain) co-express in ventral VTA. C) GAD65 only cells are found medial to the VTA. D) GAD67 and TH do not co-express in any part of the VTA. FR=foramen retroflexus, 3v=3rd ventricle, SNc=substantia nigra pars compacta.

CHAPTER 3: Expression of mGluR5 Predicts Interneuron Plasticity in the Hippocampal Stratum Radiatum

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Abstract

Changes in synaptic strength in hippocampal CA1 pyramidal cells are thought to be responsible for the acquisition and retention of short-term memory. This plasticity is modulated by feedforward inhibitory interneurons in the stratum radiatum which are composed of many subtypes including, among others, parvalbumin-containing axo-axonic cells, calretinin-containing interneuron-selective cells, and cholecystokinin/calbindin positive basket cells. While radiatum interneurons experience long-term depression (LTD), short-term depression (STD), or lack of plasticity, it is not known whether these types of plasticity correlate to specific interneuron subtypes. Using whole cell electrophysiology and real-time quantitative PCR, we characterized the plasticity expressed by different hippocampal interneuron subtypes in correlation with their mRNA expression patterns to determine cell subtype using calcium binding proteins and neuropeptide CCK. We also assessed the expression of endocannabinoid (eCB) biosynthetic enzymes including diacylglycerol lipase α, N-acyl-phosphatidylethanolamine-specific phospholipase D, and 12-lipoxygenase, as well as metabotropic glutamate receptor subunits known to mediate plasticity. Cells exhibiting LTD tended to express mRNA for at least one of the eCB biosynthetic enzymes and the metabotropic glutamate receptor subunit mGluR5. mGluR5 was not expressed by cells exhibiting STD or no plasticity. Cells that exhibited short-term depression tended to express mRNA for at least one of the eCB biosynthetic enzymes, but not mGluR5. This suggests that stratum radiatum interneuron plasticity can be predicted based

on cell subtype and mGluR expression, and that these different types of plasticity may have some importance in hippocampal function.

Introduction

Acquisition and retention of short-term memory are mediated by changes in synaptic strength between hippocampal neurons. These processes are thought to be mediated by synaptic plasticity, which is defined as alterations to neurotransmission. Traditionally, increased synaptic activity strengthens the synaptic connection, termed long-term potentiation (LTP) (Bliss and Lomo, 1973), while decreased activity weakens the synapse, termed long-term depression (LTD) (Dudek and Bear, 1992). Both LTP and LTD occur at hippocampal synapses involving excitatory pyramidal cells and inhibitory interneurons. While the excitatory pyramidal cells comprise a more homogenous population of neurons, the inhibitory interneurons that modulate their activity vary widely in morphology (Nissen et al., 2010), expression of calcium-binding proteins and other marker proteins (Freund and Buzsáki, 1996; Petilla Interneuron Nomenclature Group, 2008), and stimulus-induced plasticity (Le Duigou et al., 2011). The plasticity of CA1 pyramidal cells is modulated by feedforward inhibitory interneurons in the stratum radiatum which are composed of many subtypes including, among others, parvalbumin-containing axo-axonic cells, calretinin-containing interneuron-selective cells, and cholecystokinin/calbindin positive basket cells (Freund & Buzsáki, 1996). While radiatum interneurons exhibit long-term depression (LTD), short-term depression (STD), or lack of plasticity (McMahon & Kauer, 1997), little is known about whether these types of plasticity correlate to specific interneuron subtypes. The variety present among interneurons in the hippocampus raises questions and theories about their functional purposes, but also makes them difficult to study (Kullman & Lamsa, 2011). Understanding the specific differences in form and function that exist amongst hippocampal interneurons will make possible a more complete picture of how plasticity and memory formation occur on a cellular and molecular level in the brain.

