From Estradiol to the Endogenous Cannabinoid System: A Gateway to Drug Addiction in Females

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Dedication

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Abstract

Drug addiction is a widespread condition affecting 8-10% of the population nationwide. Although men and women are both affected by drug addiction, sex-differences in responsiveness to drugs of abuse are apparent, with women showing a greater sensitivity than men. For over 30 years, the ovarian hormone estradiol has been recognized as a key biological factor contributing to the development of drug addiction in females, with little understanding of the neurobiological underpinnings. Here we propose a neural mechanism through which estradiol primes females to respond more strongly to drugs of abuse. Estradiol can act at intracellular or membrane-localized estrogen receptors to influence cellular function. Exclusively in females, a subset of these membrane estrogen receptors are coupled to group I metabotropic glutamate receptors (mGluRs). We demonstrate that it is through group I mGluRs that estradiol structurally remodels reward circuits in the nucleus accumbens (NAc), an effect associated with heightened sensitivity to drugs of abuse in females. In the nervous system, both estradiol and group I mGluRs can influence the activity of the endogenous cannabinoid (endoCB) system, an emerging mediator of neural plasticity and behavioral responses to drugs of abuse. Interestingly, we find that estradiol enhances the activity of the endoCB system in the female NAc by rapidly altering concentrations of the two best known endoCBs and increasing cannabinoid receptor expression. Further we demonstrate that activation of the endoCB system is critical for estradiol to induce structural plasticity in the NAc and facilitate behavioral responses to repeated drug exposure. Collectively, our results suggest that estradiol, group I mGluRs and the endoCB system may be linked through a

serial pathway that is principally important in female drug addiction. These findings begin to elucidate a neural mechanism underlying female vulnerability to addiction that contributes to sex differences in drug abuse and may actually provide putative therapeutic targets that are particularly effective in treating drug abuse in women.

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CHAPTER 1

Introduction and Literature Review

Estradiol enhances female vulnerability to drug addiction

Drug addiction is a widespread disorder affecting 8-10% of the population, with 16-17% of drug users progressing to a state of addiction (SAMSA, 2014). Intriguingly, women appear to be at a heightened risk for reaching this addicted state (Becker et al., 2012; Wagner and Anthony, 2007). Sex differences in the onset and development of drug addiction support this enhanced vulnerability in women. Compared with men, women initiate psychostimulant use at an earlier age, show an enhanced acute response to drugs of abuse, more rapidly increase dose and frequency of drug use, and progress from initial exposure to addiction more rapidly (Justice and de Wit, 1999; Van Etten et al., 1999; Van Etten and Anthony, 2001). This accelerated progression towards addiction in women is especially evident with psychostimulant drugs, including cocaine and amphetamine, but also applies to other classes of abused drugs, such as opioids, nicotine, alcohol, and marijuana (Carroll et al 2004, Becker and Hu 2008).

Ovarian hormones are key biological factors that contribute to addiction vulnerability in women and underlie sex differences in the development of this condition (Festa and Quinones-Jenab, 2004; Quinones-Jenab and Jenab, 2012). Of the ovarian hormones, estradiol, the most abundant form of estrogen in females, is primarily responsible for enhancing sensitivity to drugs of abuse. Clinical research monitoring women's responses to drugs of abuse throughout the menstrual cycle and upon hormone replacement in naturally cycling women have revealed that women are more sensitive to the rewarding properties of psychostimulants when estradiol levels are high, and that

administration of exogenous estradiol further enhances these properties (Evans et al., 2002; Justice and de Wit, 1999; Maria et al., 2014)

The role of estradiol in drug addiction has been more thoroughly implicated in animal models of addiction. Removal of endogenous sources of ovarian hormones through ovariectomy in female rats attenuates sex differences in drug addiction, which are restored upon estradiol replacement (Hu and Becker, 2003; Sircar and Kim, 1999). Moreover, estradiol concentrations correlate with greater locomotor response to psychostimulants, more rapid acquisition of psychostimulant self-administration, faster escalation of psychostimulant consumption, and higher motivation to attain psychostimulants (Becker and Hu 2008, Festa and Quinones-Jenab 2004, Segarra et al 2012, Anker and Carroll 2011). Notably estradiol-facilitation of acquisition of psychostimulant self-administration can be reversed by pretreatment with an estrogen receptor antagonist (Lynch, 2006), but whether this is the case for estradiol enhancement of other psychostimulant behaviors has yet to be examined. Collectively these observations suggest that as in women, estradiol in female rodents enhances sensitivity to the stimulating and rewarding properties of psychostimulants. Although the impact of estradiol on drug addiction have been thoroughly characterized behaviorally, surprisingly few studies have attempted to elucidate the underlying neurobiological mechanisms.

Estradiol Influences Neurobiological Substrates of Addiction

Estradiol affects the function of many brain regions that drive motivated behaviors (Yoest et al., 2014), including the prefrontal cortex, hippocampus, basal

ganglia, nucleus accumbens, amygdala and ventral tegmental area (Becker and Hu, 2008; Srivastava et al., 2013; Zeidan et al., 2011; Zhang et al., 2008). The connectivity among these brain regions comprise the reward circuitry of the brain. Notably, several of the regions within the circuit converge inputs to the nucleus accumbens (NAc) (**Figure 1a**) making it a critical locus for the development and expression of motivated behaviors (Lüscher and Malenka, 2011; Nestler, 2001; Sesack and Grace, 2010). Given that estradiol impacts brain regions projecting to the NAc as well as the NAc itself, this region is a likely candidate for mediating the effects of estradiol on motivated behaviors like drug addiction.

Whereas the neurobiological underpinnings of how estradiol enhances responsiveness to drugs of abuse have received little attention to date, the neurobiological correlates of drugs of abuse have been the focus of numerous studies (Golden and Russo, 2012). These studies have revealed that the NAc is an integral brain region mediating the development and expression of addiction. Moreover, the medium spiny neurons (MSNs) that comprise >90% of neurons within this region (**Figure 1b**) manifest several signatures of addiction (Grueter et al., 2012; Kourrich et al., 2015; Nestler, 2001; Russo et al., 2010). Repeated exposure to psychostimulants enhances glutamatergic and dopaminergic input to the NAc (D'Souza, 2015; Nestler, 2001). Glutamatergic inputs are received by glutamate receptors on dendritic spine heads and dopaminergic inputs are received by dopamine receptors localized to the dendritic spine necks that protrude from the dendrites of MSNs (**Figure 1c**). Additionally, MSNs also receive local GABAergic input from other MSNs and inhibitory interneurons in the NAc (**Figure 1a,c**). Through

modulating glutamatergic, dopaminergic and GABAergic signaling in the NAc, drugs of abuse produce long lasting changes MSN excitability and structure that are thought to form a neurobiological basis for drug addiction (Kourrich et al., 2015; Russo et al., 2010; Golden and Russo, 2012).

Figure 1

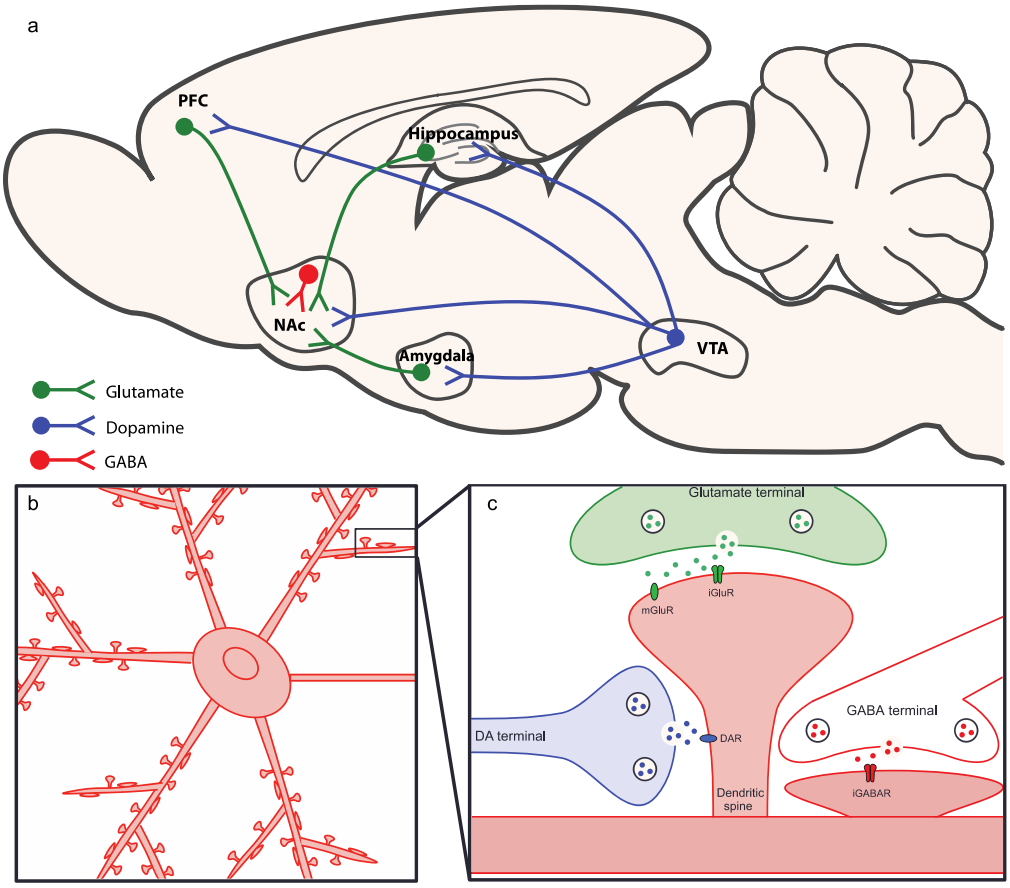

Figure 1. Synaptic connectivity of the reward circuitry to the nucleus accumbens a. Sagittal cross-section of the reward circuit of the rat brain simplified to show inputs to the nucleus accumbens (NAc). Dopaminergic projections (blue) from the ventral tegmental area (VTA) to the amygdala, NAc, prefrontal cortex (PFC), and hippocampus. Glutamatergic projections (green) from the amygdala, hippocampus and PFC to the NAc. Local GABAergic microcircuitry (red) within the NAc. b. Depiction of a Medium Spiny Neuron (MSN), the primary cell type within the NAc. c. Illustration of synaptic inputs onto dendrites of MSNs. Dopaminergic

input (blue) is received on the dendritic spine shaft by dopamine receptors (DAR). Glutamatergic input (green) is received on the head of the dendritic spine by ionotropic glutamate receptors (iGluR) and metabotropic glutamate receptors (mGluR). GABAeric input (red) is received on the shaft of the dendrite by ionotropic GABA receptors (iGABAR).

Although our study of the effects of estradiol on MSNs and the NAc are in their infancy compared to drugs of abuse, what has been shown is that there are striking similarities between the two. For example, like repeated psychostimulant exposure, estradiol enhances dopaminergic signaling and activates glutamatergic signaling within the NAc (Grove-Strawser et al., 2010; Mermelstein and Becker, 1995; Peterson et al., 2014; Thompson and Moss, 1994) that correspond to increased locomotor responses to psychostimultant (Becker and Rudick, 1999; Castner et al., 1993; Martinez et al., 2014). Similarly, estradiol also alters MSN excitability (Meitzen and Mermelstein, 2011; Mermelstein et al., 1996) and produces structural plasticity in MSNs of the NAc (Staffend et al., 2011). The similarities between psychostimulants and estradiol action in the NAc may provide a clue as to how estradiol is acting to enhance female vulnerability to addiction. Moreover, it may be through mimicking the effects of drugs that estradiol primes the female NAc to respond more strongly to the actions drugs of abuse. However, the cellular mechanisms underlying the effects of estradiol on responsiveness to drugs of abuse remain unknown.

Mechanisms of Estradiol Action

Intracellular vs. Membrane Estradiol Signaling

There are two primary ways whereby estradiol acts within the nervous system; through either intracellular estrogen receptors or membrane-localized estrogen receptors (Micevych and Mermelstein, 2008; Srivastava et al., 2013; Vrtačnik et al., 2014). In early studies, estrogen receptors were thought to be nuclear receptors. Upon binding to these nuclear receptors, estradiol regulates gene expression by interacting with estrogen response element (ERE) on the DNA (Mangelsdorf et al., 1995). These classical nuclear effects of estradiol are mediated by two known isoforms, ERα and ERβ that are coded by separate genes ESR1 and ESR2 (Walter et al 1985; Kuieper et al 1996). More recent studies have established that in addition to affecting cellular function through estrogen receptors localized to the nucleus, estradiol can also initiate rapid signaling through multiple receptors localized to the surface membrane (Gorosito et al., 2008; Micevych and Dominguez, 2009; Micevych and Kelly, 2012; Toran-Allerand et al., 2002; Vasudevan and Pfaff, 2008). Putative membrane estrogen receptors that mediate membrane estradiol signaling, have been variants of ERα and ERβ (Gorosito et al., 2008; Skipper et al., 1993), G protein coupled receptor 30 (GPER30) (Barton, 2016.; Filardo et al., 2000), a Gq-mER or STX-sensitive receptors (Qiu et al., 2003) and ER-X (Toran-Allerand et al., 2002). In addition, membrane estrogen receptors can interact with other membrane receptors including insulin-like growth factor-1 receptor (Quesada and Etgen, 2002) and metabotropic glutamate receptors (Boulware et al., 2007, 2005).

Interestingly, ERα and ERβ can reside in the nucleus or at the plasma membrane and where exactly ERα and ERβ reside is determined by the post-translational modification S-palmitoylation (Meitzen et al., 2013; Pedram et al., 2007). S-palmitoylation is a reversible lipid modification to the estrogen receptor that increases their lipophilicity of these receptors and promotes association with the plasma membrane. Thus, non-palmitoylated estrogen receptors remain intracellular, and palmitoylated ERα and ERβ interact with caveolin proteins to enable membrane estrogen signaling. Both intracellular and membrane estrogen receptors have been found in MSNs (Grove-Strawser et al., 2010; Phillips-Farfán et al., 2007; Schultz et al., 2009; Shughrue et al., 1997), and could account for the effects of estradiol within the NAc. However, the majority of estradiol effects in the region are rapid and likely mediated by membrane localized estrogen receptors (Becker and Rudick, 1999; Grove-Strawser et al., 2010; Mermelstein et al., 1996).

Membrane estrogen receptors transactivate metabotropic glutamate receptors

Membrane localized estrogen receptors physically and functionally interact with metabotropic glutamate receptors (mGluRs), wherein estradiol elicits mGluR-mediated G-protein coupled second messenger signaling (Boulware et al., 2005; Grove-Strawser et al., 2010; Meitzen and Mermelstein, 2011). As glutamate, the classical ligand of mGluR is not required, estradiol *transactivates* mGluR signaling. This coupling of ERs to mGluRs is mediated by caveolin proteins and is observed throughout the female nervous system; however, the exact pairing of ERs and mGluRs is dependent on the specific caveolin isoform associated (Boulware et al., 2005; Grove-Strawser et al., 2010; Meitzen

and Mermelstein, 2011). Moreover, coupling of either ERα or ERβ to group II mGluRs (mGluR2 and mGluR3) requires caveolin 3, whereas coupling of ERα to group I mGluRs (mGluR1 and mGluR5) requires caveolin 1 (Boulware et al., 2007). The specific coupling of estrogen receptors to mGluRs is also dependent on brain region. Specifically, in MSNs, ERα or ERβ couple to mGluR3, whereas ERα couples to mGluR5 (Grove-Strawser et al., 2010).

mGluR downstream signaling

Estradiol mediated transactivation of mGluRs can lead to a variety of cellular responses by altering ion channel physiology, transcription and translation. However, the exact nature of the cellular response elicited by estradiol depends on respective partnering with mGluR subtypes. On one hand, activation of group II/III mGluRs through ERα or ERβ stimulates $G_{i/o}$ second messenger signaling (Meitzen and Mermelstein, 2011; Yin and Niswender, 2014; **Figure 2**). In turn, activation of $G_{i/o}$ negatively regulates adenylyl cyclase (AC) and subsequent downstream protein kinase A (PKA)-dependent protein phosphorylation and cellular responses. Examples include attenuation of calcium influx through L-type calcium channels and calmodulin regulation of cyclic AMP response element (CRE)-dependent transcription, e.g. inhibition of cyclic AMP response element binding (CREB) protein phosphorylation.

Figure 2

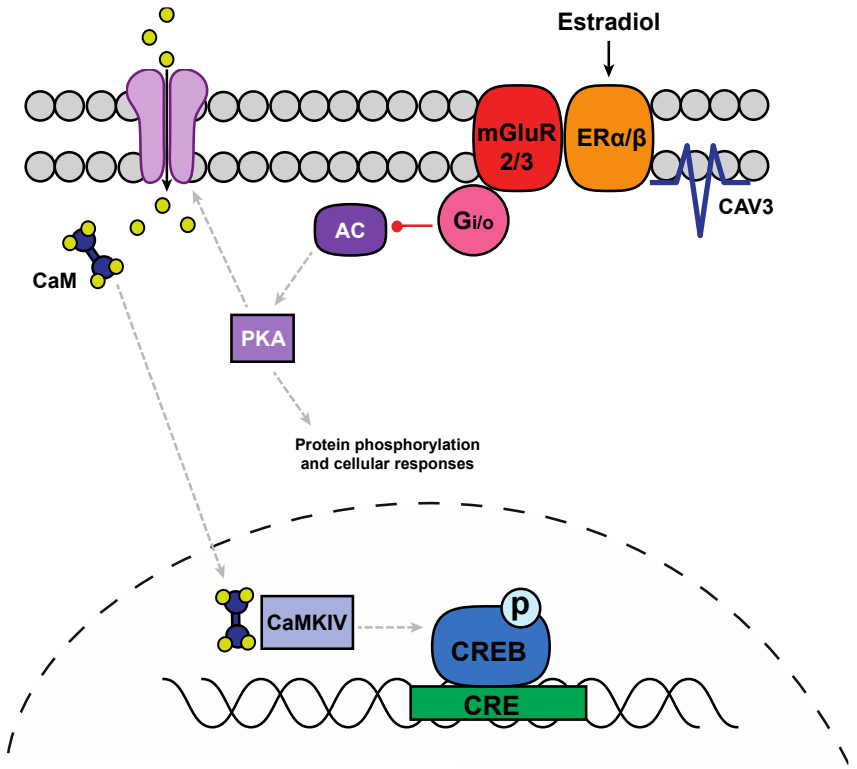

Figure 2. Schematic depicting estradiol signaling through membrane localized estrogen receptors coupled to group II mGluRs.
Estradiol activates ERα or ERβ coupled to group II mGluRs (mGluR2/3) through caveolin protein 3 (CAV3). Transactivation of mGluR2/3 initiates $G_{i/o}$ coupled second messenger signaling to inhibit adenylyl cyclase (AC) and subsequent signaling through protein kinase A (PKA) such as protein phosphorylation and calcium (yellow circles) influx through L-type calcium channels. In the absence of calcium, calcium calmodulin (CaM) does not transport calcium to the nucleus to activate calcium calmodulin kinase IV (CaMKIV)-dependent transcription of cyclic AMP response element binding (CREB) protein.

On the other hand, ligand binding to ERα, but not ERβ, activates group I mGluR signaling (Huang and Woolley, 2012; Meitzen and Mermelstein, 2011; Micevych and Christensen, 2012; **Figure 3**). Classically, group I mGluRs are generally coupled to G_q and activate phospholipase Cβ (PLCβ), which hydrolyses phosphotinositides into inositol

1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Yin and Niswender, 2014). IP3 binds to its receptors within the endoplasmic reticulum resulting in mobilization of intracellular calcium a pathway leading to calcium mobilization and activation of protein kinase C (PKC) (Yin and Niswender, 2014). In turn PKC activates the mitogen activated protein kinase (MAPK)/ extracellular signal–regulated kinases (ERK) pathway to regulate CRE-dependent transcription (e.g. enhance CREB phosphorylation) (Yin and Niswender, 2014). Activation of PLC and increases in intracellular calcium can independently or cooperatively mobilize the two best characterized endocannabinoids (endoCBs), 2-arachidonyl glycerol (2-AG) and N-arachidonoylethanolamine (anandamide or AEA). For example, increases in intracellular calcium produces N-acyl-phosphatidylethanolamine (NAPE) which is then converted by NAPE-phospholipase D (NAPE-PLD) to AEA(Alger and Kim, 2011; Wilson and Nicoll, 2002). Either direct activation of PLC by Gq or indirect activation through calcium, produces DAG that is then converted to 2-AG by DAG lipase (DAGL) (Alger and Kim, 2011; Wilson and Nicoll, 2002). EndoCBs are retrograde neurotransmitters that are produced and released postsynaptically where they travel backwards across the synaptic cleft to activate type 1 cannabinoid receptors (CB1R) on presynaptic terminals (Alger and Kim, 2011; Wilson and Nicoll, 2002).

Figure 3

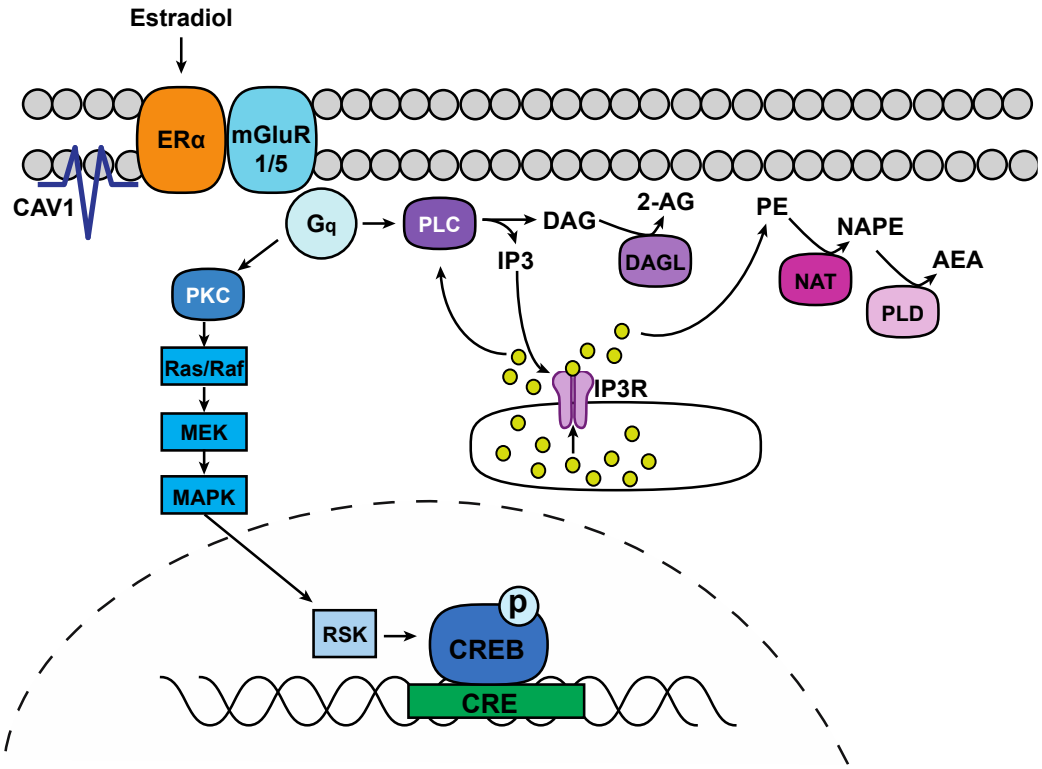

Figure 3. Schematic depicting estradiol signaling through membrane localized estrogen receptors coupled to group I mGluRs.