Comprehending the role of specific interneuron subtypes requires an understanding of the molecular mechanisms by which synapses change. Endocannabinoid (eCB) lipid signaling molecules are important and widely used transducers of synaptic plasticity (Chevaleyre et al., 2006). Made in the postsynaptic neuron from the cell membrane by biosynthetic enzymes, eCB messengers diffuse retrogradely across synapses to bind G-protein-coupled receptors (GPRs) and influence presynaptic release of neurotransmitter. For example, the eCB anadamide is made by n-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) and binds to presynaptic receptors such as CB1 and TRPV1 (Liu et al., 2006). Similarly, the eCB 2-arachidonylglycerol (2-AG) is produced by diacylglycerol lipase alpha (DAGLα) and also activates presynaptic GPRs (Tanimura et al., 2010). Finally, 12-lipoxygenase synthesizes 12-HPETE, yet another lipid-signaling molecule that can influence synaptic plasticity by acting on the presynaptic cell (Feinmark et al., 2003). Postsynaptic activation of Type I metabotropic glutamate receptors (mGluRs) initiates eCB synthesis (Jung et al., 2005; Varma et al., 2001), and Type I mGluRs are widely expressed in the hippocampus (Baude et al., 1993; Ferraguti et al., 2004).

Le Duigou et al. (2011), Gibson et al. (2008), and Edwards et al. (2012) thoroughly explored the roles of eCB and Type I mGluR-mediated plasticity at excitatory synapses onto interneurons in the hippocampus, where type I mGluR activation induces LTD. The interneurons themselves expressed the enzymes needed to produce eCBs (Merrill et al., 2012) that were directly involved physiologically in LTD-induction (Péterfi et al., 2012). Their work demonstrates the postsynaptic involvement of group I mGluRs to activate presynaptic depression of the synapse. However, it is not known how Type I mGluRs 1 and 5 specifically contribute to the induction of different types of plasticity (i.e. LTD, STD, or no plasticity) in interneurons of the CA1 stratum radiatum. Our goal was to demonstrate the relationship between hippocampal

CA1 stratum radiatum interneuron subtypes and synaptic plasticity, and the relationship between synaptic plasticity and eCB biosynthetic enzyme and type I mGluR mRNA expression. While the occurrence of eCB-mediated synaptic plasticity within the hippocampus is well documented, there is little evidence for the involvement of specific interneuron subtypes in eCB-synaptic plasticity. Using a combination of whole cell patch clamp electrophysiology and quantitative real time PCR, we discovered a relationship between the expression of Type I mGluRs and the type of plasticity that individual interneurons experienced. Expression of mGluR5 seems to predict LTD in CA1 radiatum interneurons. These data provide further evidence for the importance of interneuron synaptic plasticity within the hippocampus and the importance of eCB-mediated signaling in modulation of pyramidal cell activity.

Methods

Electrophysiology

All experiments were performed in accordance with Institutional Animal Care and Use Committee protocols and NIH guidelines for the care and use of laboratory animals. Male Sprague-Dawley rats P15-30 days old were anesthetized with isoflurane (1.5-2%) and decapitated. Brains were removed and sectioned coronally on a vibratome at 400 μm using oxygenated artificial cerebral spinal fluid (ACSF) composed of 119 mM NaCl, 26 mM $NaHCO_3$, 2.5mM KCl, 1 mM NaH_2PO_4, 2.5mM $CaCl_2$, 1.3mM $MgSO_4$, 11mM glucose, and 400 μM ascorbic acid. Recordings began at least one hour after cutting while tissue was stored in oxygenated ACSF composed of 119 mM NaCl, 26 mM $NaHCO_3$, 2.5mM KCl, 1 mM NaH_2PO_4, 2.5mM $CaCl_2$, 1.3mM $MgSO_4$, and 11mM glucose at room temperature. Inhibitory $GABA_A$ currents were blocked using 100 μM picrotoxin (Abcam) throughout. An Olympus BX51WI microscope with a 40x water immersion objective was used to visualize hippocampal radiatum CA1 cells. Interneurons in the CA1 stratum radiatum were patched with a borosilicate glass pipette (3-6 MΩ) filled with internal solution composed of 117mM cesium gluconate, 2.8mM NaCl, 20mM HEPES, 5mM $MgCl_2$, and 1 mM QX-314 (Tocris) with pH 7.28 and osmolarity 275–285 mOsm. Recording were made in voltage clamp with cells being held at -65 mV throughout the experiment. Plasticity was induced using two 100 Hz stimulations, 20 seconds apart, while still holding the cell at -65 mV in voltage clamp mode. Currents were recorded using Multiclamp 700B amplifier and digitized with an Axon 1440A digitizer (both from Molecular Devices, Sunnyvale, CA, USA). Signals were filtered at 4 kHz and recorded using Clampex 10.4 (Molecular Devices) on a personal computer. Electrophysiological data was analyzed using Clampfit software (Molecular Devices), Microsoft Excel, and Origin 10.8 (OriginLab