Estradiol activates ERα coupled to group I mGluRs (mGluR1/5) through caveolin protein 1 (CAV1). Transactivation of mGluR1/5 initiates G_q coupled second messenger signaling to regulate transcription and mobilize endcannabinoids (endoCBs). In one signaling cascade, G_q activates protein kinase C (PKC) and Ras/Raf and mitogen activated protein kinase (MAPK) signaling and ribosomal s6 kinase (RSK)-mediated phosphorylation of cyclic AMP response element binding (CREB) protein. In another signaling cascade, G_q activates protein lipase C (PLC) to produce diacylglycerol lipase (DAG) and inostitol triphosphate (IP3) to stimulate production of endoCBs. DAG is converted by DAG lipase (DAGL) to 2-arachidonyl glycerol (2-AG). IP3 binds to IP3 receptors (IP3R) to mobilize calcium (yellow circles) from intracellular calcium stores (endoplasmic reticulum). Calcium produces N-acyl-phosphatidylethanolamine (NAPE) from phosphatidylethanolamine (PE) which is then converted by NAPE-phospholipase D (NAPE-PLD) to anandamide (AEA). Calcium also acts as a positive feedback system for endoCB production by activating PLC.

Estradiol Influences the EndoCB System

Recently, Woolley and colleagues demonstrated a novel action of estradiol to mobilize endogenous cannabinoids (endoCBs) through activation of ERα coupled to mGluR1a in female rat hippocampal neurons (Huang and Woolley, 2012; Tabatadze et al., 2015). These findings expand upon previous observations wherein estradiol rapidly stimulated endoCB release from cultured human endothelial cells through membrane estrogen receptors (Maccarrone et al., 2002).

Albeit novel, the work demonstrating that estradiol directly stimulates endoCB release (Huang and Woolley, 2012; Maccarrone et al., 2002) contributes to a growing body of work highlighting that estradiol influences the activity of the endoCB system in women and female rodents. Others have demonstrated that levels of 2-AG and AEA as well as CB1R density and ligand-binding affinity fluctuate across the estrous cycle with changes in ovarian hormones (Rodriguez de Fonseca et al., 1994; Bradshaw et al., 2006; (Maccarrone et al., 2000; Waleh et al., 2002). In general, the activity of the endoCB system appears to increase with the initial rise in estradiol during the estrous and menstrual cycles (Gorzalka and Dang, 2012; El-Talatini et al., 2010). Collectively these data suggest that estradiol influences the endoCB system, with actions through group I mGluRs being principally important.

Building A Model for the Effect of Estradiol on Addiction: Through Group I mGluRs and EndoCBs?

Similar to estradiol, both group I mGluRs and the endoCB system have emerged as key mediators of drug addiction (Olive 2010; Olière et al., 2013). Although these lines of evidence were developed independently, an intriguing possibility is that within females, estradiol, group I mGluRs, and endoCBs act through a common pathway to exert effects on addiction.

Group I mGluRs (mGluR1 and mGluR5), endoCBs and CB1R are involved in the behavioral development and expression of addictive behaviors as well as their underlying neural mechanisms (Olive, 2010; Pomierny-Chamioło et al., 2014). Both Group I mGluRs and the endoCB system play critical roles in synaptic plasticity within the nervous system and are densely expressed in brain regions within the reward circuitry, namely the NAc (Herkenham, 1992; Hohmann and Herkenham, 2000; Mitrano and Smith, 2007). As previously discussed, synaptic plasticity in the reward circuitry is critical for the development and expression of addiction. Like estradiol, both group I mGluRs and endoCB system enhance glutamate activity, increase dopamine in the NAc and alter MSN excitability and structure (Asrar and Jia, 2013; Carvalho et al., 2014; Cheer et al., 2004; Fujii et al., 2005; Jung et al., 2005; Robbe et al., 2002; Sperlágh et al., 2009; Uchigashima et al., 2007). As mentioned previously, alterations in glutamatergic and dopaminergic neurotransmission in the NAc and changes in MSN excitability and structure remodel reward circuits to drive behavioral responses to drugs of abuse (Golden and Russo, 2012; Kalivas and Duffy, 1990; Kourrich and Thomas, 2009).

Compared with mGluR1, GluR5 is more highly expressed in the NAc and is more heavily implicated in reward and motivation processing that underlies drug-related behaviors (Mitrano and Smith, 2007; Olive, 2010). Furthermore, in female MSNs estradiol activates ERα specifically coupled to mGluR5 (Grove-Strawser et al., 2010). Indeed, activation of mGluR5 leads to mobilization of endoCBs in the NAc (Jung et al., 2005; Robbe et al., 2002; Uchigashima et al., 2007). It stands to reason that estradiol serially activates estrogen receptors to initiate mGluR5-dependent endoCB signaling in the NAc that ultimately rewire reward circuits and drive drug behaviors in females.

The goal of this dissertation is to begin exploring whether estradiol activates the endoCB system through group I mGluRs to enhance female vulnerability to drug addiction. Chapter 2 will first explore the *in vivo* relevance of estradiol-mediated group I mGluRs signaling in structurally remodeling NAc reward circuits. Chapter 3 will examine whether estradiol enhances activity of the endoCB system within the female rat NAc. Chapter 4 will test whether the endoCB system is involved in the estradiol induced structural rewiring of reward circuits in the NAc and facilitation of psychostimulant behavioral sensitization. This multi-level approach using morphological, physiological, and behavioral methods will help elucidate the neural mechanism through which estradiol primes females to respond more strongly to drugs of abuse and provide therapeutic targets to treat addiction in women.

CHAPTER 2

Estradiol mediates dendritic spine plasticity in the nucleus accumbens core through activation of mGluR5

Rationale

Accumulating evidence from human and rodent studies suggests that females are more sensitive to the motivating and rewarding properties of drugs of abuse. Numerous reports implicate estradiol in enhancing drug- related responses in females, yet the neurobiological mechanisms underlying this effect of estradiol are unknown. Because dendritic spine plasticity in the nucleus accumbens (NAc) is linked to the addictive effects of drugs, we examined the influence of estradiol on dendritic spines in this region. Previously our laboratory demonstrated that in female medium spiny neurons, estradiol activates metabotropic glutamate receptor subtype five (mGluR5), a G protein-coupled receptor already implicated in the etiology of drug addiction. Thus, we sought to determine whether mGluR5 is a part of the mechanism by which estradiol affects dendritic spine density in the NAc. As dendritic spine plasticity in the NAc core has behavioral consequences for drug addiction, these data may provide a clue as to how estradiol acts in females to enhance behavioral responses to drugs of abuse.

Introduction

Compared with men, women show enhanced behavioral responses to drugs of abuse and consequently are thought to be more vulnerable to addiction (Roth et al., 2004). Ovarian hormones, specifically estradiol, may underlie this vulnerability in women (Justice and de Wit, 1999; Mccance-katz et al., 2005). Consistent with the human literature, sex differences in responsiveness to drugs of abuse are apparent in animal models of addiction, with female rats exhibiting greater sensitivity to drugs of abuse than

do males (Anker and Carroll, 2011; Becker and Hu, 2008). These sex differences are eliminated upon removal of ovarian hormones and restored upon estradiol administration (Festa and Quinones-Jenab, 2004; Hu and Becker, 2003; Segarra et al., 2010; Sircar and Kim, 1999). Behaviorally, it is clear that estradiol enhances rewarding and motivating properties of drugs of abuse in females, but the neurobiological underpinnings are unknown.

Due to its prominent role in mediating reward and motivation processes, the nucleus accumbens (NAc) is integral in the development and expression of addictive behaviors. Synaptic plasticity in this region following repeated drug exposure is thought to form a neurobiological basis for addiction (Russo et al., 2010). In particular, long-lasting alterations in neuroanatomical circuitry through dynamic dendritic spine plasticity of medium spiny neurons (MSNs) are believed to underlie persistent behavioral changes following repeated drug exposure (Robinson and Kolb, 2004). A recent study from our laboratory demonstrated that estradiol treatment produces dendritic spine plasticity in the female NAc (Staffend et al., 2011) reminiscent of that induced by repeated psychostimulant exposure (Dumitriu et al., 2012). Given that dendritic spine plasticity underlies changes in behavior, the similarity in psychostimulant- and estradiol-induced dendritic spine plasticity may provide insight as to how estradiol potentiates drug responses in females.

Accumulating evidence suggests that activation of group I metabotropic glutamate receptors (mGluRs) may be a putative mechanism by which estradiol regulates dendritic spines. Both estrogen receptors (McEwen, 2001; Milner et al., 2001) and group

I mGluRs (Mitrano and Smith, 2007) are localized near dendritic spines and membrane-localized estrogen receptors directly interact with group I mGluRs (Meitzen and Mermelstein, 2011). Interestingly, this action of estradiol to transactivate group I mGluRs and initiate second-messenger signaling is specific to female neurons (Boulware and Mermelstein, 2009; Grove-Strawser et al., 2010). More recently estradiol was shown to regulate dendritic spines in the female hypothalamus through a mechanism involving group I mGluRs (Christensen et al., 2011). Whether a similar group I mGluR-dependent mechanism mediates dendritic spine plasticity in NAc remains unknown. Building off previous studies in our laboratory that implicate mGluR5 in membrane actions of estradiol in female MSNs (Grove-Strawser et al., 2010), we took a pharmacological approach to determine whether estradiol affects dendritic spine density in the female rat NAc through an mGluR5-dependent mechanism.

Materials and Methods

Animals

Ovariectomized female Sprague–Dawley rats 12 weeks of age (175–200 g) from Harlan labs (Indianapolis, IN) were housed in pairs, handled daily, and allowed to habituate to the laboratory for one week prior to experimentation. Animals were maintained on at 12-h light–dark cycle with lights on at 6:00 a.m. All animal procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the University of Minnesota.

Drugs and hormones

Estradiol (Sigma-Aldrich, St. Louis, MO) was dissolved in cottonseed oil and injected subcutaneously (s.c.) at 10 μg in 0.1 ml. Cottonseed oil (0.1 ml s.c.) was injected as the hormone vehicle control. For the first experiment, the group I mGluR receptor antagonist JNJ16259685 (JNJ, Tocris Biosciences, Minneapolis, MN) was dissolved in ethanol to make a 100 mM stock solution and stored at -20 °C. On the day of injection, the stock solution was diluted in 80 % physiological saline: 20 % ethanol to a working solution of 1 mg/kg JNJ. Whereas at low doses JNJ is a specific antagonist at mGluR1 receptors (Lavreysen et al., 2004), we administered a higher dose designed to antagonize both of the group I mGluRs, mGluR1, and mGluR5. An identical formulation without JNJ was used for the drug vehicle. In the second experiment, the specific mGluR5 receptor antagonist (Gasparini et al., 1999) 2-methyl-6-(phenylethynyl)-pyridine hydrochloride (MPEP; Tocris Biosciences, Minneapolis, MN) was dissolved in physiological saline to a working concentration of 1 mg/kg on the day of injection. Physiological saline alone was used as the drug vehicle. All drugs and drug vehicles were injected intraperitoneally (i.p.) at a volume of 1 ml/kg. For each experiment, animals were divided into four treatment groups.

1. Drug vehicle + cottonseed oil
2. Drug vehicle + estradiol
3. MPEP or JNJ + vehicle
4. MPEP or JNJ + estradiol

On the day of experimentation, animals received two injections. The first injection was administered i.p. and contained JNJ (experiment 1), MPEP (experiment 2), or the appropriate drug vehicle. After 30 min, the second injection was administered s.c. containing either estradiol or cottonseed oil. Twenty-four hours after the estradiol or oil injection, the animals were anesthetized using Beuthanasia-D (0.3 ml i.p./ animal, Schering, Union, NJ), injected with 0.25 ml heparin into the left ventricle of the heart and intracardially perfused with 25 mM phosphate buffered saline (PBS, pH = 7.2) for 3 min at 25 ml/min, followed by ice-cold 1.5 % paraformaldehyde in 25 mM PBS for 20 min. Brains were removed and coronally blocked to allow for penetration of the post-perfusion fixative (1.5 % paraformaldehyde for 1 h) into the tissue blocks containing either the NAc or hippocampus. Brains were sectioned at 300 µm in serial, coronal sections through the NAc, and hippocampus using a Vibratome (Lancer Series 1000, St. Louis, MO). Sections were placed in wells containing 25 mM PBS until DiI labeling.

DiI labeling

Prior to DiI delivery, PBS was removed from wells containing the brain sections. Microcarriers containing DiI- coated tungsten particles (2 mg lipophilic carbocyanine DiI (Molecular Probes, Carlsbad, CA) dissolved in 100 µl of dichloromethane mixed with 90 mg of 1.3 µm tungsten particles (Bio-Rad, Hercules, CA)) were made in Tefzel tubing (Bio-Rad) precoated with freshly prepared 10–15 mg/ml polyvinylpyrrolidone (PVP, Sigma-Aldrich, St. Louis, MO) and cut into 1.3 mm segments. A Helios Gene Gun (Bio-Rad) with a modified barrel, 40-mm spacer and 70-µm nylon mesh filter was used to deliver the microcarriers to the lightly fixed brain sections using helium gas at a pressure

of 100 PSI. Immediately following DiI delivery, brain sections were re-submerged in PBS for 24 h in the dark at room temperature for diffusion of DiI. After 24 h, the PBS was removed and replaced with 4 % paraformaldehyde in PBS for 1 h at room temperature. Sections were washed (3 x 10 min) in PBS then mounted on Superfrost slides and coverslipped with Fluorglo mounting media for lipophilic dyes (Spectra Services, Ontario, NY; experiment 1) or 5 % n-propyl-gallate in glycerin (w/v; experiment 2). The Fluorglo mounting media used in experiment 1 resulted in a greater resolution of individual dendritic spines compared with the glycerol-based medium used in experiment 2. The pattern of results was unaffected by the different mounting media, though there were greater spine densities in experiment 1 compared with those measured in experiment 2. We attribute this difference in spine density with the Fluorglo to a greater resolution of spines that did not protrude much past the dendritic shaft. The impact of the mounting medium was particularly evident in the CA1 region of the hippocampus because spines on CA1 dendrites do not extend as far from the dendrite as spines on medium spiny neuron dendrites.

Confocal imaging

A Leica TCS SPE confocal microscope (Leica, Mannheim, Germany) was used to acquire z-stacks of DiI-labeled whole neurons and dendritic segments (**Figure 1**). All z-stacks were maintained at an xy pixel distribution of 512 x 512 and scanned at a frequency of 400 Hz. Medium spiny neurons imaged in the nucleus accumbens shell and core were distributed throughout the rostro-caudal axis, with 80% located in the middle third of the accumbens. Additionally, CA1 pyramidal neurons were localized to the

rostral extent of the hippocampus leading to the formation of the ventral hippocampus. Whole neurons were imaged with a 20x air objective at 1.0 µm increments in the z-axis and reconstructed using Leica LAS AF software to measure the distance from the soma of target dendritic segments: 70–200 µm from soma in medium spiny neurons from the NAc or 250–400 µm from pyramidal neurons in the CA1 region of the hippocampus. Dendritic segments were imaged with a 63x oil immersion objective with a zoom of 5.61 and z-stacks were acquired in 0.12 µm increments. By adjusting the laser power and photomultiplier, each dendrite was imaged in its full dynamic range to ensure that no part of the dendritic segment was saturated. For each brain region, 2–3 neurons and 3 dendritic segments per neuron were imaged to generate a total of 6–9 segments for each brain region from every animal. Depending on the experiment and treatment conditions, a final sample size of 3–8 animals was obtained for each brain region per treatment group (see figure legends for group sample sizes).

Quantitation

Confocal z-stacks of dendritic segments were first subjected to a 3D deconvolution process using Autoquant X AutoDeblur Gold CF software (version X2.2, Media Cybernetics, Bethesda, MD). Deconvoluted images were then reconstructed in 3D using the Imaris software (version 7.6, Bitplane Inc., St. Paul, MN), with dendritic shaft and spines traced manually in the xy plane using the Filament tool and the Autodepth function to generate dendritic spine density normalized to 10 µm of dendritic length. A contrast threshold of 0.4–0.7 enabled accurate 3D reconstruction of dendritic shaft and

spines. Following 3D reconstruction, dendritic spine head diameters were obtained for each spine on a given segment.

Data analysis

For each animal, 2–3 neurons were analyzed in each brain region, though each animal was used as the unit of statistical analysis. A two-way ANOVA with subsequent Tukey HSD post hoc tests were used to evaluate the effects of drug, hormone, and drug–hormone interactions among treatment groups for each brain region using STATISTICA software (Statsoft Inc, version 9, Tulsa, OK). For all statistical tests, results were considered to be statistically significant if $p < 0.05$.

Results

Estradiol-mediated dendritic spine plasticity depends on group I mGluRs

Recent findings from our laboratory demonstrated a novel effect of estradiol on dendritic spine density in that estradiol decreased dendritic spine density in medium spiny neurons (MSN) in the NAc of female Syrian hamsters (Staffend et al., 2011). To determine whether this effect is conserved in rats, we treated ovariectomized female rats with estradiol and analyzed dendritic spine density of MSNs. Representative images of a DiI-labeled MSN and dendritic segment used to quantify dendritic spine density are shown in **Figure 4a, b**, respectively.

Figure 4

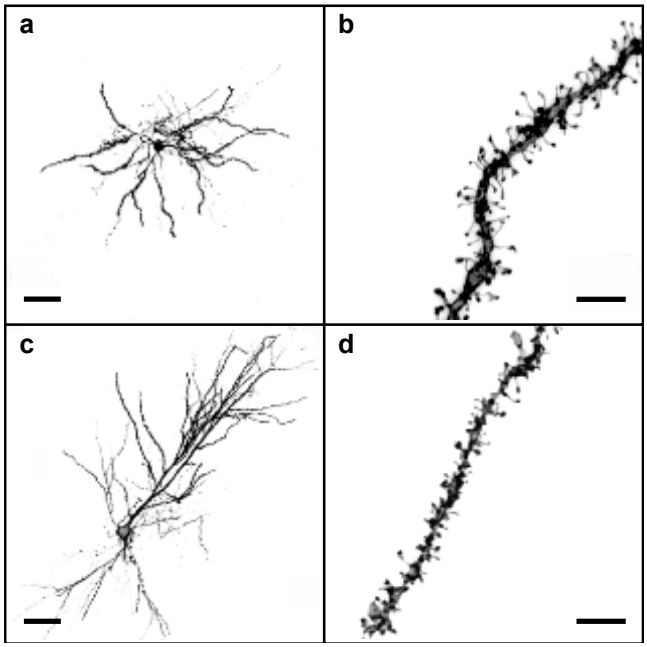

Figure 4. Representative 3D reconstructed images of DiI-labeled neurons and dendritic segments. a Low power DiI-labeled nucleus accumbens medium spiny neuron, scale bar 50 μm. b High power deconvoluted dendritic segment from an accumbal medium spiny neuron, scale bar 5 μm. c Low power DiI-labeled pyramidal neuron in CA1, scale bar 50 μm. d High power deconvoluted dendritic segment from a hippocampal pyramidal neuron, scale bar 5 μm

Consistent with previous observations, we found that estradiol treatment produced a significant decrease in dendritic spine density in NAc core MSNs (main effect of hormone: $F(1,26) = 5.77$, $p < 0.05$). Next, to determine whether estradiol decreased dendritic spine density through a mechanism involving group I mGluRs, we antagonized group I mGluRs with JNJ administration prior to estradiol treatment. The effect of group I mGluR antagonism differed across treatment groups (hormone by drug interaction: $F(1, 26) = 9.57$, $p < 0.005$). Specifically, females pretreated with JNJ prior to estradiol showed

increased dendritic spine density compared with estradiol-treated females that received vehicle pretreatment (Tukey HSD $p < 0.01$). These data indicate that pretreatment with the group I mGluR antagonist blocked the estradiol-mediated decrease in dendritic spine density in the NAc core (**Figure. 5a, d**).

Figure 5

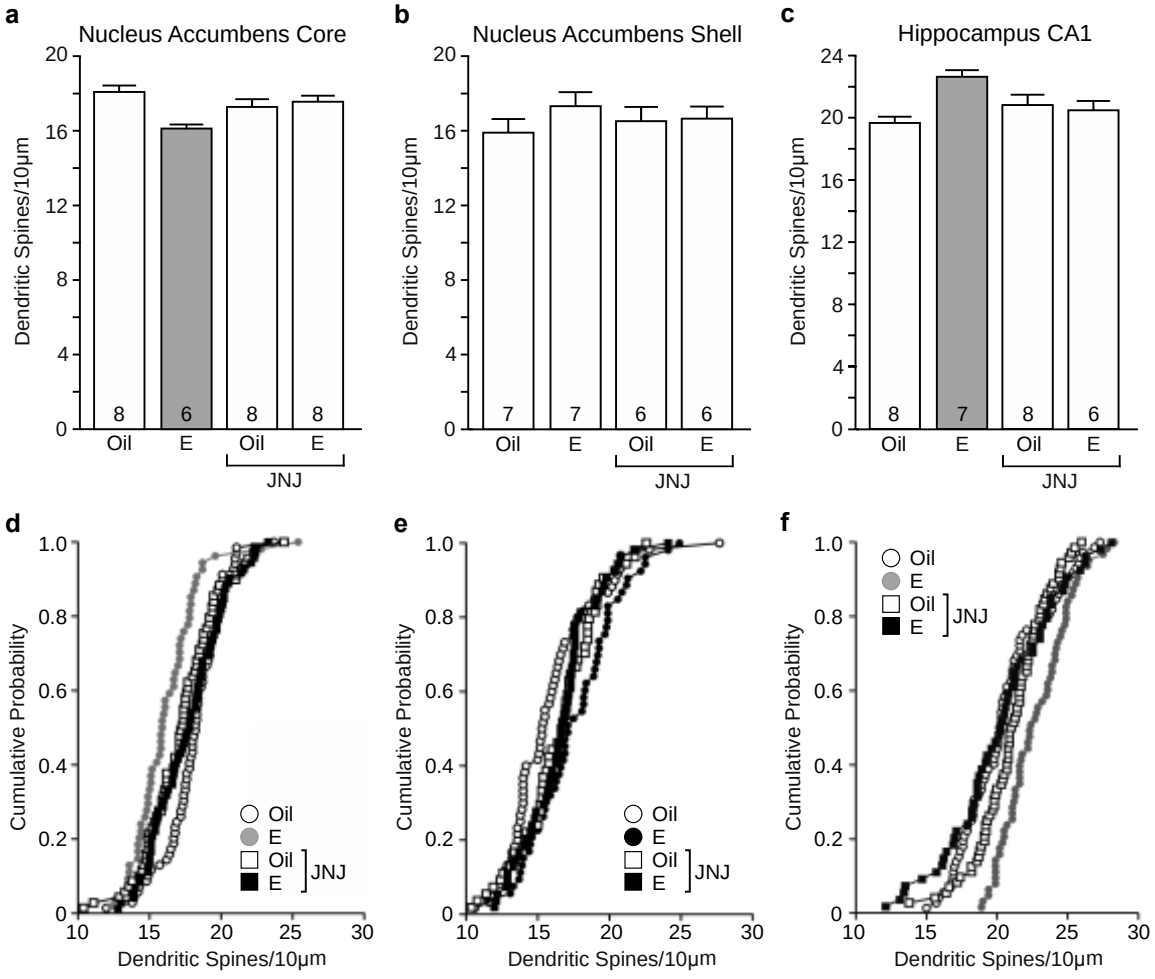

Figure 5. Estradiol-mediated dendritic spine plasticity in the nucleus accumbens core and hippocampal CA1 depends on group I mGluRs.

a Estradiol decreased dendritic spine density of medium spiny neurons in the nucleus accumbens core, and this effect was attenuated by pretreatment with JNJ. b Neither estradiol nor JNJ had a significant effect on medium spiny neurons of the nucleus accumbens shell. c Estradiol increased

dendritic spine density in hippocampal CA1 pyramidal neurons and pretreatment with JNJ blocked this effect. The number inside of each bar represents the number of animals per treatment condition. Gray bars are significantly different than white bars $p < 0.05$. Cumulative probability distribution plots of dendritic spine density within treatment groups for nucleus accumbens core (d), nucleus accumbens shell (e), and CA1 region of the hippocampus (f)

In addition to the NAc core, we examined estradiol effects on MSN dendritic spine density in the NAc shell. In this region, there was a trend toward increased dendritic spine density of MSNs following estradiol treatment and pretreatment with JNJ appeared to block increased dendritic spine density (**Figure 5b, e**). However, these effects were not significant. This is in contrast to what was observed in experiment 2 (see below). The possible variability in observance of an estrogen effect is described in the discussion.