Corporations, Northampton, MA, USA). All p-values were obtained by a Student's t-test comparing the 5 minutes before conditioning to 15-20 minutes post conditioning.

PCR

We used the PCR methods as described in our paper Merrill et al., 2015. Briefly, cells used for PCR analysis were extracted using gentle suction into the recording electrode and then placed into chilled reverse transcriptase reagents (BioRad) and processed 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 manufacturer's 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. We performed additional no-template-control tests to ensure that there were no primer dimer or hairpin interactions between primers. The samples were 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. Final PCR reactions were run in triplicate with individual primers for each target and specific FAM-TAMRA© (company) probes. The primer and probe sequences were those used in our previous study (Merrill et al., 2012). 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. Targets coming up >20 cycles after 18S were rejected as non-specific false positives.

Results

To understand how the expression of eCB biosynthetic enzymes, Type I mGluRs, and interneuron markers is correlated with the induction of specific types of plasticity in individual cells, we used a combination of whole cell patch clamp electrophysiology and real time qPCR. After performing a whole cell electrophysiology experiment with high frequency stimulation (HFS) to induce plasticity, each cell was carefully aspirated into the pipette tip and qPCR was performed on the genetic material (as described in the Methods) for the targets listed in Table 3.1. This combination of techniques allowed us to describe specific relationships between the plasticity and mRNA expression patterns in individual cells. We hypothesized that plasticity of CA1 radiatum interneurons would correlate with specific, previously described interneuron subtypes (Nissen et al., 2010; Kullman & Lamsa, 2011).

Excitatory synapses onto CA1 radiatum interneurons are known to exhibit different types of plasticity including long-term depression (LTD), short-term depression (STD), or lack of plasticity (McMahon & Kauer, 1997). Out of 27 cells, we found that about 13 exhibited LTD (48.1%), 10 exhibited STD (37%), and 4 did not exhibit plasticity (14.8%) following HFS (Figure 3.1). Regarding the stratum radiatum interneurons that do not exhibit plasticity following HFS, these neurons appear to express fewer eCB biosynthetic enzymes, such as 12-Lipo, and are normally CCK+ basket cells. They typically do not express mGluRs (Figure 3.1 No Plasticity). Interneurons exhibiting STD following HFS express higher levels of eCB biosynthetic enzymes, such as 12-Lipo and NAPE-PLD, and can be of various subtypes, including CCK+ basket cells. Some express mGluR1 (Figure 3.1 Short Term Depression). Finally, stratum radiatum interneurons exhibiting LTD in response to HFS also typically express eCB biosynthetic enzymes, but also often express mGluR5 and/or mGluR1 (Figure 3.1 Long Term Depression).