Finally, we chose to analyze dendritic spine density of hippocampal pyramidal neurons in CA1 as a positive control for the effects in the NAc because it has been repeatedly demonstrated that estradiol increases dendritic spine density of pyramidal neurons within this region (Staffend et al., 2011; Woolley, 1998). Representative images of a DiI-labeled pyramidal neuron in CA1 and dendritic segment used to quantify dendritic spine density are shown in **Figure 4c, d,** respectively. In agreement with previous findings, we showed that estradiol increased pyramidal cell dendritic spine density in the CA1 region of the female hippocampus, but only in females pretreated with vehicle (hormone by drug interaction ($F(1,24) = 13.37$, $p < 0.01$). Estradiol-treated females pretreated with JNJ exhibited lower dendritic spine density compared to estradiol-treated females that received vehicle pretreatment (Tukey HSD $p < 0.01$). These data show that antagonism of group I mGluRs prior to estradiol treatment attenuated the

estradiol-mediated increase in dendritic spine density in hippocampal CA1 pyramidal neurons (**Figure 5c, f**). In addition to analyzing dendritic spine densities in the NAc core, shell and CA1 hippocampus, we also examined dendritic spine head diameters for each dendritic segment within treatment groups. We found that there was no effect of estradiol or JNJ on dendritic spine head diameter (data not shown).

Estradiol-mediated decreases in dendritic spine density in the nucleus accumbens core depend on mGluR5

To further decipher the specific group I mGluR underlying estradiol changes in dendritic spines, we treated females with a specific mGluR5 antagonist prior to estradiol treatment. As in experiment 1, estradiol treatment decreased dendritic spine density of MSNs in the NAc core (main effect of hormone: $F(1,16) = 7.47$, $p < 0.05$). Hormone treatment conditions were affected by pretreatment with the mGluR5 antagonist (hormone by drug interaction: $F(1,16) = 10.17$, $p < 0.05$). Estradiol-treated females that received MPEP pretreatment showed increased MSN dendritic spine density in the core compared to those that received vehicle pretreatment (Tukey HSD $p < 0.01$). These data demonstrate that pretreatment with the selective mGluR5 antagonist MPEP blocked the estradiol-mediated decrease in dendritic spine density (**Figure 6a, d**).

Figure 6

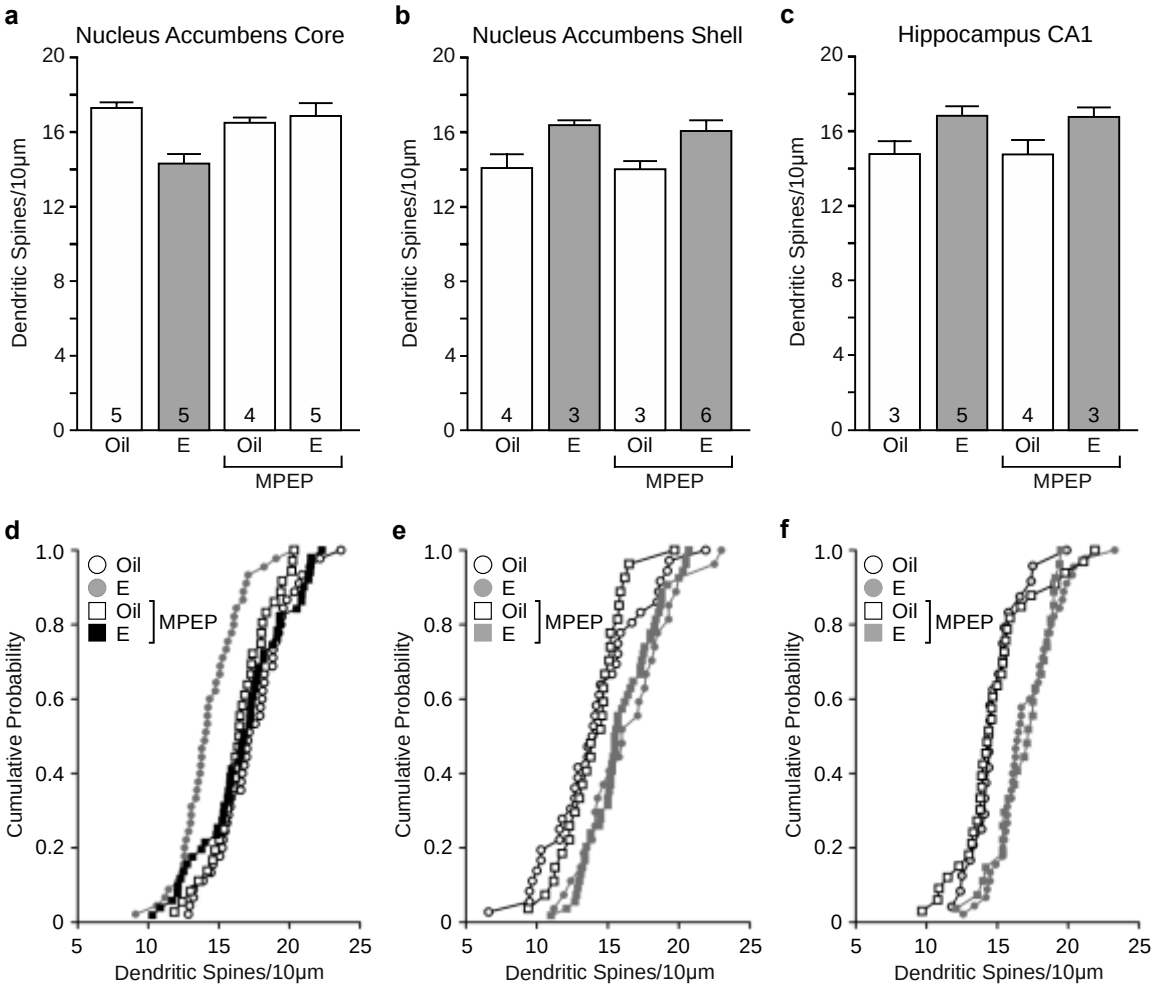

Figure 6. Estradiol-mediated decreases in dendritic spine density in the nucleus accumbens core depend on mGluR5.

a Estradiol decreased dendritic spine density of medium spiny neurons in the nucleus accumbens core, and this decrease was blocked by pretreatment with MPEP. b Estradiol increased dendritic spine density in medium spiny neurons of the nucleus accumbens shell and mGluR5 antagonism had no effect on this action of estradiol. c Estradiol increased dendritic pine density of CA1 hippocampal pyramidal neurons and pretreatment with MPEP did not affect this increase. The number inside of each bar represents the number of animals per condition. Gray bars are significantly different than white bars $p < 0.05$. Cumulative probability distribution plots of dendritic spine density within treatment groups for nucleus accumbens core (d), nucleus accumbens shell (e), and CA1 region of the hippocampus (f)

Whereas in experiment 1, we saw a non-significant trend toward an effect of estradiol on MSN dendritic spine density in the shell of the NAc, in experiment 2, estradiol produced a significant increase in MSN dendritic spine density in this region (main effect of hormone: $F(1,12) = 12.36$, $p < 0.001$). Furthermore, antagonism of mGluR5 did not affect the increased spine density observed with estradiol treatment (**Figure 6b, e**).

As before, we found that estradiol treatment significantly increased dendritic spine density in CA1 pyramidal neurons (main effect of hormone: $F(1,11) = 15.67$, $p < 0.01$) and that pretreatment with the mGluR5 antagonist, MPEP, had no effect on this increase (**Figure 6c, f**). Similar to experiment 1, neither estradiol nor MPEP affected dendritic spine head diameter (data not shown).

Discussion

Converging evidence suggests that estradiol plays a prominent role in enhancing drug-related behaviors in females and that synaptic plasticity in the NAc is thought to form a neurobiological basis for addiction. The goal of this research was to begin elucidating the mechanism by which estradiol can affect synaptic plasticity to promote rewarding/addictive properties of drugs.

The effects of estradiol on dendritic spine plasticity in the NAc have only recently been investigated (Staffend et al., 2011). In contrast, the effect of psychostimulants on dendritic spines has been the focus of numerous studies (Golden and Russo, 2012). The results of these studies suggest that patterns of dendritic spine plasticity depend on a

number of factors, including route and context of drug administration, drug administration regime, and length of withdrawal after drug cessation (Golden and Russo, 2012). However, what is particularly intriguing is that dendritic spine density in the NAc core is decreased 24 h after repeated psychostimulant exposure (Dumitriu et al., 2012). We observe the same effect in ovariectomized female rats 24 h following an acute estradiol treatment. The similarity in these findings suggests that estradiol might prime MSNs in the NAc core to respond more strongly to the actions of psychostimulants.

To examine the involvement of group I mGluRs in estradiol-mediated dendritic spine plasticity in the NAc core, we took a pharmacological approach. By blocking both mGluR1 and mGluR5 with a high dose of JNJ, we were able to attenuate the reduction in dendritic spine density produced by estradiol. Because we were able to produce the same effect with the specific mGluR5 antagonist MPEP, our assumption is that estradiol activates signaling through mGluR5 to decrease dendritic spine density in the NAc core. This assumption is supported by biochemical evidence that estradiol activates mGluR5 to phosphorylate CREB in medium spiny neurons from female rats both in vitro and in vivo (Grove-Strawser et al., 2010) and that mGluR5 alone can produce changes in dendritic spines (Asrar and Jia, 2013; Fujii et al., 2005).

We included the CA1 hippocampus as a positive control because estradiol increases dendritic spine density in this region (Staffend et al., 2011; Woolley, 1998). In the same animals that exhibited decreased dendritic spine density in the NAc core, we observed the well-established increase in spine density in hippocampal CA1 pyramidal neurons. JNJ, having mixed effects on mGluR1 and mGluR5 at the dose used, blocked

the increase in dendritic spines produced by estradiol, whereas MPEP did not have any effect in this region. These data suggest that unlike the core of the NAc where estradiol acts through mGluR5, in the hippocampus estradiol produces changes in dendritic spine density through mGluR1 signaling. This is the first indication of the mechanism by which estradiol increases dendritic spine density in CA1 and is consistent with how estradiol affects biochemical and electrophysiological properties of pyramidal neurons in this brain region (Boulware et al., 2005; Huang and Woolley, 2012). Further support that estradiol may act through mGluR1 to increase dendritic spine density in CA1 comes from the recent result that action of estradiol on mGluR1 underlies increased dendritic spine density in the hypothalamus (Christensen et al., 2011).

Based on our prior study in female hamsters (Staffend et al., 2011), we did not expect to see any changes in dendritic spine density in the NAc shell with estradiol treatment. Indeed, in the first experiment, there was no effect of estradiol on MSNs in this region. However, in the second experiment, estradiol produced an increase in dendritic spine density that was unaffected by mGluR5 antagonism. We do not know the source of this variability among the experiments in this study and our prior study in hamsters. One possibility is that the time window for observing dendritic spine plasticity following estradiol treatment may differ by brain region. In support of this idea, the time course for the expression of dendritic spine plasticity following repeated psychostimulant exposure appears to be different between the NAc core and shell, with changes in the shell occurring more rapidly (Dumitriu et al., 2012). Thus, it is possible that a similar phenomenon occurs following estradiol treatment and that our timing of sacrifice

following estradiol treatment was on the cusp of changes in dendritic spine plasticity in the shell. Clearly, a more in-depth time course analysis may provide insight into this hypothesis. That said, when estradiol effects on dendritic spines in the shell were observed, they were similar to the increases in hippocampal CA1 spine density than the decreased spine density in the NAc core. In this regard, the inability of MPEP to antagonize estradiol effects in either the hippocampus or NAc shell may suggest that, like in the hippocampus, estradiol acts through mGluR1 to affect spine density in the NAc shell.

Each subregion of the NAc is thought to serve a different function in terms of mediating drug-related behaviors. Specifically, the shell seems to be more involved in shorter-term aspects of addiction, such as reward, whereas the core seems to play a larger role in behaviors such as the development of patterned motor programs to obtain rewards (Ito et al., 2004; Meredith et al., 2008). Behaviorally, estradiol has been shown to affect both shell and core dependent responses. For example, estradiol potentiates the rewarding effects of psychostimulants, insofar as enhancing positive subjective ratings of drugs in women (Justice and de Wit, 1999; Mccance-katz et al., 2005). In addition, estradiol is known to enhance acquisition and escalation of psychostimulant intake in females as well as locomotor sensitization in female rats (Becker and Hu, 2008; Carroll and Anker, 2010; Ridenour et al., 2005). An obvious conclusion is that the estradiol-induced dendritic spine plasticity we observed in the NAc underlies the ability of estradiol to potentiate drug-related responses in females, as decreased dendritic spine density in the NAc core has been associated with locomotor sensitization (Waselus et al., 2013), and our laboratory

has recently demonstrated that mGluR5 is involved in the potentiating effect of estradiol on psychostimulant locomotor sensitization in female rats (Martinez et al. unpublished).

Our research has implications for future therapeutic approaches for addiction, especially in women. Several groups have shown that modulation of group I mGluRs have therapeutic potential for drug-related cellular and behavioral plasticity (Duncan and Lawrence, 2012; Loweth et al., 2013; Olive, 2010; Wang et al., 2013). If group I mGluRs and estradiol share a common mechanism to enhance drug-related cellular and behavioral plasticity in females, group I mGluR antagonism may serve as a viable target for developing treatments for drug addiction in women.

CHAPTER 3

Estradiol influences the endocannabinoid system in the female rat nucleus accumbens

Rationale

Activation of group I mGluRs (mGluR1 and mGluR5) leads to a variety cellular responses, one of which is the release of endogenous cannabinoids (endoCBs). EndoCBs are retrograde neurotransmitters that once released from the post-synaptic neuron bind to type 1 cannabinoid receptors (CB1Rs) on pre-synaptic terminals to produce synaptic plasticity in the CNS. Specifically, within the nucleus accumbens (NAc) activation of mGluR5 mobilizes endoCBs that bind to CB1R. Both endoCBs and CB1R have emerged as key mediators of drug addiction, thus we sought to determine whether estradiol directly mobilizes endoCBs, indirectly affects endoCB tone or effects CB1R expression within the female rat NAc.

Introduction

Sex-differences in the responsiveness to drugs of abuse are apparent, with women exhibiting a greater sensitivity than men (Roth et al., 2004). Converging evidence from both clinical and preclinical research implicate the ovarian hormone estradiol as a key biological factor in enhancing the motivating and rewarding properties of abused drugs in females (Anker and Carroll, 2011; Becker and Hu, 2008; Festa and Quinones-Jenab, 2004; Justice and de Wit, 1999; Mccance-katz et al., 2005; Sircar and Kim, 1999). However, little is known regarding the neurobiological mechanisms underlying the impact of estradiol on sensitivity to drugs of abuse in females.

The nucleus accumbens (NAc), a brain region integral to the expression of neuroanatomical and behavioral hallmarks of drug addiction, is a likely mediator of the

effects of estradiol on sensitivity to drugs of abuse. Our laboratory has recently demonstrated that estradiol produces synaptic plasticity within the NAc that we believe primes females to behaviorally respond more strongly drugs of abuse (Martinez et al., 2014; Peterson et al., 2014; unpublished). Moreover, these effects of estradiol on NAc synaptic plasticity and behavioral sensitivity to cocaine occur through activation of the group I metabotropic glutamate receptor subtype 5 (mGluR5) (Martinez et al., 2014; Peterson et al., 2014) unpublished). Our lab and others have shown that estradiol transactivates group I mGluRs to initiate rapid second-messenger signaling specifically in female neurons (Boulware et al., 2005; Boulware and Mermelstein, 2009).

Activation of group I mGluRs stimulates the production and release of endogenous cannabinoids (endoCBs) and more recently estradiol was shown to mobilize endoCBs through activating group I mGluRs in the female, but not male hippocampus (Huang and Woolley, 2012; Tabatadze et al., 2015). In addition to directly mobilizing endoCBs, estradiol has been found to increase basal endoCB tone by decreasing expression of enzymes that degrade endoCBs (Waleh et al 2002, Grimaldi et al 2012) and influence the expression of cannabinoid receptor type 1 (CB1R) throughout the brain (Rodriguez de Fonseca et al 1994). However, whether estradiol has similar effects on endoCB mobilization, synthesis, degradation, and/or binding in the NAc remains unexplored. Here we determine whether estradiol directly mobilizes endoCBs or alters expression of the components of the endoCB system that synthesize, degrade or bind endoCBs in the NAc. Given that the endoCB system is emerging as a key mediator in the

etiology of drug addiction (Olière et al., 2013), estradiol may be exerting its effects on female sensitivity to drugs of abuse by modulating the endoCB system in the NAc.

Materials and methods

Animals

Ovariectomized female Sprague Dawley rats (60 days of age) were purchased from Harlan Labs (Indianapolis, IN), and pair housed upon arrival. Females were maintained on a 12 h light-dark cycle with lights on at 7:00 am. Food and water were provided ad libitum. Females were allowed to habituate to the laboratory environment for 5 days prior to hormone treatment. All animal procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (eighth edition) and approved by the University of Minnesota Institutional Animal Care and Use Committee.

Endocannabinoid measurements

Drugs

Arachidonoyl ethanolamide-d4 (d4-AEA) was purchased from Tocris Bioscience (St. Louis, MO). Arachidonoyl ethanolamide (AEA), 2-Arachadonyl glycerol (2-AG) were purchased from Cayman Chemical (Ann Arbor, MI). HPLC-grade water and methanol were purchased from VWR International (Plainview, NY). HPLC-grade acetic acid and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO).

Treatment and Tissue punches

Estradiol (Sigma-Aldrich, St. Louis, MO) was dissolved in cottonseed oil (Sigma) at 10 µg in 0.1 ml and injected subcutaneously (s.c.). Ethyl oleate (0.1 ml) was injected s.c. as the hormone vehicle control. Females were treated with estradiol (n=18) or ethyl oleate vehicle (n=18) and rapidly decapitated 15, 30 or 60 minutes following treatment. Brains were rapidly removed and placed in a brain matrix on ice and a 2 mm coronal section at the level nucleus accumbens was taken. Bilateral punches (2 mm diameter x 2 mm thick) from the NAc were taken on ice and placed in 1.5 mL microcentrifuge tubes and immediately flash-frozen with liquid nitrogen. The tissue samples were sent overnight on dry ice from Minneapolis, MN to Bloomington, IN where they were stored in a −80°C freezer until tissue was processed for lipid extractions.

Lipid extraction

Lipid extraction was completed as previously described (Bradshaw et al., 2006). The samples were removed from the −80°C freezer. After being shocked with liquid nitrogen, they were weighed and placed in centrifuge tubes on ice. After which, 40:1 volumes of methanol were added to each tube followed by 10 µL of 1 uM d4-AEA. d4-AEA was added to act as an internal standard to determine the recovery of the compounds of interest. The tubes were then covered with parafilm and left on ice and in darkness for approximately 2 hours. Remaining on ice, the samples were then homogenized using a polytron for approximately 1 minute for each sample. The samples were then centrifuged at 19,000 ×g at 24°C for 20 minutes, the supernatants collected and placed in polypropylene tubes (15 or 50 mL), and HPLC-grade water was added making the final supernatant/water solution 25% organic. To isolate the compounds of interest,

partial purification of the 25% solution was performed on a Preppy apparatus (Sigma-Aldrich) assembled with 500 mg C18 solid-phase extraction columns (Agilent Technologies, Santa Clara, CA). The columns were conditioned with 5 mL of HPLC-grade methanol immediately followed by 2.5 mL of HPLC-grade water. The supernatant/water solution was then loaded onto the C18 column and then washed with 2.5 mL of HPLC-grade water followed by 1.5 mL of 40% methanol. The prostaglandins were then collected with a 1.5 mL elution of 70% methanol, NAGly with a 1.5 mL elution of 85% methanol, and the ethanolamides with a 1.5 mL elution of 100% methanol. All were collected in individual autosampler vials and then stored in a −20°C freezer until mass spectrometer analysis.

LC/MS/MS Analysis and Quantification

LC/MS/MS analysis and quantification of endoCBs was completed as previously described (Bradshaw et al., 2006). Samples were removed from the −20°C freezer and allowed to warm to room temperature and then vortexed for approximately 1 minute before being placed into the autosampler and held at 24°C (Agilent 1100 series autosampler, Palo Alto, CA) for LC/MS/MS analysis. Also 10–20 µL of eluants was injected separately for each sample to be rapidly separated using a C18 Zorbax reversed-phase analytical column (Agilent Technologies, Santa Clara, CA) to scan for individual compounds (mobile phase A: 20% HPLC methanol, 80% HPLC water, and 1 mM ammonium acetate; mobile phase B: 100% HPLC methanol and 1 mM ammonium acetate). Gradient elution (200 µL/min) then occurred under the pressure created by two Shimadzu 10AdVP pumps (Columbia, MD). Next, electrospray ionization was

accomplished using an Applied Biosystems/MDS Sciex (Foster City, CA) API3000 triple quadrupole mass spectrometer. A multiple reaction monitoring (MRM) setting on the LC/MS/MS was then used to analyze levels of each compound present in the sample injection. Synthetic standards were used to generate optimized MRM methods and standard curves for analysis.

Data Analysis

The amount of analyte in each sample was calculated by using a combination of calibration curves of the synthetic standards and deuterium-labeled internal standards obtained from the Analyst software. The standards provided a reference for the retention times by which the analytes could be compared. They also helped to identify the specific precursor ion and fragment ion for each analyte, which enabled their isolation. These processes provide confidence in the claim that the compounds measured were, in fact, the compounds of interest. The amount of each compound in each tissue sample was then converted to moles per gram tissue, which is how it was statistically analyzed using independent-samples t-tests. Comparisons were considered to be statistically significant if $p < 0.05$.

PCR experiment

Treatment and tissue punches

Estradiol (Sigma-Aldrich, St. Louis, MO) was dissolved in cottonseed oil (Sigma) at 10 µg in 0.1 ml and injected subcutaneously (s.c.). Cottonseed oil (0.1 ml) was injected s.c. as the hormone vehicle control. Females were treated with estradiol (n=5) or cottonseed oil (n=4) and 24-h later, euthanized via intraperitoneal injections of

Beuthanasia-D (0.4 ml; Schering, Union, NJ). Brains were rapidly removed and placed in a brain matrix on ice and a 2 mm coronal section at the level nucleus accumbens. Bilateral punches (1.07 mm diameter x 2 mm thick) from the NAc were taken on ice and placed in 1.5 mL microcentrifuge tubes containing RNAlater (Qiagen, Germantown, MD) and refrigerated overnight at 4°C until RNA extraction.

RNA extraction and cDNA synthesis

RNAlater was aspirated from the tissue and the punches were homogenized with a BulletBlender (Next Advance, Averill Park, NY) in a commercially available lysis buffer (RNeasy mini kit, Qiagen). RNA was extracted with a standard kit (RNeasy mini kit, Qiagen) by eluting with 30 µL of RNAse in DNAse free PCR grade water according to the manufacturer's instructions and the extracts stored on ice. Sample RNA was immediately reverse transcribed into cDNA using a Roche Transcriptor First Strand cDNA synthesis kit by a combination of both anchored-oligo(dt) and random hexamer primers (Roche Diagnostics, Indianapolis, IN). The template-primer mixture was denatured by heating at 95°C for 10 min in a thermal block cycler with a heated lid. The remaining components of the reverse transcription reaction were added to each PCR tube and the reaction was run for 10 min at 25°C, followed by 30 min at 55°C, and 5 min 85°C. The reaction was terminated by placing tubes immediately on ice. Sample cDNAs were diluted 1:10 with RNAse DNAse free PCR grade water, aliquotted to one-use microcentrifuge tubes and stored at -20°C until later analysis by RT-PCR.