They can be of many subtypes including CB+ or CCK/CB+ basket cells. When quantifying our results, we discovered that plasticity is not necessarily an indicator of interneuron subtype, nor is subtype indicative of plasticity (Table 3.2). While most cells exhibiting no plasticity were CCK positive, most cells that exhibited STD or LTD were categorized as "uncategorized," meaning that we were not able to categorize them into classically recognized interneuron subtypes based on our PCR results. However, real time qPCR is known to have a high rate (up to 35%) of false negatives, so it is possible that many, if not all, of our uncategorized cells actually expressed more interneuron markers than we saw or different markers we did not examine. Despite the high percentage of uncategorized cells in our sample, we discovered an important relationship between exhibition of LTD and the expression of mGluR5. Though not all cells exhibiting LTD expressed mGluR5, every cell that expressed mGluR5 exhibited LTD (Table 3.2). Therefore, mGluR5 activation could be necessary to produce the eCBs that induce LTD at this excitatory synapse onto radiatum interneurons. Additionally, mGluR1 appears to only be expressed in cells that experience LTD or STD (Table 3.2). We also observed that nearly all stratum radiatum interneuron subtypes appear to produce at least one of the eCB biosynthetic enzymes, regardless of LTD or STD plasticity. However, these enzymes are not as commonly produced in cells without plasticity (Table 3.2).

Discussion

Our data show a potential relationship between the expression of mGluR5 in CA1 radiatum interneurons and the exhibition of LTD: it appears that cells expressing mGluR5 exhibit LTD preferentially over other types of plasticity observed in radiatum interneurons. Furthermore, nearly all the cells in our study expressed mRNA for eCB biosynthetic enzymes as demonstrated previously (Merrill et al., 2012), confirming that eCB-mediated plasticity is likely a common and important mechanism of presynaptic plasticity in the CA1 stratum radiatum of the hippocampus.

Our recent study demonstrating the differential distribution of eCB biosynthetic enzyme mRNA (Merrill et al., 2012) is important, as it suggests that eCB-mediated processes do not occur equally at CA1 stratum radiatum interneuron synapses. In fact, in studies of eCB-mediated LTD, all interneurons tested did not respond equally to high-frequency stimulation (McMahon and Kauer, 1997, Gibson et al., 2008). McMahon & Kauer (1997) first described plasticity of excitatory inputs to hippocampal interneurons in the stratum radiatum. They reported both bistratified and basket cell morphology among interneurons, though concluded plasticity expression could not be correlated with interneuron subtype. Our data also supports that interneuron plasticity is not necessarily cell-type specific, but rather plasticity is determined by the expression of eCB-biosynthetic enzymes and mGluRs.

Our results also indicate a qualitative effect on plasticity—cells that express mGluR5 can exhibit LTD while those that do not express mGluR5 do not exhibit LTD. These hippocampal interneurons also express mRNA for eCB biosynthetic enzymes including DAGLα, which is expressed in high levels in pyramidal cells and lower levels in interneurons (Péterfi et al., 2012). Péterfi et al. showed that higher stimulation frequencies and higher concentrations of DHPG

were required to induce plasticity in interneurons than in pyramidal cells, indicative of a more quantitative effect. Though we did not test whether increasing the number of stimulus trains increased the incidence of LTD, we call attention to expression of other eCB biosynthetic enzymes besides DAGLα in hippocampal interneurons of the CA1 stratum radiatum. NAPE-PLD and 12-LO are also frequently expressed in CA1 radiatum interneurons (Merrill et al., 2012). These postsynaptically-located enzymes produce anadamide and 12-HPETE, respectively, which can also activate CB1 presynaptically and induce plasticity. Though a quantitative effect as demonstrated by Péterfi et al. may be real and present, we acknowledge that other eCB biosynthetic enzymes may be also contributing to the plasticity we observed, either synergistically or on their own. Additionally, we point out that the qualitative effect we describe is specific to the Type 1 GPR mGluR5, not an eCB biosynthetic enzyme. Moreover, our data do not suggest that expression of any particular eCB biosynthetic enzyme correlates with a certain type of plasticity—NAPE-PLD and 12-LO were expressed in cells exhibiting any type of plasticity, and DAGLα was present in cells expressing STD or LTD.