Primers

Real-time PCR assays were designed using the Roche ProbeFinder online software (www.Universalprobelibrary.com). Assays were designed for a reference gene, ribosomal protein L13a (RPL13a), and four genes of interest: diacylglycerol lipases a (DAGla) and N-acyl- phosphatidylethanolamine-specific phospholipase D (NAPE-PDL). monoacylglycerol lipase (MAGL), fatty acid amide hydrolase (FAAH) and cannabinoid receptor type 1 (CB1R). Primer sequences were checked using a BLAST search to verify specificity. Additionally the efficiency of each primer set was calculated by using standard curves of serially diluted template cDNA. As expected, all efficiencies were found to be approximately two. The forward and reverse primer sequences used are as follows: *napepld* (GenBank accession number XM_006235914.1) TGGAGCTTATGAGCCAAGGT and CGTCAATGTGAATCCTTACAGC; *faah* (GenBank accession number XM_003750001.2) CAGAAGTTACAGAGTGGAGAGCTG and CACGCAGTTGGTCCCTTT; *dagla* (GenBank accession number XM_006231037.1) AAGCACCAAGCCCAAATG and AGCTCCGACTTGGGGATAC; *mgll* (GenBank accession number XM_006236861.1) CACCGTCCAGAAGGACTACC and AGCTGCTAGGATGGAGATG; *cn1r* (GenBank accession number XM_006237985.1) CAAGACGGTGTTTGCCTTCT and GAGCATAGATGATGGGGTTCA; *rpl13a* (GenBank accession number NM_173340.2) CCCTCCACCCTATGACAAGA and GGTACTTCCACCCGACCTC.

Real Time PCR

Real-time PCR reactions were carried out in a 20 µL reaction consisting of 4 µL RNAse DNAse free PCR grade water, 200 nM of appropriate forward and reverse

primers, LightCycler 480 SYBR Master Mix and 4 μL diluted (1:10) cDNA template. Real-time PCR was measured using a LightCycler 480 machine (Roche). PCR for individual cDNA samples was performed in triplicate. Threshold values were calculated using the second derivative max method, and standardized to the ribosome-related gene rpl13a, using LightCycler 480 software version 1.5. The thermal cycling program used a preincubation step at 95°C for 5 min, followed by 45 cycles consisting of a 10 s denaturing step at 95°C, an annealing step for 10 s at 60°C, an extension step for 10 s at 72°C, and a measurement of fluorescent intensity. At the end of each cycling program, a melting curve was run.

Data Analysis

Crossing point (Cp) values were averaged across triplicate samples for the gene of interest (CB1R, FAAH, DAGLa, MAGL, NAPEPDL) and normalized to the reference gene (RPL13a) (ΔCt) and then averaged Cp values were analyzed with the ΔΔCt method to determine relative changes in gene expression between oil- and estradiol-treated females. All data were examined using Prism 5 for Windows, Version 5.04 (GraphPad Software, Inc., La Jolla, CA) using independent-samples t-tests. Comparisons were considered to be statistically significant if $p < 0.05$.

Results

Estradiol modulates 2-AG and AEA in the nucleus accumbens

Recent studies from Woolley and colleagues demonstrated that *in vitro* application of estradiol to hippocampal slices from adult female rats stimulated the release of endogenous cannabinoids (endoCBs) (Huang and Woolley, 2012; Tabatadze et

al., 2015). To determine whether this effect occurs in the adult rat nucleus accumbens, we treated ovariectomized female rats with estradiol and analyzed endoCB levels from NAc tissue samples 15, 30 and 60 minutes later. We were particularly interested in whether estradiol could alter levels of the two best-characterized endoCBs 2-arachidonoylglycerol (2-AG) and N-arachidonoylethanolamine (anandamide or AEA). Relative to oil treated females; estradiol-treated females exhibited a decrease in 2-AG within the NAc 15 minutes after the injection ($t(10) = 2.968$; $p < 0.05$) that did not persist through 30 and 60 minutes (**Figure 7a**). In contrast, the profile for AEA was opposite to that of 2-AG; there were no differences in NAc AEA content between estradiol and vehicle treated females at 15 or 30 minutes, but estradiol treatment decreased AEA by 60 minutes ($t(10) = 2.373$; $p < 0.05$ **Figure 7b**).

Figure 7

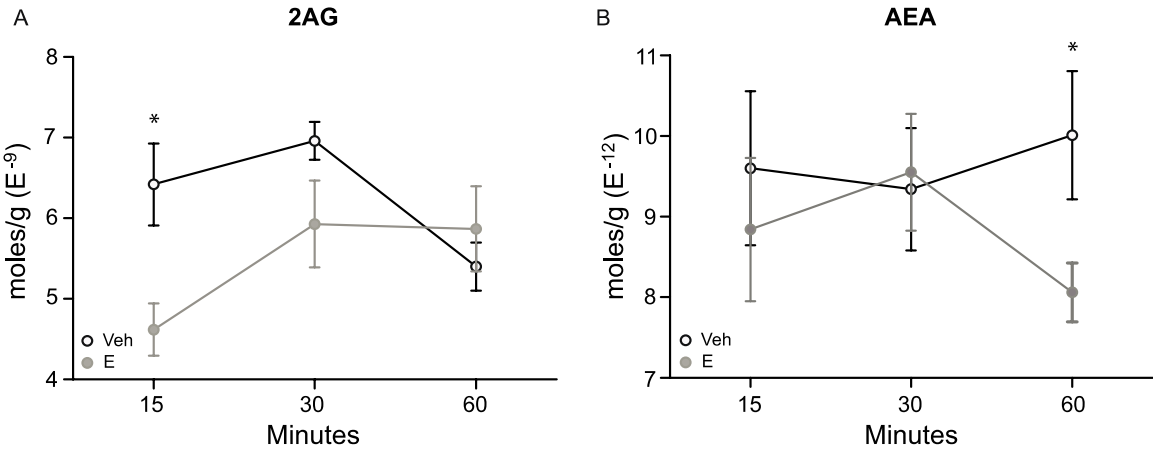

Figure 7. Changes in endocannabinoid content within the female rat nucleus accumbens (NAc) following estradiol treatment.

Estradiol treated female rats sacrificed 15 minutes after the injection exhibited decreased 2AG content in the NAc compared with vehicle treated females. There were no differences in NAc 2AG content between estradiol and vehicle treated females when sacrificed 30 or 60 minutes after

treatment. Estradiol treatment did not effect AEA levels 15 or 30 minutes after the injection, but decreased AEA 60 minutes after treatment. Filled grey circles represent estradiol treated females, whereas open white circles represent vehicle treated females; n = 6 per treatment condition; * $p < 0.05$.

Estradiol upregulates CB1R expression in the nucleus accumbens

In addition to examining whether estradiol treatment alters NAc endoCB content on a rapid timescale, we also wanted to determine whether estradiol modulates basal endoCB tone or endoCB receptor expression over a longer timescale. To test this, we treated ovariectomized female rats with estradiol and analyzed expression of mRNA transcripts for the enzymes that synthesize and degrade AEA (N-acyl-phosphatidylethanolamine-specific phospholipase D or NAPEPLD and fatty acid amide hydrolase or FAAH, respectively), the enzymes that synthesize and degrade 2-AG (diacylglycerol lipase α or DAGLα and monoacylglycerol lipase or MAGL, respectively), and the receptor that binds both AEA and 2-AG, cannabinoid receptor type 1 (CB1R) 24 hours after the injection (**Figure 8**).

Figure 8

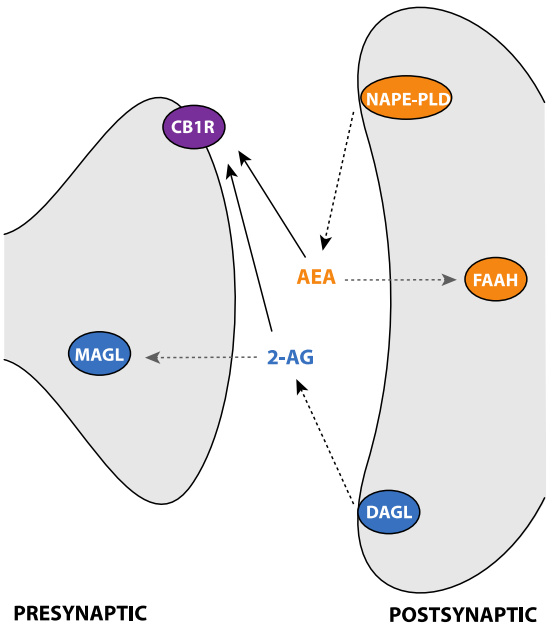

Figure 8. Schematic depicting synthesis, degradation and binding of the endocannabinoids (endoCBs).
2-arachidonoylglycerol (2-AG, blue) and anandamide (AEA, orange). 2-AG is synthesized from the postsynaptic neuron through diacylglycerol lipase (DAGL) and released out to the synaptic cleft where it can bind to presynaptic type 1 cannabinoid receptors (CB1R) and/or be taken up into the presynaptic terminal where is is degraded by monoacylglycerol lipase (MAGL). AEA is synthesized from the postsynaptic neuron through N-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and released out to the synaptic cleft where it can bind to presynaptic CB1R and/or be taken back up by the postsynaptic neuron where it is degraded by fatty acid aminde hydroxylase (FAAH).

Relative to vehicle treated females, estradiol treated females exhibited increased CB1R expression in the NAc ($t(6) = 3.049$; $p < 0.05$; **Figure 9e**), but no effects on the expression of synthesis of degrading enzymes for either AEA or 2AG were observed (**Figure 9a-d**).

Figure 9

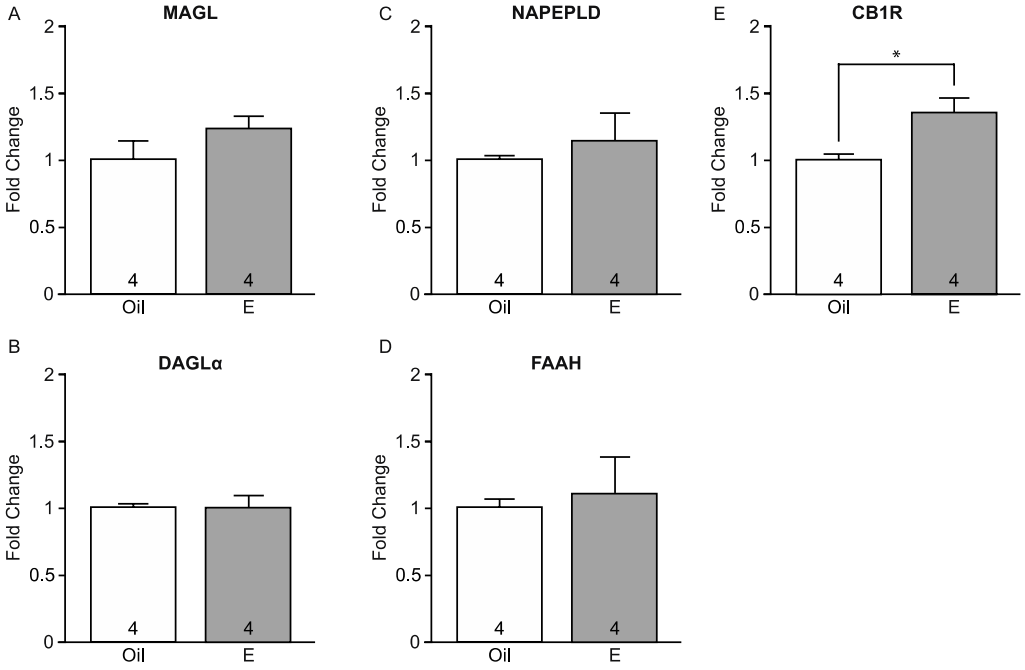

Figure 9. Changes in gene expression of components of the endocannabinoid system following estradiol treatment in the female rat nucleus accumbens (NAc).

Female rats sacrificed 24 hours after estradiol treatment did not show any differences in the synthesizing enzyme (MAGL, a) or degrading enzyme (DAGLα, b) for 2AG compared with vehicle treated females. Similarly, estradiol treatment was not found to effect the expression of the synthesizing enzyme (NAPEPLD, c) or degrading enzyme (FAAH, d) for AEA. However, compared with vehicle treated females, estradiol increased expression of the endocannabinoid receptor, CB1R (e). Grey bars represent estradiol treated females, whereas white bars represent oil vehicle treated females. The number inside of each bar represents the number of animals per treatment condition; * $p < 0.05$.

Discussion

Actions of estradiol within the nucleus accumbens (NAc) appear to be important in mediating female sensitivity to drugs of abuse (Peterson et al., 2014). Here we demonstrate that in the NAc, estradiol elicits both rapid and long-term effects on the

endogenous cannabinoid (endoCB) system, an emerging moderator of addiction (Olière et al., 2013). Specifically, we show that estradiol rapidly (less than 1 hour) alters concentrations of the two best studied endoCBs, 2-arachidonly glycerol (2-AG) and anandamide (AEA) in the female rat NAc. In addition, this rapid action of endoCB mobilization, estradiol treatment produced a longer-lasting (at least 24 hours) increase in cannabinoid type 1 receptor (CB1R) expression within this brain region.

These findings are of the first to demonstrate that estradiol influences the endoCB system in the NAc. Moreover, they complement a growing body of work suggesting that ovarian hormones, particularly estradiol, affect the activity of the endoCB system in both women and female rodents. Initial observations found that across the estrous cycle, CB1R density and ligand-binding affinity fluctuate in multiple brain regions; effects that are sensitive to ovariectomy and restored following hormone replacement (Rodriguez de Fonseca et al., 1994). In parallel, CNS levels of 2-AG and AEA, fluctuate in concert with ovarian hormones (Bradshaw et al., 2006). Notably, evidence from *in vitro* (Huang and Woolley, 2012) and *in vivo* (Scorticati et al., 2004) studies demonstrate that AEA levels are increased in the CNS following treatment with estradiol. The activity of the endoCB system appears to increase with the rise in plasma estradiol during the estrous cycle in female rodents (Gorzalka and Dang, 2012) and menstrual cycle in women (El-Talatini et al., 2010).

Although there are several ways through which estradiol can signal to influence the endoCB system, previous data suggest membrane estrogen receptor activation of mGluR signaling to be important. Moreover, estradiol rapidly stimulates AEA, but not 2-

AG release *in vitro* through surface membrane estrogen receptors (Maccarrone et al., 2002), particularly membrane-localized estrogen receptor alpha (ERα) coupled to mGluR1a (Maccarrone et al., 2002). Consequently, we were somewhat surprised to see decreases in 2-AG and AEA following estradiol treatment *in vivo*. However, because endoCBs are very transient molecules, it is likely that our experimental design did not allow sufficient temporal resolution to adequately capture the endoCB mobilization profiles. *In vitro* the physiological effects of estradiol mediated by endoCBs are observed within a few minutes of application (Huang and Woolley, 2012). These data suggest that increases in endoCBs may have occurred within the minutes before our initial 15 min time point or before our final 60 min time point, in the case 2-AG and AEA, respectively. Intriguingly, Woolley and colleagues observed a similar decrease in endoCBs when measuring levels from hippocampal tissue (personal communication Dr. Bradshaw who measured the samples). This is in stark contrast to their electrophysiological studies where they observed an increase in endoCBs following estradiol activation of group I mGluRs (Huang and Woolley, 2012; Tabatadze et al., 2015). As electrophysiological methods are able to measure effects on a finer timescale, this may be a contributing factor to the discrepancy in results. These findings shed light on ours in that perhaps we missed the initial increase in endoCBs and that decreases we observed may reflect an increase in endoCB degradation or turnover. Future studies will be necessary to fully characterize the endoCB profiles following estradiol treatment in the NAc.

As mentioned above, the existing literature demonstrate that only AEA is regulated by estradiol (El-Talatini et al., 2010; Huang and Woolley, 2012; Maccarrone et

al., 2002; Tabatadze et al., 2015), whereas here we show estradiol affects 2-AG in addition to AEA. In hippocampal pyramidal neurons, where the mechanism through which estradiol mobilizes AEA was first characterized, ERα couples to mGluR1a (Boulware et al., 2005; Huang and Woolley, 2012; Tabatadze et al., 2015), However, in medium spiny neurons (MSNs) of striatum, including the NAc, ERα couples to mGluR5 instead of mGluR1a (Grove-Strawser et al., 2010). Interestingly, at least in male subjects, activation of mGluR5 in the NAc stimulates mobilization of 2-AG rather than AEA (Jung et al., 2005; Seif et al., 2011). Thus whether estradiol mobilizes 2-AG or AEA may depend on the exact pairing of membrane localized ERα with group I mGluRs. That ERα differentially couples to mGluR1a and mGluR5 in the NAc shell and core (Peterson et al., 2014) may underlie that estradiol effects both 2-AG and AEA in the NAc. Moreover, it may be that ERα-mGluR1a activation in the NAc shell mobilizes AEA, whereas ERα-mGluR5 activation mobilizes 2-AG in the NAc core.

Interestingly, estradiol altered 2-AG and AEA on different time courses (i.e. 2-AG by 15 minutes and AEA by 60 minutes). This could be a product of differential coupling of ERα to group I mGluRs in the NAc core and shell as mentioned in the previous paragraph, wherein ERα-mGluR5 mobilization of 2-AG in the core precedes ERα-mGluR1 mobilization of AEA in the shell. Another possibility is that the delayed mobilization of AEA is directly stimulating the metabolism of 2-AG as has been observed in the striatum (Maccarrone et al., 2008). Regardless, estradiol rapidly modulates endoCBs in the NAc and although not explicitly tested here, this action of

estradiol likely occurs through membrane ERα and group I mGluRs as has been observed in other biological systems (Jung et al., 2005; Seif et al., 2011; Maccarrone et al., 2002).

In addition to regulating endoCBs in the NAc through actions at the membrane, estradiol also exerts genomic effects on their receptors. Moreover, estradiol increases CB1R mRNA expression in the female NAc within 24 hours after treatment. Although we did not determine whether the estradiol-mediated increase in NAc CB1R mRNA corresponds to increases in protein in this study, this relationship has been observed *in vitro* in cell cultures and depends on estrogen receptors (Notarnicola et al., 2008). These increases in CB1R mRNA and protein were both observed as early as 1 hr following estradiol treatment and persisted through at least 24 hrs (Notarnicola et al., 2008). Given this time course, it is unclear whether estradiol upregulates CB1R through actions at membrane-localized estrogen receptors and subsequent second messenger signaling or through nuclear estrogen receptors. In the future, delineating the mechanism whereby estradiol regulates CB1R expression may be of interest.

In contrast to the CB1R, we did not observe any effects of estradiol in regulating the expression of the primary enzymes responsible for maintaining endoCB tone. This was somewhat surprising given that others have localized estrogen response elements to the fatty acid amide hydroxylase (FAAH) gene, the enzyme responsible for the degradation of AEA (Maccarrone et al., 2000; N S Waleh et al., 2002). Activation of genomic estrogen receptors down-regulates the expression FAAH resulting in elevated AEA tone. That we did not see any changes in mRNA for the synthesizing or degrading enzymes for AEA or 2AG in the NAc could be due to the fact that we only only examined mRNA at a

single time point 24 hours after estradiol treatment. It is possible that we would have observed changes had we looked at earlier or later time points. Additionally, it could be that effects of estradiol on gene expression is NAc subregion specific and consequently were washed out through combining the NAc core and shell in our analysis.

In summary, here we demonstrate that estradiol influences the endoCB system within the NAc on both short- and long-term timescales. As the endoCB system in the NAc is a key mediator of drug addiction, that estradiol enhances its function may provide insight as to how estradiol potentiates female sensitivity to drugs of abuse. Repeated exposure to drugs of abuse produces long lasting changes in endoCB system function, such as increases CB1R density in the NAc (Orio et al., 2009). Moreover, it may be that by mobilizing endoCBs and upregulating their respective receptors, that estradiol places the female NAc in a receptive state to respond more strongly to the future actions of drugs of abuse.

CHAPTER 4

Estradiol impacts the endocannabinoid system in female rats to influence behavioral and structural responses to cocaine

Rationale

Compared with men, women show enhanced responses to drugs of abuse, and consequently are thought to be more vulnerable to addiction. The ovarian hormone estradiol has emerged as a key facilitator in the heightened development of addiction in females and these actions of estradiol appear mediated by estrogen receptor (ER) interactions with metabotropic glutamate receptor type 5 (mGluR5). However, the downstream effectors of this ER/mGluR5 activity are unknown. Here we investigate whether cannabinoid 1 receptor (CB1R) activation is a part of the mechanism whereby estradiol influences behavioral and synaptic correlates of addiction in female rats. Relative to oil control females, estradiol-treated rats exhibited sensitized locomotor responses and decreased dendritic spine density in nucleus accumbens core medium spiny neurons following repeated cocaine injections. These effects of estradiol were blocked by AM251, a CB1R inverse agonist. These results demonstrate that part of the signaling mechanism through which estradiol impacts behavioral and synaptic correlates of addiction in female rats requires activation of CB1Rs.

Introduction

There are sex differences in the etiology of drug addiction, with women progressing more rapidly to an addicted state. This is particularly true for psychostimulants, but applies to multiple classes of abused drugs (Becker and Hu, 2008; Carroll et al., 2004). The ovarian hormone estradiol is a key biological factor contributing to this vulnerability in females (Hedges et al., 2010). Clinical research demonstrates that

women are more sensitive to the rewarding properties of psychostimulants when endogenous estradiol levels are elevated, and administration of exogenous estradiol further enhances these properties (Justice and de Wit, 1999; Maria et al., 2014; McCance-Katz et al., 2005). Animal models of addiction parallel human reports, in that high estradiol concentrations correspond with greater locomotor responses to psychostimulants, a more rapid acquisition of psychostimulant self-administration, enhanced motivation for psychostimulants, and increased total psychostimulant consumption (Anker and Carroll, 2011; Becker and Hu, 2008; Festa and Quinones-Jenab, 2004; Segarra et al., 2010) Yet, despite the abundance of behavioral observations of the effects of estradiol in females on responsiveness to psychostimulants, little is known regarding the cellular mechanisms underlying these phenomena.

While estradiol was once thought to act solely through a single nuclear estrogen receptor (Couse and Korach, 1999; Klinge, 2001), recent studies have established that estradiol can affect cellular function through multiple receptors, many localized to the surface membrane (Micevych and Kelly, 2012). In addition to GPER-1 (GPR30) and the putative ER-X, and the STX-sensitive receptors (Barton, 2016; Filardo and Thomas, 2012; Qiu et al., 2003; Toran-Allerand et al., 2002), palmitoylation of both ERα and ERβ promotes receptor trafficking to the surface membrane (Meitzen et al., 2013; Pedram et al., 2007). Within the nervous system, these two estrogen receptors functionally couple to metabotropic glutamate receptors (mGluRs) leading to glutamate-independent mGluR signaling (Boulware et al., 2007, 2005).

Recent work from our lab has identified a putative signaling mechanism through which estradiol can impact structural and behavioral substrates of drug addiction, which in turn may promote a greater vulnerability for female drug abuse. Within medium spiny neurons (MSNs) of the striatum and nucleus accumbens (NAc), estradiol activates ERα coupled to mGluR subtype 5 (mGluR5) (Grove-Strawser et al., 2010). Specific to the NAc core, estradiol induces synaptic plasticity via decreases in dendritic spine density through transactivation of mGluR5 (Peterson et al., 2014). In general, synaptic plasticity within the NAc core is thought to be a neurobiological substrate of drug addiction, and to underlie psychostimulant behavioral sensitization (Dumitriu et al., 2012; Li et al., 2004; Robinson and Kolb, 2004; Waselus et al., 2013). Related, estradiol enhances psychostimulant locomotor sensitization through membrane ER coupling to mGluR5 (Martinez et al., 2014).

Activation of group I mGluRs leads to a variety of other cellular responses, one of which is the release of endogenous cannabinoids (endoCBs) (Alger and Kim, 2011; Wilson and Nicoll, 2002). Upon release from post-synaptic membranes, endoCBs within the CNS retrogradely bind to presynaptic type 1 cannabinoid receptors (CB1Rs). Interestingly, multiple lines of research demonstrate that estradiol enhances endoCB activity in the nervous system of both women and female rodents (Bradshaw et al., 2006; El-Talatini et al., 2010; Gorzalka and Dang, 2012; Huang and Woolley, 2012; Rodríguez de Fonseca et al., 1994; Scorticati et al., 2004). Moreover, similar to estradiol, both endoCBs and CB1Rs have emerged as key endogenous moderators of drug addiction (Olière et al., 2013). Although these lines of evidence were developed independently, an

intriguing possibility is that within females, estradiol and endoCBs may act through a common pathway to exert effects on structural plasticity and addiction. At least in male animals, activation of mGluR5 leads to mobilization of endoCBs in the NAc (Jung et al., 2005; Robbe et al., 2002; Uchigashima et al., 2007). Based on these observations, we hypothesized that estradiol activates ERs to initiate mGluR5-dependent endoCB signaling, and that it is the endoCB system that is ultimately responsible for the effects of estradiol on structural and behavioral substrates of drug addiction.

Materials and Methods

Animals

Female Sprague Dawley rats 12 weeks of age (175-200g) were ovariectomized at Harlan labs (Indianapolis, IN). Upon arrival at our animal facility animals were housed in pairs, handled daily, and allowed to habituate for one week prior to experimentation. Animals were maintained on a 12 hr light-dark cycle (lights on at 6:00 am) with all behavioral testing occurring between 8:30 am and 1:30 pm. Food and water were available ad libitum. Animal procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the University of Minnesota.

Drugs and hormones

17β-estradiol (Sigma-Aldrich, St. Louis, MO) was dissolved in cottonseed oil (Sigma-Aldrich) and injected s.c. at 2 μg in 0.1 ml oil. Cottonseed oil (0.1 ml s.c.) was injected as the vehicle control. The type 1 cannabinoid receptor inverse agonist N-

(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; Tocris Biosciences, Minneapolis, MN) was dissolved in DMSO to make a 100 mM stock solution and stored at -20 oC. On the day of injections, the stock solution was diluted in 0.5% methylcellulose (Sigma-Aldrich) in deionized water to a working solution of 1 mg/kg/ml. We chose a low dose of AM251 because it does not affect locomotor activity on its own (Shearman et al., 2003; Thiemann et al., 2008). This low dose of AM251 allowed us to examine the role of CB1R activation on estradiol-induced locomotor responses to cocaine, without compromising general locomotor responses.

Throughout the experiment, ovariectomized (OVX) females were injected s.c. with estradiol or oil on a 2 days on-2 days off schedule to mimic the estrous cycle of an intact female rat (Butcher et al., 1974). Thirty min prior to each hormone injection, females received an i.p. injection of AM251 or vehicle. Beginning on the third day of the experiment, females were injected i.p. with 15 mg/kg/ml cocaine (cocaine hydrochloride; Covidien, St. Louis, MO) in saline or a comparable volume of the saline vehicle for 5 consecutive days. This pattern of cocaine injections produces a modest (if any) sensitization in untreated OVX females (Segarra et al., 2010; Sircar and Kim, 1999), allowing us to more clearly isolate a synergistic effect of estradiol on cocaine-induced locomotor sensitization and test whether this effect is attenuated by AM251 pretreatment.

Locomotor Activity

Locomotor activity was assessed according to previously published methods (Martinez et al., 2014) with the following modifications: Locomotor chambers were

placed inside a sensing frame (Kinder Scientific, Poway, CA) to measure activity in the XY plane (i.e. ambulations). Animals were in locomotor chambers for 2 hrs each day for the 7 days of the experiment. For the 5 consecutive days that females were injected with cocaine, animals were in the chamber 1 hr before (habituation session) and 1 hr following (test session) the injection.

Activity data analyses

For each animal, locomotor data were obtained for the entire 1 hr test period on the first and fifth test sessions. Depending on treatment condition, a final sample size of 14-20 animals was obtained for each treatment group (see Fig 2 for treatment group sample sizes). As an index of sensitization, locomotor activity was compared on first vs. fifth test sessions using paired-samples t-tests with Bonferroni correction for multiple comparisons (SPSS version 20.0, IBM Corp, Armonk, NY). For all statistical tests, results were considered to be statistically significant if $p < 0.0125$.

Tissue preparation for dendritic spine measurements

A subset of animals that completed the behavioral study was examined for neuronal morphology. Perfusions, DiI labeling, confocal imaging, quantification and data analysis followed previously published methods (Staffend and Meisel, 2011a) with some refinements (Peterson et al., 2015, 2014).

Twenty-four hrs following the final cocaine injection, animals were anesthetized using Beuthanasia-D (0.3 ml i.p./animal, Schering, Union, NJ), injected with 0.25 ml heparin into the left ventricle of the heart and intracardially perfused with 25 mM phosphate buffered saline (PBS, pH = 7.2) for 3 min at 25 ml/min followed by ice-cold

1.5% paraformaldehyde in 25 mM PBS for 20 min. Perfusion with 1.5% paraformaldehyde results in more complete and clearer visualization of DiI-labeled neurons when compared to perfusion with the standard 4% paraformaldehyde (Staffend and Meisel 2011b). Brains were removed and coronally blocked to allow for penetration of the post-perfusion fixative (1.5% paraformaldehyde for 1 hr) into the tissue blocks containing the striatum. Brains were sectioned at 300 μm in serial, coronal sections through the striatum using a Vibratome (Lancer Series 1000, St. Louis, MO) and placed in wells containing 25 mM PBS until DiI labeling.

DiI labeling

Instructions for DiI labeling are described in detail by Staffend and Meisel (2011b). Briefly, microcarriers containing DiI-coated tungsten particles (2 mg lipophilic carbocyanine DiI; Molecular Probes, Carlsbad, CA) dissolved in 100 μl of dichloromethane mixed with 90 mg of 1.3 μm tungsten particles (Biorad, Hercules, CA) were made in Tefzel tubing (Biorad) pre-coated with freshly prepared 10-15 mg/ml polyvinylpyrrolidone (PVP, Sigma-Aldrich) and cut into 1.3 mm segments. A Helios Gene Gun (BioRad) with a modified barrel, 40 mm spacer and 70 μm nylon mesh filter was used to deliver the microcarriers to the lightly fixed brain sections using helium gas at a pressure of 100 PSI. Prior to DiI delivery, PBS was removed from wells containing the brain sections. Immediately following DiI delivery, brain sections were re-submerged in PBS for 24 hrs in the dark at room temperature for diffusion of DiI. After 24 hrs, the PBS was removed and replaced with 4% paraformaldehyde in PBS for 1 hr at room temperature.

Confocal Imaging

Following DiI labeling, coronal sections through the nucleus accumbens (NAc) were washed in PBS, mounted on Superfrost slides (Brain Research Laboratories, Newton, MA) and coverslipped with Fluorglo mounting medium for lipophilic dyes (Spectra Service, Ontario, NY). Dendritic segments were imaged using Type LDF immersion oil (refractive index: 1.515; Cargille, Cedar Grove, NJ) and a Leica PLAN APO 63X, 1.4 NA oil immersion objective (11506187; Leica, Mannheim, Germany). Based on our recent analyses, this immersion oil is critical to resolve dendritic spines in the axial plane when used with this objective lens (Peterson et al 2015). All z-stacks were maintained at an xy pixel distribution of 512 x 512 and scanned at a frequency of 400 Hz. Whole neurons were imaged with a 20x air objective at 1.0 µm increments in the z-axis and reconstructed using Leica LAS AF software to measure the distance from the soma of target dendritic segments: 70–200 µm from soma in medium spiny neurons. Dendritic segments were imaged with a 63x oil immersion objective with a zoom of 5.61 and z-stacks were acquired in 0.12 µm increments. By adjusting the laser power and photomultiplier, each dendrite was imaged in its full dynamic range. Within the NAc core, 3 neurons and 3 dendritic segments per neuron were imaged to generate a total of 9 segments from every animal. Depending on the treatment conditions, a final sample size of 4-6 animals was obtained per treatment group (see **Figure 12** for treatment group sample sizes).

Confocal z-stacks of dendritic segments were first subjected to a 3D-deconvolution process using Autoquant X AutoDeblur Gold CF software (version 3.0;

Media Cybernetics, Bethesda, MD). Deconvoluted images were then reconstructed in 3D using the Imaris software (version 7.7; Bitplane Inc., St. Paul, MN).

Dendritic spine data analysis

All analyses were completed by an experimenter blinded to the treatment conditions. Nine dendritic segments (from 3 separate neurons) were averaged to generate a single dendritic spine density value for each animal. In cocaine-treated females, a two-way ANOVA with subsequent Tukey HSD post hoc tests were used to evaluate the effects of drug, hormone and drug-hormone interactions among treatment groups in the NAc core using SPSS software (version 20.0, IBM Corp). For all statistical tests, results were considered to be statistically significant if $p < 0.05$.

Results

Estradiol-facilitated psychostimulant locomotor sensitization depends on CB1R

Several laboratories have demonstrated that estradiol treatment in females facilitates cocaine-induced sensitization of locomotor responses (Segarra et al., 2010; Sircar and Kim, 1999) More recently, we demonstrated that this effect of estradiol is dependent on activation of mGluR5 (Martinez et al., 2014). As the endoCB system lies downstream of mGluR5 signaling (Jung et al., 2005; Robbe et al., 2002; Uchigashima et al., 2007), we sought to determine whether this effect of estradiol depends on endoCB signaling, specifically through activation of CB1R. To examine this question, females received the CB1R inverse agonist, AM251 (or vehicle) 30 min prior to estradiol (or oil) treatment. This treatment regimen occurred for two consecutive days before the first and

fifth cocaine locomotor test days (**Figure 10**). Importantly, on each locomotor test day cocaine was administered 24 hrs after other drug and hormone manipulations, to eliminate direct effects of these agents on locomotor activity following cocaine.

Figure 10

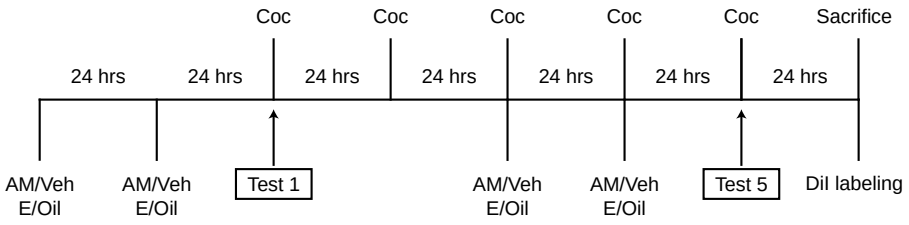

Figure 10. Timeline of experimental manipulations.
Ovariectomized female rats were injected with the cannabinoid type 1 receptor inverse agonist, AM251, or vehicle (veh) followed by estradiol (E) or oil, on days 1, 2, 5, and 6 (n = 14-19 per group). Cocaine (Coc) was injected on days 3-7 and locomotor activity was assessed on day of the first and fifth Coc injections (Test 1 and Test 5 respectively). 24 hrs after the final Coc injection a subset of females that were tested for behavior were sacrificed for DiI labeling.

In accordance with previous studies (Martinez et al., 2014; Segarra et al., 2010; Sircar and Kim, 1999), females treated with estradiol exhibited robust locomotor sensitization to cocaine when comparing ambulations after the fifth cocaine injection to the first cocaine injection ($t(13) = 3.043$, $p < 0.0125$; **Figure 11**). Pretreatment with AM251 blocked the effect of estradiol. AM251 or vehicle treatment in the absence of estradiol did not significantly affect ambulatory movement responses to repeated cocaine injections.

Figure 11

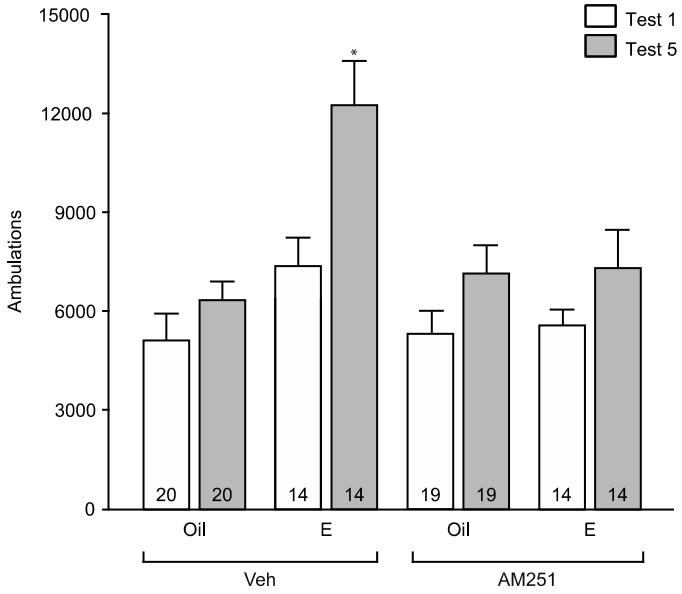

Figure 11. The estradiol-mediated increase in cocaine-induced ambulations depends on type 1 cannabinoid receptors.

In estradiol (E) treated females, ambulations (mean + SEM) were higher in response to the fifth cocaine (Coc) vs. first Coc injection (Test 5 vs. Test 1). This effect of estradiol was not observed when females were treated with the cannabinoid type 1 receptor inverse agonist, AM251, 30 min prior to estradiol. Oil or AM251 treatment alone did not affect Coc-induced ambulations. The number inside of each bar represents the number of animals per treatment condition. * $p < 0.0125$ Veh+E Test 1 vs Veh+E Test 5

Estradiol-induced dendritic spine plasticity depends on CB1R

As synaptic plasticity within the NAc core is thought to form a neurobiological basis for pyschostimulant-induced locomotor sensitization (Dumitriu et al., 2012; Li et al., 2004; Robinson and Kolb, 2004; Waselus et al., 2013), we examined the effect of AM251 and estradiol treatment on dendritic spine plasticity of NAc core MSNs in cocaine-treated females. Twenty-four hrs after the fifth and final cocaine injection, a

subset of females from each treatment group was arbitrarily selected for dendritic spine analyses. Representative images of a DiI-labeled MSN and dendritic segment used to quantify dendritic spine densities are shown in **Figure 12a** and **Figure 12b**, respectively. The effects of estradiol and AM251 differed across treatment groups (two-way ANOVA: hormone x drug interaction: $F(1,21) = 10.6$, $p < 0.05$). Estradiol treatment produced a significant reduction in dendritic spine density in the NAc core ($p < 0.05$, Tukey HSD post hoc test). In contrast, estradiol-treated females that received AM251 did not exhibit this change in the density of dendritic spines (**Figure 12c,d**). Compared with estradiol or oil treatment, females that received AM251 alone did not significantly differ in dendritic spine density within the NAc core.

Figure 12

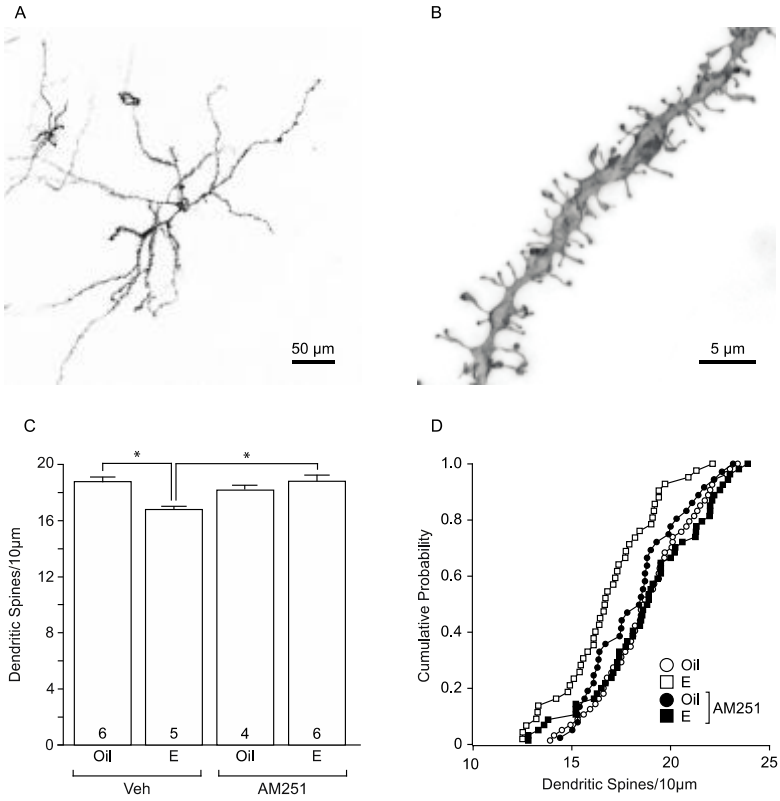

Figure 12. Estradiol-mediated dendritic spine plasticity in the nucleus accumbens core depends on type 1 cannabinoid receptors.
a Low power DiI-labeled nucleus accumbens medium spiny neuron, scale bar 50 μm. b High power dendritic segment from an NAc core medium spiny neuron, scale bar 5 μm. c In cocaine-treated females, estradiol treatment decreased dendritic spine density of medium spiny neurons in the NAc core, and this effect was attenuated by pretreatment with the cannabinoid type 1 receptor inverse agonist, AM251. Dendritic spine density in the NAc core of females treated with AM251 alone did not differ from estradiol or oil treatment alone. d Cumulative probability distribution plot of dendritic spine density within treatment groups for nucleus accumbens core highlights the effect of estradiol on dendritic spines. The number inside of each bar represents the number of animals per treatment condition. * $p < 0.05$ Veh+Oil vs Veh+E and Veh+E vs AM251+E

Discussion

For women, estradiol is a key biological factor contributing to the etiology of addiction. Yet, our understanding of the underlying neural mechanisms through which estradiol acts is still in its infancy. Recent findings indicate that estradiol, through activation of metabotropic glutamate receptor type 5 (mGluR5), structurally remodels nucleus accumbens (NAc) reward circuits (Peterson et al., 2014) and imparts a greater sensitivity to psychostimulants (Martinez et al., 2014). Here we extend these findings by demonstrating that activation of the endogenous cannabinoid (endoCB) system is necessary for these effects. Specifically, CB1R signaling is required for estradiol potentiation of cocaine-induced locomotor responses and alterations to the dendritic spine density of NAc core medium spiny neurons (MSNs). While it is currently unknown whether these results generalize beyond psychostimulant use, they are relevant, as recent reports indicate ~50% more women than men receive treatment for psychostimulant abuse (SAMHSA, 2014).

The interplay between estradiol and the endoCB system in the context of repeated exposure to cocaine complement and extend a growing body of work that in the absence of drugs of abuse, ovarian hormones, particularly estradiol, influence the endoCB system. Initial observations found that across the estrous cycle, CB1R density and ligand-binding affinity fluctuate in multiple brain regions; effects that are sensitive to ovariectomy and restored following hormone replacement (Rodriguez de Fonseca et al., 1994). In parallel, CNS levels of 2-arachidonly glycerol (2-AG) and anandamide (AEA) fluctuate in concert with ovarian hormones (Bradshaw et al., 2006) and evidence from *in vitro* (Huang and Woolley, 2012) and *in vivo* (Scorticati et al., 2004) studies suggests that AEA levels are increased in the CNS following treatment with estradiol. The activity of the endoCB system appears to increase with the initial rise in plasma estradiol during the estrous cycle (Gorzalka and Dang, 2012). Similarly, plasma endoCB levels are highest with rising estradiol in the follicular phase of the menstrual cycle (El-Talatini et al., 2010).

While estradiol can act through nuclear or membrane-localized estrogen receptors (ERs) to influence the endoCB system, membrane ER activation of mGluR signaling appears to be principally important. Physiological concentrations of estradiol rapidly stimulate AEA release from cultured human endothelial cells through surface membrane estrogen receptors (Maccarrone et al., 2002). Furthermore, in female rat hippocampal neurons membrane-localized ERα couples to mGluR1a, promoting AEA activation of CB1R, and leading to an attenuation of GABAergic neurotransmission (Huang and Woolley, 2012; Tabatadze et al., 2015). Coupling of ERs to mGluRs is in fact observed throughout the female nervous system, though the exact pairing of ERs and mGluRs is

brain-region dependent. For example, whereas in CA1 hippocampal pyramidal neurons ERα activates mGluR1a (Boulware et al., 2005), in MSNs of the caudate and NAc core, ERα couples to mGluR5 (Grove-Strawser et al., 2010; Martinez et al., 2014; Peterson et al., 2014).

While ER/mGluR signaling plays an essential role in the regulation of endoCBs, other forms of estrogen signaling also appear to regulate this neurotransmitter system. For example, ERs down-regulate the expression of fatty acid amide hydroxylase (FAAH), the enzyme responsible for the degradation of AEA (Maccarrone et al., 2000; Waleh et al., 2002). Transcriptional repression of FAAH through ER binding traditional estrogen response elements results in elevated AEA tone. Presumably it is through this mechanism that estradiol elevates AEA to elicit anxiolytic effects in female rats, which are blocked with a CB1R antagonist (Hill et al., 2007). Moreover, this nuclear action of estradiol converges with membrane actions at the same functional endpoint to increase AEA (Huang and Woolley, 2012; Tabatadze et al., 2015). These findings parallel other biological systems, where both membrane and nuclear ERs work cooperatively to control various physiological processes (Levin, 2014, 2005; Pedram et al., 2016).

Estradiol regulation of AEA release and metabolism provide evidence that this endoCB is critical for the effects of estradiol; however, as mentioned previously, 2-AG is also regulated by ovarian hormones (Bradshaw et al., 2006). Intriguingly, at least in male subjects, the effects of mGluR5 activation in the NAc are primarily dependent on 2-AG (Jung et al., 2005; Seif et al., 2011), a more potent activator of the CB1R (Sugiura et al., 2006). Because the vast majority of studies utilize only male subjects, it would be of

interest to determine if there is a sex difference between mGluR regulation of 2-AG and AEA signaling.

Notwithstanding which endoCB is (primarily) activating CB1Rs in response to estradiol, converging lines of evidence begin to outline how estradiol acts at a circuit level to enhance female sensitivity to cocaine. In their pioneering study, Woolley and colleagues determined that estradiol mobilization of endoCBs activated CB1R on GABAergic terminals to inhibit presynaptic GABA release, and thus indirectly increase post-synaptic pyramidal neuron excitability (Huang and Woolley, 2012; Tabatadze et al., 2015). While these data were from the hippocampus, CB1Rs are also primarily localized on GABAergic terminals in the NAc (Winters et al., 2012), suggesting a similar effect may occur here. Moreover, activation of CB1Rs in the NAc locally disinhibits dopamine release in this region (Cheer et al., 2004; Chen et al., 1993; Sperlágh et al., 2009) as does estradiol in the female NAc and caudate resulting in increased locomotor responses to psychostimulants (Becker and Rudick, 1999; Schultz et al., 2009; Thompson and Moss, 1994). Sustained enhancement of dopamine neurotransmission in the NAc and changes in MSN excitability are recognized as a key factors to the onset of drug addiction through structural remodeling of reward circuits leading to enhanced behavioral responses to psychostimulants (Golden and Russo, 2012; Kalivas and Duffy, 1990; Kourrich and Thomas, 2009).

In contrast to the long-lasting effects on NAc structure and function imposed by repeated exposure to drugs of abuse, the effects of estradiol are both more transient and cyclical. This is true not only for the effects of estradiol on neurotransmission and

excitability, but for structural plasticity as well. We observe decreased dendritic spine density in the NAc core 24-48 hours after estradiol (Peterson et al., 2014; Staffend et al., 2011). These effects reverse over several days in the absence of further hormone treatment (Woolley, 1998). Regardless of duration, group I mGluRs and CB1R are necessary for the effects of estradiol on structural plasticity (Christensen et al., 2011; Peterson et al., 2014;). In addition, activation of group I mGluRs or CB1Rs alone are sufficient to decrease dendritic spine density in the NAc core (Gross et al., 2015; Carvalho et al., 2014). Thus group I mGluRs and CB1R are both necessary and sufficient to produce dendritic spine plasticity in the NAc core.