The highly heterogeneous nature of hippocampal interneurons has historically made them difficult to study. Expression of classic interneuron markers can correlate with morphology and/or plasticity (Freund & Buzsáki, 1996; Nissen et al., 2010; Péterfi et al., 2012), and to effectively study these interneurons, investigators must carefully define the population they are studying, often making it challenging to compare the findings of different studies. We concede that the present study is limited in that we were unable to study cell morphology and correlate projection patterns to plasticity and Type I mGluR expression. To reduce the incidence of false negatives in PCR, we pulled the entire cell following the plasticity experiment, making it prohibitively difficult to also image cells for morphology. We limited our study to interneurons

of the CA1 stratum radiatum. Our data describes interneurons that likely fall into classically described subtypes including parvalbumin-containing axo-axonic cells, calretinin-containing interneuron-selective cells, and cholecystokinin/calbindin positive basket cells (Freund & Buzsáki, 1996), but subtype does not necessarily correlate with the type of plasticity that a cell will exhibit. Furthermore, in our study, many neurons where classified as being "uncategorized" cell types because their expression pattern of interneuron markers did not match classic descriptions. We recognize that PCR is known to have at least a 35% rate of false negatives as well as false positives, though ACSF controls were used to decrease the rate of false positives. We believe that the high rate of uncategorized cells is due to the technical limitations of single-cell, quantitative real time PCR or targets we selected to investigate.

The mechanisms behind the plasticity we observed in excitatory afferents onto CA1 radiatum interneurons have already been explored. While we used a high frequency stimulus to induce plasticity (two bursts of 100 Hz, similar to Nissen et al., 2010), LTD can also be elicited using DHPG (Péterfi et al., 2012; Edwards et al., 2012; Le Duigou et al., 2011) or a paired spiking protocol (Péterfi et al., 2012; Gibson et al., 2008) described TRPV1-dependent, HFS-induced interneuron plasticity in the stratum radiatum. Edwards et al. (2012) and Le Duigou et al. (2011) describe a DHPG-induced LTD at this synapse that appears to be modulated presynaptically via the production of a postsynaptically produced retrograde messenger that is not dependent on CB1, $GABA_B$, or TRPV1. Conversely, Péterfi et al. (2012) describe a paired spiking-induced plasticity that is dependent on mGluR5, postsynaptic calcium influx, DAG lipase activity, and CB1, though the cells record from in this study were in the CA1 field, but not specifically the stratum radiatum. Our data confirm that LTD is inducible at excitatory synapses onto interneurons in the CA1 radiatum and point to the importance of mGluR5 activation when

inducing LTD. mGluR1 appears to be less vital for long term plasticity not only in our study, but also in others (Edwards et al., 2012; Le Duigou et al., 2011; Péterfi et al., 2012). eCB biosynthetic enzymes appear to be dispersed ubiquitously among CA1 radiatum interneurons, and eCB synthesis likely plays an important role in hippocampal interneuron plasticity. Collectively, these data further demonstrate the importance of CA1 stratum radiatum interneurons in modulation of pyramidal cell activity and the potential of distinct interneuron populations to differentially modulate learning and memory processing within the hippocampus.

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Table 3.1: qPCR Targets for Each Cell. CA1 radiatum interneurons were run for classic interneuron markers, eCB biosynthetic enzymes, and Type I mGluRs.

Gene Targets		
Interneuron Markers	eCB Enzymes	Type I mGluRs
GAD65	12-Lipoxygenase	mGluR5
GAD67	NAPE-PLD	mGluR1
Parvalbumin (PV)	DAGLα	
Calbindin (CB)		
Calretinin (CR)		
CCK		

Table 3.2: Expression of eCB Biosynthetic Enzymes and Type I mGluRs as a Function of Plasticity and Interneuron Subtype.

Plasticity	Cell Type	DAGLα	NAPE-PLD	12-LO	mGluR5	mGluR1
None	CCK (75%)		X	X		
	PV (25%)					
STD	CCK (38%)	X	X	X		
	CCK-CB (13%)			X		
	Uncategorized (50%)		X	X		X
LTD	CCK (11%)		X			X
	CCK-CB (11%)			X	X	
	CB (11%)	X				
	PV (11%)			X		X
	Uncategorized (56%)	X		X	X	X