At the same time that the effects of estradiol on dendritic spine plasticity in the NAc that have received little attention (Peterson et al., 2014; Staffend et al., 2011), numerous studies have investigated the effect of psychostimulants. Although initial studies indicated that repeated exposure to psychostimulants increased dendritic spine density within the NAc core (e.g., Li et al., 2004; Norrholm et al., 2003), more recent studies using advanced staining techniques and 3-D imaging, thought to more accurately represent neuroanatomical structure (Golden and Russo, 2012), have observed a decrease in spine density within this brain region following repeated cocaine administration (Dumitriu et al., 2012; Waselus et al., 2013). Independent of methodological issues, dendritic spine plasticity of MSNs in the NAc are associated with enhanced behavioral responsiveness to psychostimulants. While changes in dendritic spines may be necessary for behavioral sensitization, our findings suggest that alone they are not sufficient as

estradiol enhances the effects of psychostimulants, but does not typically produce sensitization on its own.

In summary, it has been previously postulated that the endoCB system is an intermediary between estradiol and drug addiction (Maccarrone, 2004). Here, we outline a putative mechanism linking estradiol to the endoCB system in remodeling reward circuits, imparting a greater vulnerability for drug abuse in females. Whereas endoCB signaling via group I mGluR occurs in both males and females, the coupling of ERs to group I mGluRs appears relatively exclusive to the female nervous system. This provides a sex-specific mechanism that may account for the accelerated onset of female drug addiction. Hence, decoupling ERs to mGluR activation of endoCB in the reward system may be considered as a potential therapeutic means to mitigate hormonal influences on female vulnerability to addiction.

CHAPTER 5

Overall Discussion and Conclusions

Summary and Significance

For the past 30 years, the steroid hormone estradiol has been recognized as a key biological factor contributing to the development of drug addiction in females. The work within this dissertation has begun to elucidate a putative underlying neurobiological mechanism through which estradiol potentiates female vulnerability to drug addiction. We demonstrate that estradiol rewires reward circuits in the nucleus accumbens (NAc) and enhances behavioral responding to psychostimulants through group I metabotropic glutamate receptors (mGluRs) and the endogenous cannabinoid (endoCB) system. Whereas estradiol, group I mGluRs and the endoCB system have each independently been associated with addition (Becker and Hu, 2008; Olière et al., 2013; Olive, 2010), our work suggests that they may be linked through a serial pathway that is principally important in female drug addiction. Such a mechanism where estradiol activates group I mGluRs then the endoCB system in females has been implicated previously *in vitro* (Huang and Woolley, 2012; Tabatadze et al., 2015), but here we provide the first evidence suggesting that this mechanism may occur *in vivo* to drive behavior. Additionally, characterizing this mechanism may actually provide putative therapeutic targets that are particularly effective in treating drug abuse in women.

The significance of this dissertation, however, extends beyond drug addiction. As drugs of abuse usurp the brain's reward circuitry, which evolved to support naturally rewarding behaviors such as sex (an estradiol-regulated behavior), the mechanism described here undoubtedly plays a role in naturally rewarding behaviors as well. Furthermore, this signaling mechanism is not specific to areas of the brain involved in

reward and reinforcement; rather, it appears to be conserved across the female nervous system. It may be, then, that the effects of estradiol on numerous other functions, including nociception, cognition, mood, motor control, and learning and memory, are also mediated by this signaling pathway. Consequently, this dissertation research extends beyond the field of drug addiction and provides a better understanding of the fundamental neural mechanisms driving sex differences in neurobiology and behavior.

PART I: Evidence for the Model

Estradiol Rewires Reward Circuits to Enhance Female Sensitivity to Drugs

Plasticity in the NAc drives behavioral responses to drugs of abuse

The general consensus is that rewiring of neural circuits through structural and synaptic remodeling drive changes in behavior. How reward circuits are rewired to drive addictive behaviors has been the focus of numerous studies (Golden and Russo, 2012). Within this literature, several hallmarks of an addicted brain have emerged, one of which is the structural remodeling of medium spiny neurons (MSNs) within the nucleus accumbens (NAc) (Golden and Russo, 2012), a hub within the reward circuit. Whereas the exact nature of this structure-function relationship is still a topic of debate, it is generally accepted that long-lasting changes in dendritic spines, the synaptic contact points within these circuits, form a neurobiological basis for addiction (Robinson and Kolb, 2004).

The NAc is made up of a core and shell and these two subregions appear to have distinct roles in mediating drug addiction. Specifically, the shell seems to be more

involved in shorter-term aspects of addiction, such as reward, whereas the core seems to play a larger role in behaviors such as the development of patterned motor programs to obtain rewards (Ito et al., 2004; Meredith et al., 2008). Despite that these subregions comprise the same primary cell type, MSNs, they are oppositely affected by repeated exposure to drugs of abuse (Dumitriu et al., 2012; Kourrich and Thomas, 2009). Interestingly, this disparity between core and shell is thought to precipitate addiction-related behavior (Kourrich et al., 2015; Kourrich and Thomas, 2009).

Estradiol primes the NAc to respond more strongly to drugs of abuse

Whereas the effects of psychostimulants on plasticity in the NAc has been extensively studied (Golden and Russo, 2012), the effects of estradiol on have only recently been investigated (Peterson et al., 2014; Staffend et al., 2011). Here we find that like repeated exposure to drugs of abuse (Dumitriu et al., 2012), estradiol treatment in ovariectomized female rats induces opposite changes in dendritic spine density between the NAc core and shell. Moreover, the directionality of the changes that we observe in the core and shell in response to estradiol are identical to those produced by repeated psychostimulant exposure (Dumitriu et al., 2012; Waselus et al., 2013). The dichotomies between the core and shell are thought to mediate psychostimulant-induced behavioral plasticity (Kourrich et al., 2015; Kourrich and Thomas, 2009). Indeed, estradiol does affect both shell- and core-dependent behavioral responses in females. Estradiol potentiates the rewarding effects of psychostimulants (Justice and de Wit, 1999; McCance-Katz et al., 2005; presumably through actions in the shell), facilitates psychostimulant-induced locomotor sensitization (Hu and Becker, 2003; Sircar and Kim, 1999; presumably through actions in

the core), and enhances acquisition and escalation of psychostimulant intake (presumably through both actions in the core and shell; Anker and Carroll, 2011; Becker and Hu, 2008; Ridenour et al., 2005). Our data suggest that estradiol, in the absence of drugs, primes the reward circuits in the female NAc to respond more strongly to the future actions of psychostimulants (Martinez et al., 2014; Peterson et al., 2014).

An important distinction between the effects of estradiol on structural plasticity and drugs of abuse is how long they last. Whereas, the effects of drugs of abuse are long-lasting, those of estradiol are more transient (Hedges et al., 2010). Rewiring of brain circuits by estradiol was first observed and are better characterized outside the NAc (Cooke and Woolley, 2005; Woolley, 1998). From these studies we know that structural remodeling parallels fluctuations in estradiol levels through the hormone cycle of intact females and is mimicked by estradiol replacement in ovariectomized females (Woolley, 1998; Woolley and McEwen, 1993). Moreover, in ovariectomized females, estradiol-mediated dendritic spine plasticity reverse over several days in the absence of further hormone treatment (Woolley, 1998; Woolley and McEwen, 1993). Thus remodeling of reward circuits by estradiol is likely cyclical and transient compared with that of drugs of abuse.

Estradiol Activates Group I mGluRs to Rewire Reward Circuits and Enhance Female Sensitivity to Drugs

Estradiol activates group I mGluRs to prime the NAc to respond more strongly to drugs of abuse

In addition to demonstrating a bidirectional effect on dendritic spine plasticity between subregions of the NAc we found that the opposing changes occur through differential activation of mGluR1a vs. mGluR5 (Peterson et al., 2014). Although it was once thought that group I mGluRs elicit identical cellular responses, our findings contribute to a growing body of literature suggesting that although homologous, mGluR1 and mGluR5 can in fact have differential or even opposite effects (Bonsi et al., 2008; Mannaioni et al., 2001; Poisik et al., 2003, Gross et al., unpublished). Moreover, we found that estradiol-mediated decreases in dendritic spine density in the core occur through mGluR5; whereas the increases in the shell occur through mGluR1a (Peterson et al., 2014; Gross et al., unpublished). It was somewhat surprising that estradiol increased shell dendritic spine density through mGluR1a, as in MSNs, ERa was found to couple specifically with mGluR5 and not mGluR1a (Grove-Strawser et al., 2010). However, these results were obtained *in vitro* from female neonatal striatal cultures comprising MSNs from the entire striatum including core, shell and caudate. Thus it is plausible that coupling of ERa to mGluRs is developmentally regulated or simply different *in vivo*. Regardless, that estradiol increases dendritic spine density in the NAc shell through mGluR1a is consistent with the mechanism whereby estradiol increases spine density in other brain regions, including the hippocampus and hypothalamus (Boulware et al., 2005;

Christensen et al., 2011; Peterson et al., 2014). Interestingly, positive allosteric modulation of either mGluR1 or mGluR5 results in identical changes in dendritic spine density in the core and shell (i.e. increases with mGluR1 activation and decreases with mGluR5 activation; Gross et al unpublished). Consequently, these opposite changes between the core and shell appear to be mediated by the specific coupling of group I mGluR subtype to membrane-localized estrogen receptors in these regions. Collectively our data suggest that it is through group I mGluRs that estradiol primes the reward circuits in the female NAc to respond more strongly to the future actions of psychostimulants. Recently our lab demonstrated that group I mGluRs are critically important in the potentiating effects of estradiol on addictive behaviors (Martinez et al., 2014; Martinez et al., unpublished) providing support that estradiol-mediated changes in dendritic spine plasticity in the NAc are behaviorally relevant.

Estradiol activates group I mGluRs in the NAc

Although the neural site of action for the interaction between estradiol and group I mGluR signaling on the neuroanatomical and behavioral hallmarks of addiction was not directly identified within this dissertation, actions of estradiol directly in the NAc likely play a critical role. Estrogen receptors are expressed in the NAc (Phillips-Farfán et al., 2007; Shughrue et al., 1997) and site specifically blocking them in the female rat NAc attenuates the reward-enhancing properties of estradiol (Walf et al., 2007). Compared with mGluR1, mGluR5 has been more thoroughly investigated and consequently more heavily implicated in the drug addiction literature. However, this may in part be due to a lack of mGluR1 specific pharmacological agents. To date, studies suggest a role for

mGluR1 outside of the NAc in development and expression of addiction (Olive, 2010; Xie et al., 2010) and a role of mGluR1 in the NAc to temporarily reverse psychostimulant-mediated synaptic plasticity (Loweth et al., 2013). In contrast, mGluR5 in the NAc core has emerged as a mediator of drug responsiveness. Specifically, blockade of mGluR5 in the NAc core decreases cocaine seeking behavior (Knackstedt et al., 2014; Wang et al., 2013), whereas activation of mGluR5 in the NAc core enhances cocaine seeking (Wang et al., 2013). Recent work from our lab implicate that it is through mGluR5 acting on MSNs in the NAc core that estradiol decreases dendritic spine density in this region (Gross et al., unpublished). Moreover, like estradiol treatment, site-specifically activating mGluR5 in the NAc decreased dendritic spine density in the core (Gross et al., unpublished). Collectively these studies suggest that actions of estradiol and mGluR5 signaling in the NAc core play an important role in mediating responsiveness to drugs of abuse. Furthermore, they provide support for an interaction between estrogen receptors and group I mGluRs within the NAc *in vivo*.

Estradiol activates membrane ERα*/group I mGluRs in the NAc*

While there are several ways in which estradiol could impact female sensitivity to drugs of abuse, membrane estradiol signaling appears to be principally important. Activation of ERα in females enhance locomotor responses to cocaine (Van Swearingen et al., 2013), an effect that is exacerbated by overexpression of ERα at the membrane of MSNs (Schultz et al., 2009). Through classical estrogen receptors (Xiao et al., 2003), like ERα, and group I mGluRs (Bruton et al., 1999), estradiol enhances dopamine release within the NAc (Becker, 1990; Thompson and Moss, 1994) a key factor underlying

behavioral responses to drugs of abuse and consequently the onset of addiction. Moreover, in parallel with rapid increases in dopamine, estradiol rapidly enhances psychostimulant-induced locomotor activity in females (Castner et al., 1993). To expand upon this work, our lab has shown that blocking mGluR5 prior to estradiol treatment attenuates the effect of estradiol on structure in the NAc core (Peterson et al., 2014) and to enhance stimulating properties and intake of psychostimulants (Martinez et al., 2014; Martinez et al., unpublished). Collectively, these studies provide strong support for a membrane mechanism of estradiol action, though membrane and genomic actions of estradiol often work cooperatively to achieve the same functional endpoint (Levin, 2014, 2005; Pedram et al., 2016).

Estradiol Activates the EndoCB System to Rewire Reward Circuits and Enhance Female Sensitivity to Drugs

There are several possibilities as to how estradiol signaling through group I mGluRs could initiate the development of an addicted phenotype. Here, we specifically highlight the involvement of the endogenous cannabinoid (endoCB) system. Although, it has been previously postulated that the endoCB system is an intermediary between estradiol and drug addiction (Maccarrone, 2004), we provide the first demonstration that this is indeed the case. Specifically, we show that estradiol influences the endoCB system in the NAc to remodel reward circuits and impart a greater sensitivity to the stimulating properties of psychostimulants in females.

Estradiol enhances activity of the endoCB system

Estradiol rapidly modulates levels of the two best studied endogenous cannabinoids, 2-arachidonyl glycerol (2-AG) and anandamide (AEA) in the NAc. Given that estradiol activates group I mGluRs that in turn mobilize endoCBs, it was somewhat surprising that we saw a decrease in endoCB levels following estradiol treatment. However, because endoCBs are very transient molecules, perhaps our experimental design did not allow the temporal resolution to adequately capture the dynamic endoCB mobilization profiles. In support of this idea, using electrophysiological methods, Woolley and colleagues observed endoCB mobilization in the hippocampus within minutes after estradiol application (Huang and Woolley, 2012; Tabatadze et al., 2015), however, when measuring endoCBs from estradiol-treated hippocampal tissue, they observed a decrease similar to what we see in the NAc (personal communication with Dr. Bradshaw who measured endoCBs from the samples). Future studies will be necessary to fully characterize the endoCB profiles following estradiol treatment in the NAc. Regardless, estradiol rapidly modulates endoCBs in the NAc and although not explicitly tested here, this action of estradiol likely occurs through membrane ERα and group I mGluRs as has been observed in human endothelial cells and rat hippocampal slices (Maccarrone et al., 2002; Huang and Woolley, 2012; Tabatadze et al., 2015).

Whereas estradiol modulated endoCBs levels in the NAc on a rapid time course, it did not affect endoCB tone through genomic actions. This was somewhat surprising given that others have localized genomic estrogen response elements to fatty acid amide hydroxylase (FAAH), the enzyme responsible for the degradation of AEA (Maccarrone et

al., 2000; Waleh et al., 2002). Activation of genomic estrogen receptors down-regulates the expression FAAH resulting in elevated AEA tone. That we did not see any changes in mRNA for the synthesizing or degrading enzymes for AEA or 2AG in the NAc could be due to the fact that we only only examined mRNA at a single time point 24 hours after estradiol treatment. It is possible that we would have observed changes had we looked at earlier or later time points. Additionally, it could be that effects of estradiol on gene expression is NAc subregion specific and consequently were washed out through combining the NAc core and shell in our analysis.

Although estradiol did not affect endoCB tone in the NAc, it did exert genomic effects on the cannabinoid receptors. Moreover, estradiol increased CB1R mRNA expression in the female NAc within 24 hours after treatment (unpublished observations). These findings compliment other observations highlighting CB1R density and ligand-binding affinity are regulated by estradiol *in vivo* in multiple brain regions (Rodriguez de Fonseca et al., 1994). *In vitro,* increases in CB1R mRNA is accompanied by increases in protein within 1-24 hr of estradiol treatment (Notarnicola et al., 2008). Given this time course it is unclear mechanistically how estradiol upregulates CB1R, whether through membrane actions of estradiol and subsequent second messenger signaling or via direct genomic actions of estradiol. In the future, delineating the mechanism whereby estradiol regulates CB1R expression may be of interest.

Our findings demonstrate that estradiol enhances the activity of the endoCB system in the female NAc, complementing a growing body of literature wherein estradiol influences the endoCB system in both women and female rats (El-Talatini et al., 2010;

Gorzalka and Dang, 2012). It is likely that estradiol is acting through both nuclear and membrane localized receptors to impact the endoCB system, as both membrane and nuclear estrogen receptors work cooperatively to control various physiological processes in several biological systems (Levin, 2014, 2005; Pedram et al., 2016).

Estradiol activates the endoCB system to prime the NAc to respond more strongly to drugs of abuse

To corroborate our findings that estradiol enhances endoCB system function in the NAc, we further demonstrate its importance in mediating female vulnerability to drug addiction. Moreover, we show that estradiol remodels NAc reward circuits and potentiates responses to cocaine through activating CB1Rs (Peterson et al., 2016). Although we showed the necessity of CB1R in mediating the effects of estradiol on structure and behavior through blocking CB1R, we did not show the sufficiency of CB1R activation. However, others have shown that CB1R activation produces the same effect on NAc dendritic spine density as estradiol (Carvalho et al., 2014). Interestingly, whereas blockade of CB1R appears to be relatively consistent in attenuating behavioral responsiveness to drugs of abuse, the effects of CB1R activation are more variable (Olière et al., 2013). The reason for this may lie in that the majority of these studies focused on male subjects. In fact, it appears that the few studies, including our own, investigating the role of the endoCB system in female addiction have demonstrated a more consistent role for CB1R in enhancing behavioral responses to drugs of abuse (Higuera-Matas et al., 2007; Lee et al., 2014). It may be that the interaction between estradiol and the endoCB system in the NAc underlies these consistent behavioral effects.

Taken together our data show that estradiol enhances endoCB system function in the NAc that likely contributes to the structural remodeling of reward circuits and addiction vulnerability in females. Future studies will need to determine whether estradiol is acting directly in the NAc to influence endoCB system function, changes and structure and drug-behaviors. Additionally, it would be of interest to more thoroughly examine how and which endoCBs are responsible for the rewiring reward circuits and driving behavioral responses to psychostimulants. Our preliminary work shows that estradiol influences both 2-AG and AEA in the NAc. This is in contrast to the existing literature demonstrating rapid mobilization of AEA and not 2-AG by estradiol (Maccarrone et al., 2002; Huang and Woolley, 2012; Tabatadze et al., 2015). However, the effects of mGluR5 activation in the NAc are primarily mediated by 2-AG (Jung et al., 2005; Seif et al., 2011), a more potent activator of the CB1R (Sugiura et al., 2006). Given that the effects we see on NAc structure and behavioral responding are CB1R dependent, 2-AG appears to be a likely candidate. Of course both 2-AG and AEA could both be involved in these processes and may in fact have distinct roles as they have been shown to differentially regulate synaptic plasticity depending on neuron type and properties (Mathur et al., 2013). It may be that the roles of AEA and 2-AG are region dependent, wherein activation of ERα/mGluR1a in the NAc shell mobilizes AEA as it does in the hippocampus (Huang and Woolley, 2012; Tabatadze et al., 2015) and activation of ERα/mGluR5 in the NAc core mobilizes 2-AG. Future studies are necessary to determine how exactly these two endoCBs interact in response to estradiol to promote an addictive phenotype.

PART II: Speculation on Function of the Model

How the Model Induces Synaptic Plasticity to Drive Behavioral Responses to Drugs of Abuse

EndoCBs are retrograde neurotransmitters that are well-known for their role in synaptic plasticity, particularly short synaptic depression and long term synaptic depression (STD and LTD, respectively) in the CNS. Upon release from the post-synaptic membrane, endoCBs activate presynaptic CB1Rs to initiate G-protein ($G_{i/o}$) coupled second messenger signaling that inhibits neurotransmitter release (Wilson and Nicoll, 2002). How CB1R-mediated synaptic depression ultimately affects function of the post-synaptic neuron depends on localization of CB1R to GABAergic or glutamatergic terminals.

Estradiol activates group I mGluRs and the endoCB system in the hippocampus

Given the role of endoCBs in regulating synaptic input and cellular excitability, they clearly have the capacity to modulate circuit function thus contributing to circuit remodeling and behavioral effects of estradiol. In the brain the effects of estradiol have been most thoroughly examined in the hippocampus. Here ERα-mGluR1a signaling mobilizes AEA that bind pre-synaptic CB1R on GABAergic terminals to initiate inhibitory-LTD (iLTD) (Huang and Woolley, 2012; Tabatadze et al., 2015). iLTD suppresses inhibitory influence on CA1 pyramidal neurons and enhance neuronal excitability. In turn, decreased inhibitory neurotransmission and increased pyramidal neuron excitability have both been associated with increases in dendritic spine density in these neurons that correlate with hippocampal-dependent behaviors (Boulware et al.,

2013; Murphy et al., 1998; Woolley, 2007, 1998). Interestingly, estradiol induces iLTD through AEA in females, whereas studies in males implicate 2-AG, suggesting that there may be a fundamental sex difference in how endoCBs govern synaptic plasticity (Huang and Woolley, 2012; Tabatadze et al., 2015). In addition to the role of the endoCB system in neuron-neuron signaling, an emerging literature in the hippocampus demonstrates its involvement in glial regulation of neuronal communication. Moreover, through the endoCB system, astrocytes regulate neuronal synaptic transmission and morphology (Navarrete et al., 2014; Perez-Alvarez et al., 2014). Together these observations suggest that estradiol-mediated changes in synaptic input through the endoCB system may lead to structural changes in the hippocampus that are important for hippocampal-dependent behaviors.

Estradiol activates group I mGluRs and the endoCB system in the NAc

Compared with the hippocampus where effects of estradiol have been extensively studied, less work has been done in the NAc. Like in the hippocampus, CB1R are localized to GABAergic terminals in the NAc where they elicit iLTD (Winters et al., 2012) to influence microcircuits and behavioral outputs. It appears that how endoCBs signal in the striatum is complex given the two distinct MSN subpopulations and the two excitability states that they exhibit (Mathur et al., 2013). The two subpopulations of MSNs, D1 and D2, make up two distinct pathways that differentially regulate behavioral output and sensitivity to drugs of abuse. Moreover, D1 neurons comprise the direct pathway that positively regulate motor output and sensitivity to drugs of abuse, whereas D2 neurons make up the indirect pathway and negatively regulate motor output and

sensitivity to drugs of abuse (Grueter et al., 2012). Furthermore, both D1 and D2 neurons can electrically exist in an up or down state that reflects their intrinsic excitability and the likelihood of firing action potentials to drive their respective circuits. Intriguingly, endoCB modulation of circuits depend on MSN subpopulation and electrical state and which endoCB is present (Mathur et al., 2013). In the upstate endoCB-iLTD occurs at both the indirect and direct pathways through release of 2-AG and solely involves MSN-MSN synapses (Mathur et al., 2013). Given that this iLTD equally effects D1 and D2 MSNs, the net result is no change in behavioral output. In contrast, in the downstate, endoCB-iLTD occurs through AEA only in the direct pathway at both MSN-MSN synapses as well as MSN synapses at fast spiking interneurons (FSIs) (Mathur et al., 2013). This suppression of inhibitory input to D1 MSNs likely drives the direct pathway-meditated motor output and sensitivity to drugs of abuse.

In addition to GABAergic terminals, CB1R have been also been localized to glutamatergic terminals and elicit excitatory-LTD (eLTD) and (Robbe et al., 2002). This endoCB eLTD occurs at glutamatergic synapses from the prelimbic cortex (Robbe et al., 2002) and within the indirect D2 pathway through mGluR5 (Grueter et al., 2010). As prelimbic cortex projections specifically to the NAc core drive cocaine seeking (Grueter et al., 2012) it may be that this synapse specific endoCB-mediated eLTD inhibits glutamatergic input to D2 MSNs to decrease the output of the indirect pathway thereby enhancing drug seeking behavior. In support of this idea, blocking LTD at glutamatergic synapses in the NAc is sufficient to block psychostimulant sensitization (Brebner et al., 2005). Interestingly, eLTD in D2 MSNs of the NAc core depend on both CB1R and

TRPV channels (Grueter et al., 2010). 2-AG is a full agonist at CB1R, whereas AEA is a partial agonist of CB1R and TRPV channels (Dietz et al., 2009; Maccarrone et al., 2015; Pertwee et al., 2010; Sugiura et al., 2006), thus either 2-AG, AEA or both may be involved in eLTD in indirect pathway. In summary, AEA induces iLTD at D1 MSNs to the drive direct pathway, whereas 2AG and/or AEA induces eLTD at D2 MSNs to inhibit indirect pathway and both work in concert to drive drug seeking behavior.