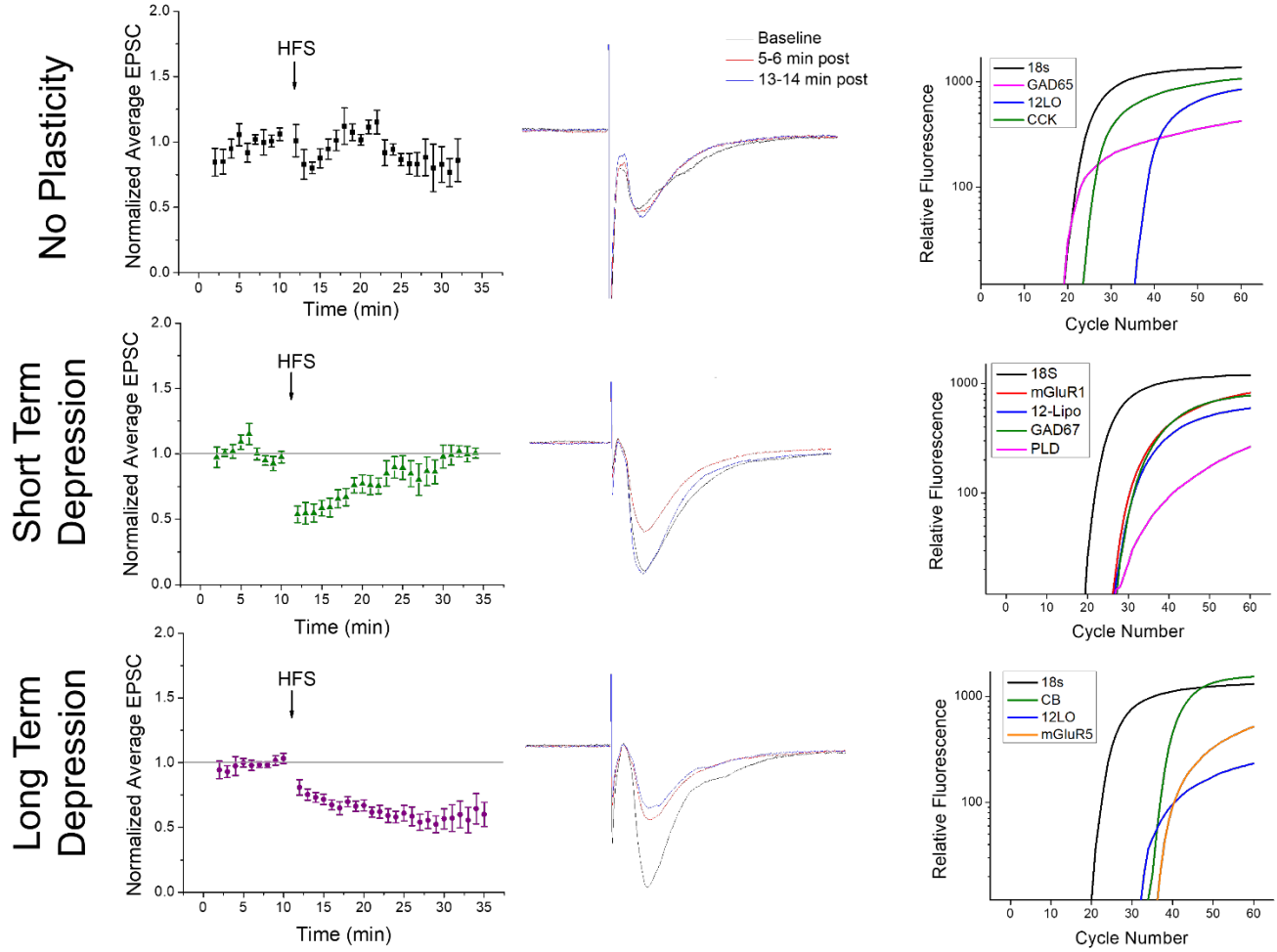

Figure 3.1: Gene Expression and Plasticity Profiles of Stratum Radiatum Interneurons. Approximately 15% of cells studied displayed no plasticity (n = 4, $p>0.05$) and many of these expressed mRNA for eCB biosynthetic enzymes, but not Type I mGLuRs. Next, 37% of interneurons expressed short term depression (LTD) which returned to baseline approximately 10-15 minutes post-conditioning (n = 10). These cells often expressed mRNA for eCB biosynthetic enzymes and occasionally for mGluR1. Finally, nearly 50% of cells displayed long term depression (n = 13, $p<0.05$ between baseline and 10-15 minutes post-conditioning). These cells always expressed mRNA for at least one eCB biosynthetic enzyme and often for Type I mGluRs, especially mGluR5.