Perhaps through activating group I mGluRs, estradiol mobilizes endoCBs to drive drug seeking behavior through endoCB-LTD by selectively stimulating output of direct pathway MSNs and inhibiting output of indirect pathway MSNs as outlined above. In females, which endoCB is released at a particular synapse may be mediated by the exact coupling of ERα with group I mGluRs. Since ERα-mGluR1a signaling mobilizes AEA in female hippocampal neurons (Huang and Woolley, 2012), perhaps ERα-mGluR1a signaling in MSNs (as observed in the NAc shell) also stimulates AEA, whereas ERα-mGluR5 signaling in MSNs (as observed in the NAc core) mobilize 2-AG. As repeated group I mGluR-mediated LTD results in the structural remodeling of synapses (Shinoda et al., 2010, 2005), the changes in synaptic input through the endoCB system may underlie structural changes observed in MSNs that are thought to drive behavior.

How Relationships Between Neuronal Excitability and Structure Drive Behavioral Responses to Drugs of Abuse

Whereas the general consensus is that experience-dependent changes in synapses underlie behavioral responses, drawing causal links between these phenomena remains a

significant challenge in the field of neuroscience. Both synaptic and intrinsic (away from synapse) factors regulate cellular excitability to drive structural remodeling and behavior. These relationships in the NAc are best understood in the context of repeated exposure to drugs of abuse. However, identifying exact nature of the relationships among MSN excitability, structure and drug-related behavior has been clouded by inconsistencies among experimental methods within the literature such as route, dosage, context and duration of psychostimulant administration, period of abstinence following drug cessation, neuronal population and subregion of NAc examined (Golden and Russo, 2012), as well as neuronal labeling and dendritic spine analysis methods (Dumitriu et al., 2011).

If a snapshot was taken 24 hours following a regimen of repeated psychostimulant exposure that induce a sensitized locomotor behavioral response, neuronal excitability (intrinsic and synaptic) appears to oppose structural plasticity in the NAc (Dumitriu et al., 2012; Kourrich et al., 2007; Kourrich and Thomas, 2009; LaPlant et al., 2010). These observations provide support for the permissive function hypothesis for MSN excitability**,** wherein changes in intrinsic excitability allow for synaptic plasticity to develop (Kourrich et al., 2015). Additionally, these observations suggest that a change in excitability in one direction results in an opposite or compensatory change in structure to normalize neuronal activity (Huang et al., 2011; Kourrich et al., 2015; Russo et al., 2010). However, psychostimulant induced plasticity in MSN excitability and structure change over time during abstinence from drug exposure (Dumitriu et al., 2012; Kourrich et al., 2007; Kourrich and Thomas, 2009). Regardless, it has been postulated that by

inducing such plasticity in the NAc, repeated cocaine exposure establishes a new baseline level of MSN activity and lowers the threshold necessary for future cocaine-induced synaptic plasticity (Kourrich et al., 2007).

Interestingly changes in MSN excitability and structure are opposite in the core and shell and persist for different time courses following cessation of drug exposure (Dumitriu et al., 2012; Kourrich et al., 2007; Kourrich and Thomas, 2009; LaPlant et al., 2010). As stated earlier, it is thought that the disparity between core and shell may precipitate drug-related behaviors. Here we demonstrate that estradiol induces opposite dendritic spine plasticity in the NAc core and shell, mirroring those observed 24 hours following repeated exposure to drugs of abuse (Peterson et al., 2014). Future studies will be necessary to determine how these structural changes related to MSN function. However, estradiol regulates potassium channels (Du et al., 2014; Woolley, 2007), which underlie opposing changes in intrinsic excitability between the core and shell following repeated psychostimulant exposure (Kourrich and Thomas, 2009), and modulates calcium currents that contribute to neuronal excitability and firing capacity through membrane-localized estrogen receptors (Mermelstein et al., 1996; Woolley, 2007). Moreover, group I mGluR receptor activation alters the spike firing and excitability of MSNs in the NAc (D'Ascenzo et al., 2009) and are required for estradiol-mediated dendritic spine plasticity in the NAc (Peterson et al., 2014). Collectively, these data suggest by activating group I mGluRs, estradiol alters MSN excitability and structure similar to the adaptations that have been shown following repeated exposure to psychostimulants. It may be then that by inducing such plasticity in the NAc, estradiol establishes a new baseline level of MSN

activity and lowers the threshold necessary for future cocaine-induced synaptic plasticity.

How the Model Functions in the NAc to Rewire Reward Circuits and Drive Drug Behavior

Our work has begun to outline a potential mechanism for how estradiol acts in the female nervous system to enhance vulnerability to drug abuse through group I mGluRs and the endoCB system. Whereas estradiol, group I mGluRs and the endoCB system have all independently been implicated in the etiology of drug addiction, our work outlines a putative mechanism linking these systems into one single pathway. Moreover, that estradiol activates membrane localized ERα coupled group I mGluRs to activate the endoCB system. As described above, endoCBs induce long-term synaptic plasticity (i.e. LTD) at both GABAergic and glutamatergic synapses. It may be through initiating LTD at specific synapses in the NAc via the endoCB system, that estradiol regulates MSN excitability and morphology to weaken specific connections while strengthening others to ultimately enhance sensitivity to drugs of abuse.

Postsynaptically, both estrogen receptors (McEwen, 2001; Milner et al., 2001) and group I mGluRs (Mitrano and Smith, 2007) localize to membranes near synapses on dendritic spines where they directly interact with mGluRs (Meitzen and Mermelstein, 2011; **Figure 13**). Estradiol mediated transactivation of group I mGluRs mobilize endoCBs that bind presynaptic CB1R (Huang and Woolley, 2012; Tabatadze et al., 2015). CB1R have been localized to the terminals of both local GABAergic interneurons (GABA IN), MSNs and glutamatergic projection neurons (Glu PN) (Herkenham, 1992; Hohmann and Herkenham, 2000; Mathur et al., 2013; Wilson and Nicoll, 2002; Winters

et al., 2012; Wu et al., 2015; **Figure 13**). Although CB1Rs are not localized to terminals of dopaminergic projection neurons (DA PN) within the NAc (Cheer et al., 2007; Herkenham, 1992; Lupica and Riegel, 2005), activation of CB1R influences DA in the NAc likely through an intermediate neuron such as GABA IN (Cheer et al., 2004; Chen et al., 1993; Sperlágh et al., 2009; **Figure 13**).

The effects of estradiol-induced endoCB signaling in the NAc depends on localization of CB1R to excitatory or inhibitory terminals and whether iLTD or eLTD is induced. On one hand, endoCB binding to CB1R on Glu PN terminals would result in eLTD, inhibition of glutamate input to the postsynaptic MSN and decreased excitability of the MSN (**Figure 13**). On the other hand, endoCB binding to CB1R on either MSNs or GABA IN terminals would result in iLTD, inhibition of GABA input to the postsynaptic MSN and increased excitability of the MSN (**Figure 13**). In addition to directly influencing MSN excitability, iLTD at GABA IN can also indirectly influence MSN excitability through actions at DA terminals. Specifically, activation of CB1Rs on GABA IN decreases GABA input onto DA PN terminals resulting in a disinhibition of dopamine release and increase in dopamine concentrations (Cheer et al., 2004; Chen et al., 1993; Sperlágh et al., 2009; **Figure 13**).

As D1 and D2 MSNs make up two distinct pathways that oppositely regulate behavioral output and sensitivity to drugs of abuse, it is important to consider which neuron populations are effected by estradiol-induced endoCB-dependent LTD. Given that D1 MSNs of the direct pathway positively regulate motor output and sensitivity to drugs of abuse, whereas D2 MSNs of the indirect pathway negatively regulate motor output and

sensitivity to drugs of abuse (Grueter et al., 2012), estradiol likely acts to increase the activity of D1 MSNs and decrease the activity of D2 MSNs (**Figure 13**). To do this estradiol could induce endoCB-dependent eLTD at Glu PN synapses onto D2 MSNs to decrease D2 MSN excitability and induce endoCB-dependent iLTD at MSN or GABA IN synapses onto D1 MSNs to increase D1 MSN excitability (**Figure 13**). Estradiol could also decrease activity of D2 MSNs and increase activity of D1 MSNs through disinhibition of DA terminals and DA release onto D2 or D1 MSNs, respectively (**Figure 13**). These changes in D1 and D2 MSN excitability likely contribute to the structural changes induced by estradiol in the NAc core in shell (i.e. increases in dendritic spine density in the NAc shell and decreases in dendritic spine density in the NAc core) (**Figure 13**). Perhaps estradiol changes MSN excitability and structure through endoCB-dependent LTD that activates D1 direct pathway and inhibits the D2 indirect pathway to ultimately enhance sensitivity to drugs of abuse (**Figure 13**).

Figure 13

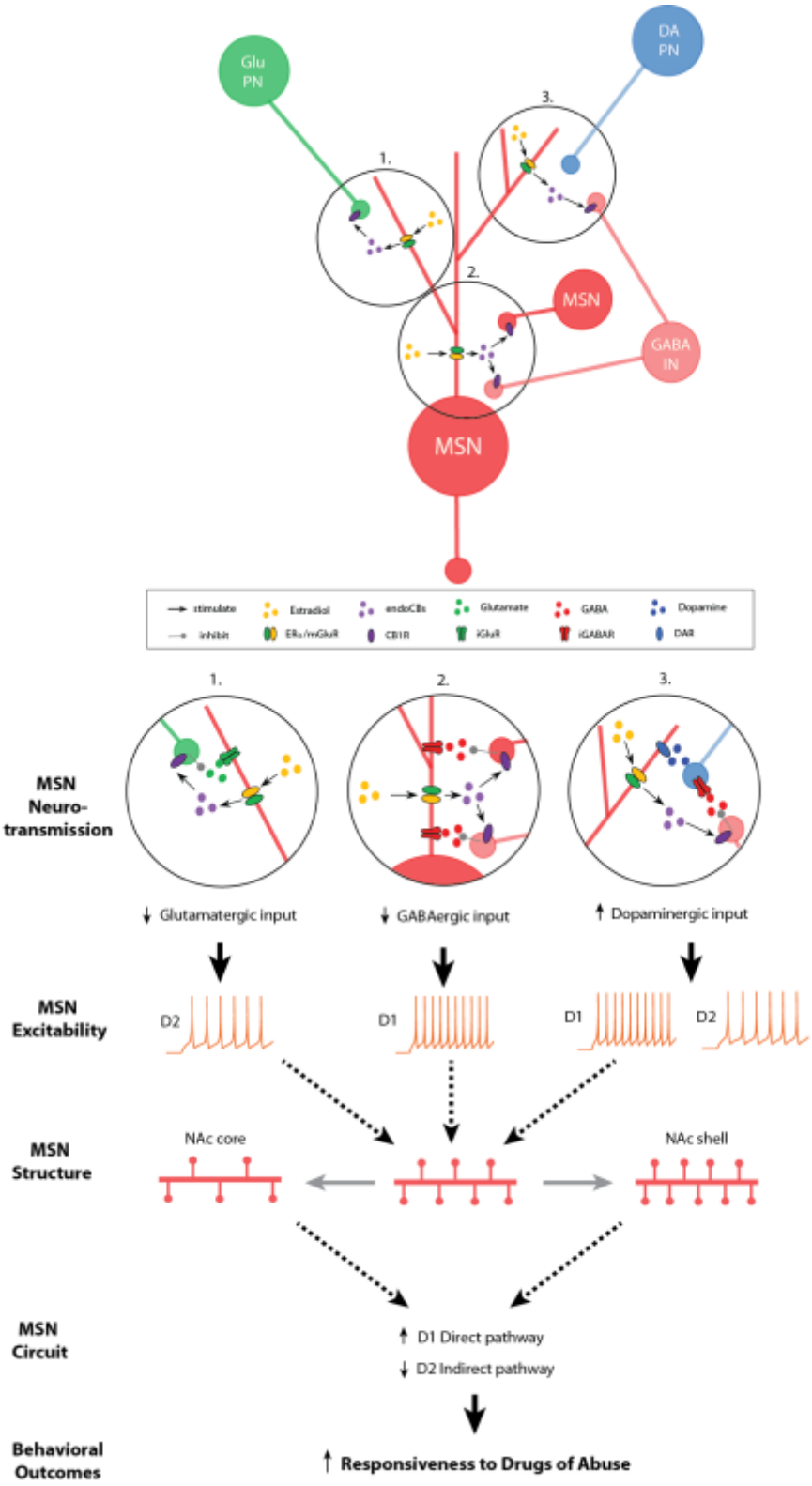
Glu PN
DA PN
1.
2.
3.
MSN
GABA IN
MSN
stimulate
inhibit
Estradiol
ERα/mGluR
endoCBs
CB1R
Glutamate
iGluR
GABA
iGABAR
Dopamine
DAR
MSN Neuro-transmission
Glutamatergic input
GABAergic input
Dopaminergic input
MSN Excitability
D2
D1
D1
D2
MSN Structure
NAc core
NAc shell
MSN Circuit
D1 Direct pathway
D2 Indirect pathway
Behavioral Outcomes
Responsiveness to Drugs of Abuse

Figure 13. Model depicting how estradiol enhances female responsiveness to drugs of abuse through activation of group I mGluR-mediated endocannabinoid (endoCB) mobilization in the nucleus accumbens (NAc).
Estradiol activation of estrogen receptors (ERs) coupled to group I mGluRs mobilizes endoCBs to alter glutamatergic (1), GABAergic (2) and dopaminergic (3) input to medium spiny neurons (MSNs). 1. endoCBs bind to cannabinoid type 1 receptors (CB1R) localized to terminals of glutamatergic projection neurons (glu PN) and inhibit glutamate release onto D2 MSNs thereby decreasing their excitability. 2. endoCBs bind to CB1R localized to terminals of local GABAergic MSNs or GABAergic interneurons (GABA IN) and inhibit GABA release onto D1 MSNs thereby increasing their excitability. 3. endoCBs bind to CB1R localized to terminals of local GABAergic interneurons (GABA IN) and inhibit GABA release onto terminals from dopamine projection neurons (DA PN) to increase dopaminergic input to MSNs thereby increasing excitability of D1 MSNs and decreasing excitability of D2 MSNs (i.e. functionally remodeling reward circuits). These changes in NAc neurotransmission and excitability of D1 and D2 MSNs result in increased dendritic spine density in the NAc shell and decreased dendritic spine density in the NAc core (i.e. structurally remodeling reward circuits). Collectively changes in neurotransmission, excitability and structure of MSNs results in enhanced activity of D1 direct pathway and blunted activity of D2 indirect pathway to drive responsiveness to drugs of abuse.

This model suggests that local actions of estradiol in the NAc are responsible for the effects we observe on the endoCB system, MSN structure and behavioral responses to drugs of abuse. However, estradiol acts throughout the female nervous system, particularly in several areas in the reward circuitry. On one hand, that all of the studies in here have utilized systemic injections of estradiol, group I mGluR antagonists and CB1R antagonists limits our model because we cannot be certain that the NAc is the site of action. In the future, it would be of great interest to systematically test whether this signaling is indeed a single unified pathway acting directly in the NAc. However, on the other hand that systemically blocking group I mGluRs and CB1Rs attenuated estradiol-mediated NAc circuit remodeling and sensitivity to psychostimulants holds therapeutic

potential. Several groups have shown that modulation of group I mGluRs and the endoCB system have therapeutic potential for drug-related cellular and behavioral plasticity (Duncan and Lawrence, 2012; Loweth et al., 2013; Olière et al., 2013; Olive, 2010; Olive et al., 2012; Wang et al., 2013). Thus that estradiol, group I mGluRs and the endoCB system share a common mechanism to enhance drug-related cellular and behavioral plasticity in females, group I mGluR or endoCB antagonism may serve as a viable target for developing treatments for drug addiction in women.

Importantly, coupling of estrogen receptors to group I mGluRs is sex specific, occurring only in the female nervous system (Boulware et al., 2005; Boulware and Mermelstein, 2009; Grove-Strawser et al., 2010; Huang and Woolley, 2012; Tabatadze et al., 2015). Likewise, endoCB mobilization through this coupling is specific to females (Huang and Woolley, 2012; Tabatadze et al., 2015). Several studies have demonstrated a role for group I mGluRs and the endoCB system in the etiology of drug addiction, however there are a number of studies that have failed to do so. A contributing factor to this variability may lie in the fact that the majority of these studies used only male subjects. Future studies using female subjects may reveal a more consistent role for group I mGluRs and endoCB system in addiction. Moreover, manipulation of group I mGluR signaling and the endoCB system may in fact be more effective in treating drug addiction in women compared with men.

Conservation of the Mechanism Throughout the Nervous System

Here we provide evidence for a mechanism whereby estradiol activates group I mGluR-mediated endoCB signaling in the NAc to remodel reward circuits and drive psychostimulant sensitivity. Given that the reward circuitry usurped by drugs of abuse, evolved to drive naturally motivated behaviors such as sex, feeding and social behaviors, the mechanism described here undoubtedly plays a role in these behaviors as well. However, in addition to influencing the NAc to drive motivated behaviors, estradiol acts throughout the female nervous system to affect a whole host of other neurobiological processes. Given that the endoCB system is highly expressed throughout the nervous system and implicated in several of these processes, activation of group I mGluR-mediated endoCB signaling by estradiol may underlie other effects of estradiol on the brain and behavior.

Some evidence suggesting that this mechanism is conserved throughout the nervous system is beginning to emerge. As discussed in detail above, estradiol transactivates group I mGluRs to mobilize endoCBs, alter pyramidal neuron excitability and structure that drive hippocampal-dependent processes, such as learning and memory (Boulware et al., 2013; Huang and Woolley, 2012; Peterson et al., 2014; Tabatadze et al., 2015). Similarly, in the hypothalamus, estradiol transactivates group I mGluRs, regulates the endoCB system, produces structural plasticity and drives reproductive behaviors in females (Christensen et al., 2011; Micevych and Sinchak, 2013; Nguyen and Wagner, 2006). Coupling of estrogen receptors to group I mGluRs occurs in other areas of the central nervous system, including the basal ganglia, amygdala, and cortex, where

estradiol regulates corresponding neurobiological processes underlying motor control, stress, fear, anxiety, cognition and disease states (Becker and Hu, 2008; De Jesús-Burgos et al., 2012; Gillies and McArthur, 2010; Spampinato et al., 2012; Srivastava et al., 2013; Zeidan et al., 2011; Zhang et al., 2008). In the future it will be of great interest to pursue this question further and determine the degree to which this mechanism is conserved throughout the nervous system and whether it is limited to females.

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APPENDIX I

Impact of immersion oils and mounting media on the confocal imaging of dendritic spines

Rationale

Structural plasticity, such as changes in dendritic spine morphology and density, reflect changes in synaptic connectivity and circuitry. Procedural variables used in different methods for labeling dendritic spines have been quantitatively evaluated for their impact on the ability to resolve individual spines in confocal microscopic analyses. In contrast, there have been discussions, though no quantitative analyses, of the potential effects of choosing specific mounting media and immersion oils on dendritic spine resolution. Using medium spiny neurons from the rat striatum and nucleus accumbens, we seek to quantitatively determine the impact of these variables on resolving dendritic spines in 3D confocal analyses. It may be that choice of immersion oil and mounting media is critical for obtaining the best resolution and more accurate measures of dendritic spine densities.

Introduction

Dendritic spines are small structures that protrude from the dendritic shaft where they receive the majority of glutamatergic inputs for a neuron (Alvarez and Sabatini, 2007). They are highly dynamic structures (Bhatt et al., 2009), for which alterations in structure or density imply changes in neuroanatomical circuitry, neuronal function and consequently behavior. Given these properties, confocal-based dendritic spine analyses are common in the neuroscience literature, despite the challenges of resolving small spine structures with varying geometries that are located in close proximity to the dendritic shaft and other spines. Following confocal laser scanning microscopy, optical sections

through dendritic segments are reconstructed into representative 3D images. However, light scattering can obscure distinct structures, and limit the ability to distinguish between individual dendritic spines.

Recently, there has been greater attention toward systematically testing and quantitatively evaluating procedural variables used in preparing tissue for confocal 3D measurements of dendritic spines (Dumitriu et al., 2012; Staffend and Meisel, 2011a, 2011b). There has been additional focus on the imaging parameters necessary to achieve optimal resolution of dendritic spines (Dumitriu et al., 2011). In addition to optimizing imaging parameters, these authors suggested that experimenter-controlled variables such as proper selection of mounting medium and immersion oil may have important consequences for resolution (Dumitriu et al., 2011). Surprisingly, experiments that empirically test the impact of choice of immersion oil and mounting medium on resolution of small biological structures, such as dendritic spines are limited.

Throughout our experimentation over the last several years, we have examined how mounting media and immersion oils of different refractive indices affect the resolution of individual dendritic spines and consequently dendritic spine densities of dialkylcarbocyanine (DiI)-labeled striatal medium spiny neurons (MSNs). We have examined two separate choices for mounting media: glycerin, a versatile non-hardening compound widely used in fluorescent microscopy and Fluorglo, a recently developed hardening com- pound compatible with fluorescence. Likewise, we examined two distinct immersion oils: Type FF, with a refractive index that closely matches the mounting

media versus Type LDF, with a refractive index that matches the glass through which the sample is visualized.

Here we report that the choice of mounting medium has a small effect on optical resolution, and dependent on brain region, may influence the ability to resolve individual dendritic spines. In contrast, immersion oil has a larger impact on 3D resolution. Specifically, matching the immersion oil refraction index to the glass reliably enhanced the ability to visualize dendritic spines.

Materials and Methods

Point spread function analyses of microspheres

Preparation of FluoSpheres

Instructions for preparing sub-resolution fluorescent micro- spheres are detailed in (Cole et al., 2011). Briefly, number 1.5 coverglass (Zeiss) and slides (Brain Research Laboratories; Newton, MA) were washed in 70% ethanol and flamed to dry, after which 100nm red fluorescent microspheres (FluoSpheres carboxylate- modified microspheres 580/605; Invitrogen, Molecular Probes; Eugene, OR) diluted at 1:25,000 in dH2 O were sonicated for 20 min, vortexed and applied in 20 µl aliquots to the coverglass. FluoSpheres were allowed to dry onto the coverglass (~2h) and mounted to slides using 5% n-proply-gallate in glycerin (w/v) or Fluorglo mounting medium for lipophilic dyes (Spectra Services, Ontario, NY) with a 120 µm secure seal spacer (Electron Microscopy Sciences; Hatfield, PA).

Confocal imaging of FluoSpheres

A Leica TCS SPE confocal microscope (Leica, Mannheim, Germany) was used to acquire z-stacks through FluoSpheres using a 63× oil immersion objective with a 5.61 zoom factor. Lateral and axial sampling parameters were 61 nm and 130 nm, respectively, and were identical to those used to image our dendritic segments. All z-stacks were maintained at a xy pixel distribution of 512 × 512 and scanned at a frequency of 400 Hz. FluoSpheres were imaged using either Type FF immersion oil (refractive index: 1.47; Cargille, Cedar Grove, NJ) or Type LDF immersion oil (refractive index: 1.515; Cargille, Cedar Grove, NJ).

Point spread function analysis

FluoSphere z-stacks were opened in FIJI (National Institute of Health; Bethesda, MD) where a single FluoSphere was cropped and analyzed using the MetroloJ plug-in. MetroloJ generated a PSF report detailing the full width and half maximum (FWMH), the standard measurements for fluorescent objects, in the lateral and axial directions. PSF reports were generated for 4–5 FluoSpheres in each condition and the average was reported for FWHM in **Figure 14**.

Analyses of dendritic spine densities

Animals

Female Sprague Dawley rats 12 weeks of age (175–200 g) from Harlan labs (Indianapolis, IN) were housed in pairs, handled daily, and allowed to habituate to the laboratory for one week prior to experimentation. Animals were maintained on at 12 h light–dark cycle with lights on at 6:00 am. All animal procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory

Animals and were approved by the Animal Care and Use Committee at the University of Minnesota.