CHAPTER 4: Conclusion

Synaptic plasticity is amazing. Our brains are wired with individualized methods of coding information so that we can learn, remember, and survive. We engage in hundreds of activities and tasks every day that literally shape our brains. Even the way that we think about ourselves and our surroundings can alter who we are on a neurological level.

Comprehending the molecular mechanisms of plasticity is key to understanding behavioral modifications. We understand uniqueness and creativity in the brain as we learn about synaptic plasticity. We understand the neural roots of many brain pathologies as we learn about plasticity. Drug addiction, mental illness, traumatic brain injury, aging, stress, and many other states of mind and body alter synaptic plasticity. From the LTP-like changes that form memories to the mechanisms of stress-induced hippocampal atrophy, clearly the physiology of our brains determines our behavior. Psychologists should find it difficult to talk about behavior without talking the brain, just as neuroscientists should remember the changes in behavior that result from changes in neural physiology.

The projects presented here have focused on how synaptic plasticity functions at specific synapses in the VTA and hippocampus. We highlight a novel form of plasticity at the synapse of inhibitory inputs to VTA GABA cells. We also report on how interneurons of the hippocampus express eCB biosynthetic enzymes and mGluRs in order to exhibit neural plasticity. Practically, these studies expand our understanding of the how synapses are modified on a cellular level and how that modulation can vary from cell to cell. Our results are unique in that we report multiple forms of plasticity occurring at the same synapses. We believe that these findings highlight the heterogenous character of GABAergic interneurons as well as the potential for GABA modifications to influence behavior in specialized ways. We speculate that GABA cells may act

as “switch points,” where neural outcomes are summated, measured, and executed to ultimately influence behavior.

One important behavioral consequence of plasticity in the mesolimbic dopamine circuit is addiction. Normally, this circuit helps us discern pleasurable, species-perpetuating stimuli from aversive, harmful stimuli, but addiction is the sustained disruption of this processing center such that an individual can no longer process reward normally. This disruption is the result of an artificial increase in dopamine release, commonly caused by drugs of abuse. Behaviorally, addiction is manifested by cravings, withdrawals, and compulsive drug-seeking behavior. A picture of the true pathology of addiction is slowly emerging as various synapses are studied at different phases of addiction using sophisticated techniques. Addiction involves changes to VTA dopamine and GABA neurons, as well as their efferents and afferents. My contribution to the field of molecular addiction research helps to paint a more complete picture of the effects that drugs of abuse have (and don’t have) on the brain. Using our knowledge of molecular plasticity mechanisms within the mesolimbic dopamine circuit, I hope that a pharmacological treatment for narcotic addiction will one day become available.

In conclusion, inhibitory neurons in the brain have long been underappreciated and understudied. However, new research argues that inhibitory GABA neurons may be the hidden ringmasters who orchestrate neural and behavioral outcomes. This study of GABA neurons in the brain contributes to the growing body of evidence suggesting the vital role of inhibitory neurotransmission in modulating both synaptic plasticity and behavior.

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CURRICULUM VITAE

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Education

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Research Experience

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I studied inhibitory inputs to GABA cells in the ventral tegmental area in mouse brain slices using whole cell electrophysiology.

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I studied the role of TRPV1 receptor in modulating plasticity in the hippocampus using field electrophysiology.

Publications and Abstracts

Co-authored the following article with Jeff G. Edwards and four others:
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Abstract accepted to and presented at Society for Neuroscience 2017:
"Long-term potentiation of inhibitory inputs onto VTA GABA neurons"

Abstract accepted to and presented at Society for Neuroscience 2016:
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Abstract accepted to and presented at Society for Neuroscience 2015:
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Co-authored the following article with Jeff G. Edwards and fourteen others (published under my maiden name St. Pierre): "Transient receptor potential vanilloid 1 agonists modulate hippocampal CA1 LTP via the GABAergic system." Neuropharmacology 61. 730-738. (2011) doi: 10.1016/j.neuropharm.2011.05.018

Teaching Experience

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- Taught physiology lectures to a class of ~150 students.
- Prepared supplemental material for lectures.
- Received humbling feedback about my performance.

Neuroscience Teaching Assistant Sept. 2014– April 2015
Brigham Young University, Provo, UT

- Led literature discussions with ~20 students.
- Held review sessions for literature discussions and tests.
- Responded to student questions during reviews and via email.
- Attended lecture and assisted the professor.

Chemistry Teaching Assistant January- April 2014
Brigham Young University, Provo, UT

- Taught chemistry principles to students in a tutorial lab setting four hours/week.
- Mentored over 50 chemistry students once a week in two "recitation" classes.
- Worked closely with the professor to revise exams before the students took them.
- Received the distinguished Garth L. Lee Undergraduate Teaching Award.

Writing Tutor Sept. 2013- Mar. 2014
Brigham Young University Writing Center, Provo, UT Sept. 2010- Apr. 2011

- Met with students from various disciplines, diagnosed their papers, and taught writing principles
- Wrote proposals and created presentations for peer tutoring conferences

Awards and Honors

Grants

Graduate Research Travel Award October 2015
I wrote and submitted a request for travel funds that detailed the purpose of and experiences that I hoped to have at the 2015 Society for Neuroscience Meeting in Chicago, IL. I was awarded $400 to help cover travel expenses for that trip.

Graduate Research Fellowship April 2015
I wrote a detailed research proposal including methods, expected results, preliminary data, and a statement of academic merit. I received $15,000 to support my research for an entire year so that I wouldn't have to work as a Teaching Assistant. This competitive fellowship was only awarded to about 28% of applicants.

Office of Research and Creative Activities (ORCA) Grant February 2014
I wrote a two-page grant proposal outlining an intended research project and was awarded $1500 to use for mentored research with Dr. Jeff Edwards.

Scholarships

Neuroscience Fellowship May 2014-June 2018
The Department of Physiology and Developmental Biology paid my tuition in full each semester/term.

Abrelia Clarissa Hinckley Scholarship Sept. 2013 - Apr. 2014
I was awarded $3153 each semester of the academic year based on my cumulative GPA and university faculty recommendations.

Karl G. Maesar Scholarship Sept. 2010 – Apr. 2011
I was awarded $2652 each semester of the academic year based on my cumulative GPA.

Brigham Young Scholarship Sept. 2009 – Apr. 2010
I was awarded $2145 each semester of the academic year based on my cumulative GPA.

Awards

Grad FAD Retreat 3rd Place Oral Presentation December 2016
Received a cash prize for a competitive presentation competition amongst graduate students of BYU's PDBio Department. Presented on "Modulation of Inhibitory Inputs to VTA GABA Neurons."

Garth L. Lee Undergraduate Teaching Award May 2014
The BYU Department of Chemistry awards one chemistry teaching assistant (TA) with this award every semester. I was chosen out of over 20 TAs to receive this award which included a cash prize of $150.

Leadership/Service

Volunteer July 2015-February 2017
Utah Valley Regional Medical Center

- Directed patients and their families to different areas in the hospital.
- Delivered flowers and other gifts to patients.
- Accompanied patients and their families to the emergency room.
- Helped admit patients in the wound care department.

Volunteer Representative Aug. 2011 – Feb. 2013
The Church of Jesus Christ of Latter-day Saints

- Taught religious principles in the Spanish language in Costa Rica.
- Conducted daily and weekly planning sessions and reported achievements.
- Trained new volunteers and taught them necessary skills.

Professional Affiliations

Society for Neuroscience May 2015-January 2018