The animals were anesthetized using Beuthanasia-D (0.3ml i.p./animal, Schering, Union, NJ), injected with 0.25ml heparin into the left ventricle of the heart and intracardially perfused with 25 mM phosphate buffered saline (PBS, pH = 7.2) for 3 min at 25 ml/min followed by ice-cold 1.5% paraformaldehyde in 25 mM PBS for 20 min. Perfusion with 1.5% paraformaldehyde results in more complete and clearer visualization of DiI-labeled neurons when compared to perfusion with the standard 4% paraformaldehyde (Staffend and Meisel, 2011a,b). Brains were removed and coronally blocked to allow for penetration of the post-perfusion fixative (1.5% paraformaldehyde for 1 h) into the tissue blocks containing the striatum. Brains were sectioned at 300 μm in serial, coronal sections through the striatum using a Vibratome (Lancer Series 1000, St. Louis, MO). Sections were placed in wells containing 25 mM PBS until DiI labeling.

DiI labeling

Instructions for DiI labeling are described in detail by Staffend and Meisel (2011a). Briefly, microcarriers containing DiI-coated tungsten particles (2 mg lipophilic carbocyanine DiI (Molecular Probes, Carlsbad, CA) dissolved in 100 μl of dichloromethane mixed with 90mg of 1.3 μm tungsten particles (Biorad, Hercules, CA)) were made in Tefzel tubing (Biorad) pre-coated with freshly pre- pared 10–15 mg/ml polyvinylpyrrolidone (PVP, Sigma-Aldrich, St. Louis, MO) and cut into 1.3 mm segments. A Helios Gene Gun (Bio- Rad) with a modified barrel, 40 mm spacer and 70 μm nylon mesh filter was used to deliver the microcarriers to the lightly fixed brain

sections using helium gas at a pressure of 100 PSI. Prior to DiI delivery, PBS was removed from wells containing the brain sections. Immediately following DiI delivery, brain sections were re-submerged in PBS for 24 h in the dark at room temperature for diffusion of DiI. After 24 h, the PBS was removed and replaced with 4% paraformaldehyde in PBS for 1 h at room temperature. Sections were washed in PBS then mounted on Superfrost slides and coverslipped with 5% n-proplygallate (w/v) in glycerin or Fluorglo mounting media for lipophilic dyes.

Confocal imaging

A Leica TCS SPE confocal microscope (Leica, Mannheim, Germany) was used to acquire z-stacks of DiI labeled whole neurons and dendritic segments. Whole neurons were imaged using a Leica PLAN APO 20×, 0.7 NA air objective (11506166; Leica, Mannheim, Germany), a pixel distribution of 512×512, at a frequency of 400 Hz, with pinhole size set to 1 Airy. Each neuron was scanned at 1.0 µm increments in the z-axis. Leica LAS AF software was used to reconstruct z-stacks of whole neurons in order to trace the actual length of a dendrite from the soma. Target dendritic segments 70–200µm from the soma in medium spiny neurons of the striatum were imaged with a Leica PLAN APO 63×, 1.4 NA oil immersion objective (11506187; Leica, Mannheim, Germany), a pixel distribution of 512×512, at a frequency of 400Hz, with pinhole size set to 1 Airy and a zoom of 5.61. Z-stacks of dendritic segments were acquired with a lateral sampling of 61nm at 0.13 µm increments in the z-axis. By adjusting the laser power and photomultiplier, each dendrite was imaged in its full dynamic range where the saturation threshold was achieved at dendritic spine heads. Separate dendritic segments were

imaged using either Type FF immersion oil (refractive index: 1.47; Cargille, Cedar Grove, NJ) or Type LDF immersion oil (refractive index: 1.515; Cargille, Cedar Grove, NJ). For each brain region, 2–3 neurons and 3 dendritic segments per neuron were imaged to generate a total of 6 to 9 segments for each brain region from every animal. Depending on the experiment and treatment conditions, a final sample size of 3–8 animals was obtained for each brain region per treatment group (see figure legends for group sample sizes).

Quantitation

Confocal z-stacks of dendritic segments were first subjected to a 3D-deconvolution process using Autoquant X AutoDeblur Gold CF software (Media Cybernetics, Bethesda, MD; version 2.2 to evaluate the effect of mounting medium on dendritic spines and version 3.0 to evaluate the effect of immersion oil on dendritic spines). Deconvoluted images were then reconstructed in 3D using the Imaris software (Bitplane Inc., St. Paul, MN; version 7.1 in experiment 1 and version 7.6 in experiment 2), with dendritic shaft and spines traced manually in the xy plane using the Filament tool and the Autodepth function to generate dendritic spine density normalized to 10 μm of dendritic length. A contrast threshold of 0.4–0.7 enabled accurate 3D reconstruction of dendritic shaft and spines.

Data analysis

For each animal, 2–3 neurons were analyzed in each brain region, though each animal was used as the unit of statistical analysis. Independent sample t-tests were used to evaluate statistical the effects of mounting medium and immersion oil for each brain

region using Graph Pad Prism 5 software (La Jolla, CA). For all statistical tests, results were considered to be statistically significant if $p < 0.05$.

Results

Quantitative and qualitative analysis of point spread functions from FluoSpheres mounted with two different mediums and imaged with two different immersion oils

Initially, we took a materials approach using fluorescent beads to evaluate how mounting media and immersion oils influenced imaging of a point source of light. The 3D distortion pattern, i.e., point spread function, generated by confocal microscopy determines the resolution limit of an optical system (Pawley, 2010). The point spread function can be measured by imaging an object smaller than the resolution limit, such as a 100nm fluorescent microspheres (FluoSphere) used in our study. To determine the point spread function generated by our imaging parameters of dendritic segments, we followed previously published protocols (Cole et al., 2011). First, we prepared FluoSphere samples mounted in either glycerin-based mounting medium (5%-n-propylgallate in glycerin) or Fluorglo mounting medium. Next, we imaged the samples under dendritic segment imaging parameters using either Type FF or Type LDF immersion oil. **Figure 14** shows qualitative and quantitative measures of point spread function in lateral and axial planes for glycerin-based mounting medium with Type FF immersion oil (left), Fluorglo mounting medium with Type FF immersion oil (middle) and Fluorglo mounting medium with Type LDF immersion oil (right). Quantitative measures of point spread function from these images were reported in the standard measurement for size of object in

fluorescent images, full width at half maximum (FWHM). In the first analysis, the lateral and axial FWHM of FluoSpheres imaged with Type FF immersion oil was found to be numerically higher in the glycerin-based mounting medium compared to the Fluorglo mounting medium. In the second analysis, FluoSpheres mounted in Fluorglo had lower lateral and axial FWHM values when imaged with Type LDF immersion oil compared with FluoSpheres imaged with Type FF immersion oil.

Figure 14

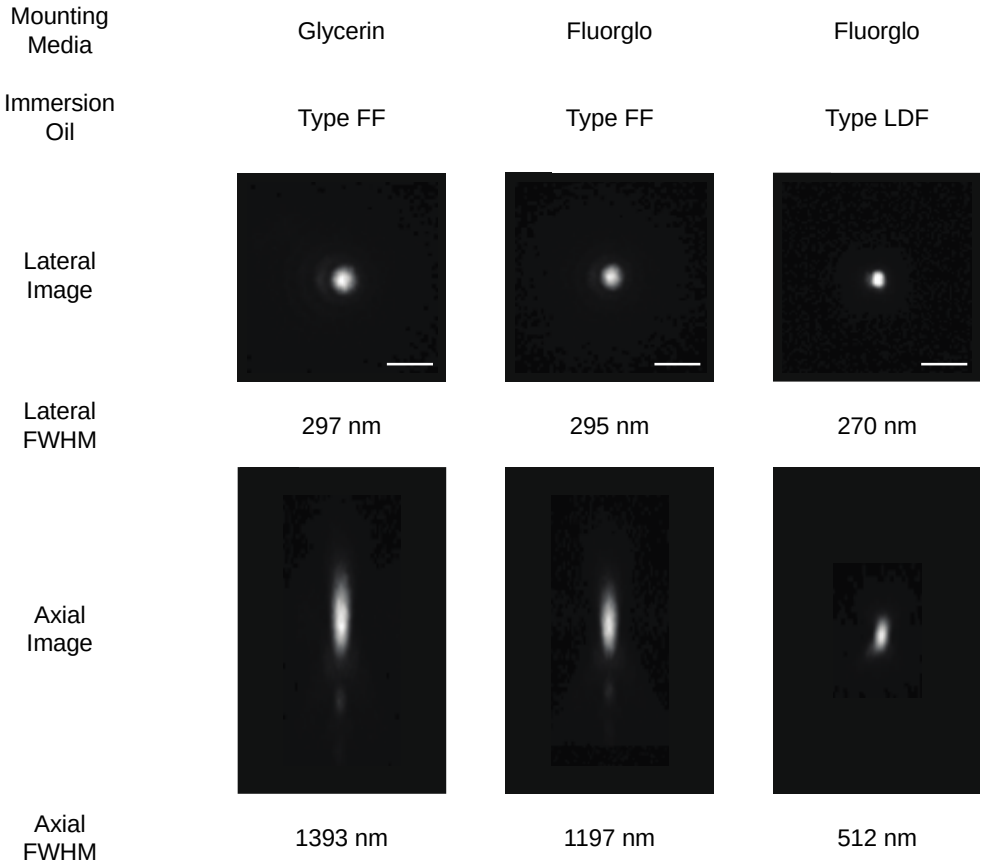

Figure 14. Mounting medium has an effect on axial, but not lateral resolution of imaged FluoSpheres, while immersion oil affects both lateral and axial resolution of imaged FluoSpheres. Whether FluoSpheres imaged with Type FF immersion oil, were mounted with glycerin or Fluorglo had no effect on lateral FWHM. Contrastingly, FluoSpheres imaged with Type FF immersion oil exhibited a slight decrease in axial FWHM when mounted with Fluorglo compared

with glycerin. FluoSpheres mounted with Fluorglo that were imaged with the LDF immersion oil showed a small decrease in lateral FWHM and a large decrease in axial FWHM compared to FluoSpheres imaged with Type FF immersion oil. The full width at half maximum (FWHM) or standard measurement for fluorescent object value is the average obtained from 4 to 5 individual FluoSpheres. Scale bar is 1 μm.

Qualitative point spread function analyses of dendritic segments from striatal medium spiny neurons mounted with two different mediums and imaged with two different immersion oils

Next, we sought to determine whether the effects of mounting medium and immersion oil that we observed from imaging FluoSpheres were applicable to imaging dendritic segments. **Figure 15** shows a visual depiction of point spread function in lateral and axial planes for the same three conditions outlined in **Figure 14**. Consistent with the data obtained from the FluorSpheres, changing the mounting media from 5%-n-propylgallate in glycerin to Fluorglo reduced the light scatter in the z-plane. Furthermore, use of the Type LDF immersion oil further improved the quality of the axial image.

Figure 15

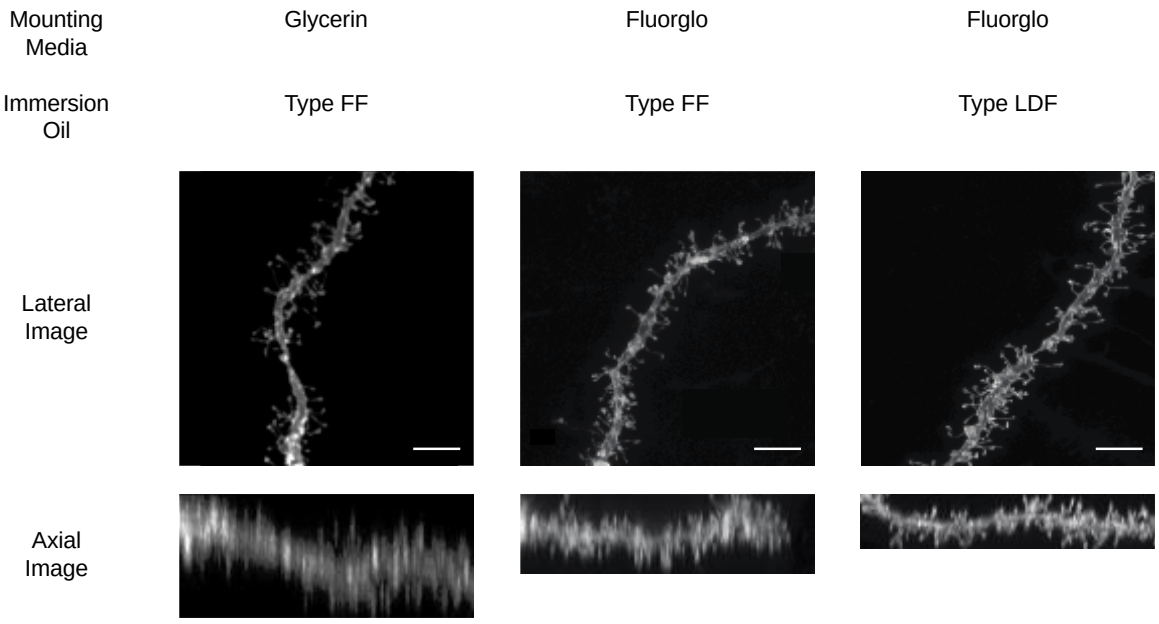

Figure 15. Mounting medium and immersion oil each have a small effect on lateral resolution and a large effect on axial resolution of imaged striatal medium spiny neuron dendritic segments. When dendritic segments were imaged with Type FF immersion oil, sections mounted with Fluorglo medium showed a slight improvement in lateral resolution and a more dramatic improvement axial resolution compared to sections mounted with glycerin. Similarly, when sections were mounted with Fluorglo, dendritic segments imaged with Type LDF immersion oil showed a slight improvement in lateral resolution and a more dramatic improvement axial resolution compared to dendritic segments imaged with Type FF immersion oil. Scale bar is 50 µm.

Mounting medium under some conditions affects the ability to accurately quantify spine densities

While the information obtained from both the FluoSpheres and the axial images of dendritic spines indicate improvement of the 3D reconstruction using Fluorglo mounting medium, we wanted to empirically test whether this would translate into an increased ability to resolve dendritic spines. To do so, we compared dendritic spine

densities of medium spiny neurons (MSNs) imaged from striatal sections that were mounted in either 5%-n-propylgallate in glycerin or in Fluorglo medium. We found that the Fluorglo media increased the ability to visualize dendritic spines from MSNs of the caudate-putamen (CPu) (**Figure 16**; independent sample t-test; $t(13) = 2.28$, $p < 0.05$). In contrast, altering the mounting media did not significantly affect the quantification analyses of the nucleus accumbens core (NAcC) and nucleus accumbens shell (NAcSh).

Figure 16

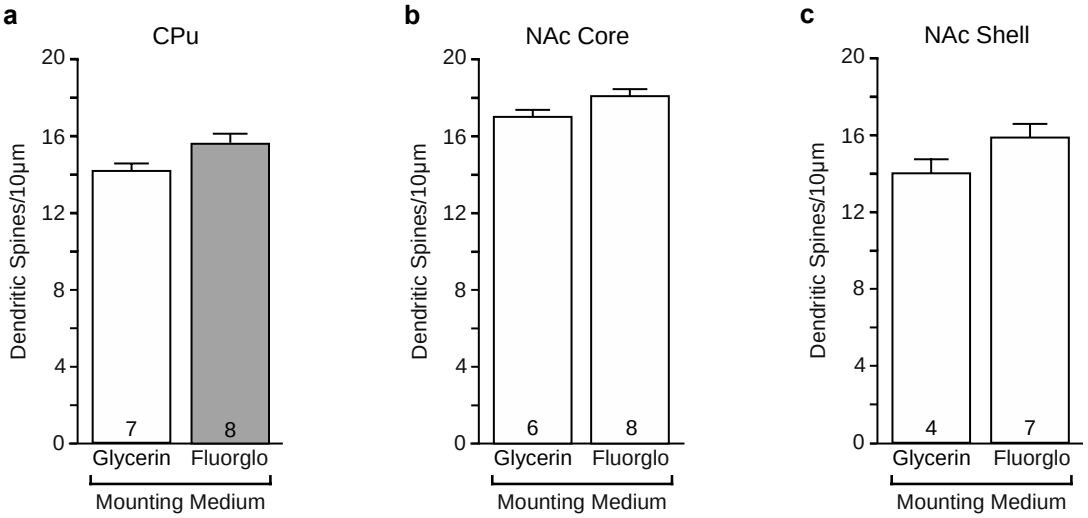

Figure 16. Mounting medium affects striatal dendritic spine densities obtained from DiI-labeled medium spiny neurons in the caudate putamen (CPu), but not the nucleus accumbens core (NAcC) or nucleus accumbens shell (NAcSh).

a Compared with sections mounted with glycerin-based medium, sections mounted with Fluorglo mounting medium showed increased dendritic spine density of medium spiny neurons in the CPu. b Dendritic spine densities of medium spiny neurons of the NAcC were unaffected by mounting medium selection. c Dendritic spine densities of medium spiny neurons of the NAcSh were unaffected by mounting medium selection. The number inside of each bar represents the number of animals per treatment condition. Gray bars are significantly different than white bars $p < 0.05$.

Immersion oil has a consistent effect on dendritic spine densities obtained from medium spiny neurons in three striatal regions

In the last experiment, we determined whether mounting medium affects the ability to resolve individual dendritic spines within these three brain regions. Dendritic spine densities from striatal and nucleus accumbens sections were mounted with Fluorglo medium and imaged using either Type FF or Type LDF immersion oil. The ability to resolve individual dendritic spines in all three brain regions was affected by the immersion oil. Specifically, LDF immersion oil increased the ability to visualize individual spines (**Figure 17**, independent sample t-test; CPu: $t(6) = 2.52$, $p < 0.05$; NAcC: $t(6) = 3.76$, $p < 0.05$; NAcSh: $t(6) = 4.27$, $p < 0.05$).

Figure 17

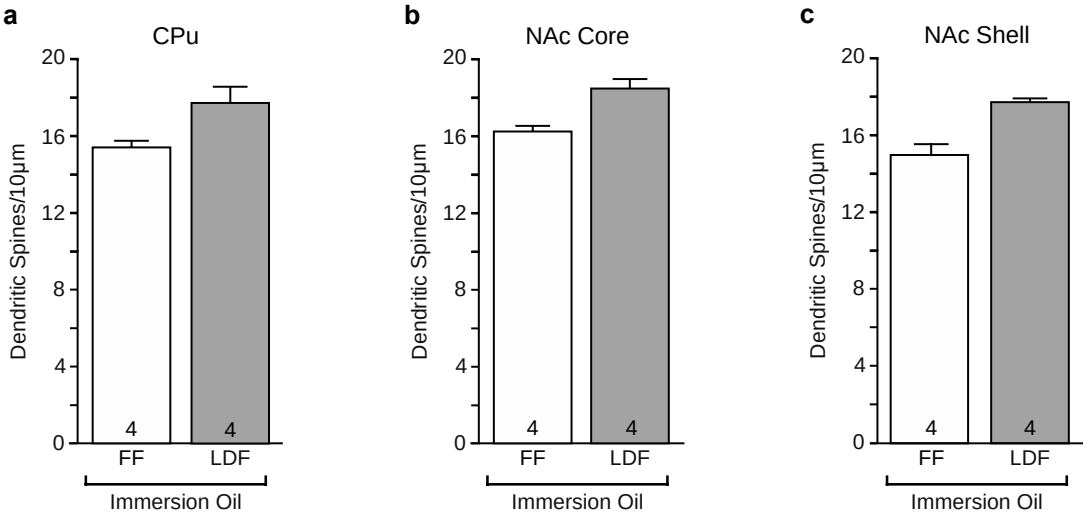

Figure 17. Immersion oil influences striatal dendritic spine densities obtained from DiI-labeled medium spiny neurons in the caudate putament (CPu), nucleus accumbens core (NAcC) and nucleus accumbens shell (NAcSh).

a Compared with dendritic segments of medium spiny neurons imaged with Type FF immersion oil, dendritic segments medium spiny neurons imaged with Type LDF immersion oil showed increased dendritic spine density in the CPu. b Compared with dendritic segments of medium

spiny neurons imaged with Type FF immersion oil, dendritic segments medium spiny neurons imaged with Type LDF immersion oil showed increased dendritic spine density in the NAcC. c Compared with dendritic segments of medium spiny neurons imaged with Type FF immersion oil, dendritic segments medium spiny neurons imaged with Type LDF immersion oil showed increased dendritic spine density in the NAcSh. The number inside of each bar represents the number of animals per treatment condition. Gray bars are significantly different than white bars $p < 0.05$.

Discussion

The resolution of 3D images obtained from confocal microscopy not only depends on the ability to detect a light source, but also the ability to resolve different sources of light. Thus, selection of mounting media and immersion oil would seem to be of great importance, particularly when imaging small, spatially constricted structures such as dendritic spines. While the appropriate optimization of these variables may seem apparent, these variables are often overlooked (and rarely reported) when quantitative analyses of dendritic spines are published. Alternatively, with a lack of quantitative data to validate the impact of mounting media and immersion oil on the ability to resolve distinct biological structures, altering these variables could be considered to reflect theoretical limitations of an imaging system, and not germane from a practical standpoint. Hence, our goal was to understand the effect of these two variables using confocal microscopy in a biologically relevant context.

First, we compared two frequently utilized mounting medias. In biological preparations, glycerin-based medias are quite adaptable – allowing various optics and chromophores to be utilized. This was compared to Fluorglo, an example of a fluorescent-compatible hardening compound. The hardening of the media helps limit

movement of the sample when imaging, but this compound is less versatile than glycerin-based medias. Data from both the FluoSpheres and dendritic spines indicate Fluorglo slightly improved optic performance. In one brain region (i.e. caudate putamen) this translated into an increased ability to resolve dendritic spines, whereas in both the core and shell regions of the nucleus accumbens, our quantitative analyses were unaffected. Our impression is that the improved resolution with Fluorglo may be a function of the hardening properties of this mounting medium, as media that hardens as it dries minimizes the distortion of the z-plane caused by the weight of the coverglass and pressure of the objective. Alter- natively, the refractive index of the glycerin-based mounting media is 1.47. This compares to Fluorglo, with a refractive index of 1.42. This difference may also contribute to the disparity in resolution. Nevertheless, it would seem use of a hardening compound would be the preferred mounting media in these types of studies, although glycerin-based medias offer a reasonable alternative if required.

In contrast, selection of the appropriate immersion oil seems essential. There has been the question regarding whether the refractive index of the immersion oil should match the tissue and mounting media, or the glass through which the sample is imaged. Both Type FF and Type LDF are commonly used immersion oils for these reasons. Specifically, in our setup, the refractive index of the Type FF immersion oil (RI: 1.47) matched that of the tis- sue and mounting medium, whereas the refractive index of the Type LDF (RI: 1.515) immersion oil matched that of the glass. From our quantitative data, there is marked improvement of the optical image when using an immersion oil (Type LDF) matching the glass through which the sample is visualized. In all three

striatal subregions, dendritic spine densities from sections imaged with Type LDF immersion oil were greater than those imaged with Type FF immersion oil. Differences in the point spread functions in the lateral plane and more dramatically in the axial plane of the FluorSpheres imaged with Type LDF immersion oil support our measurements in the dendritic segments. Thus we attribute the increased spine density observed with Type LDF immersion oil to an increase in ability to resolve individual spines especially in the axial plane. Our data emphasize that to obtain the best optical resolution, the refractive index of immersion oil should match that of the glass through which the sample is visualized rather than the refractive index of the medium that a sample is mounted within. Consequently, selecting immersion oil based on refractive index of the microscopic objective is crucial in reliably resolving individual dendritic spines.

Our data highlight the importance of mounting medium and immersion oil selection in confocal imaging. Yet, the choice of mounting medium or immersion oil is commonly omitted in experimental methods. This is key because differences in dendritic spine density values across laboratories are often greater than a significant treatment outcome within a single laboratory. Dumitriu et al. (2012) noted that divergent data trends have been reported depending on whether dendritic spine analyses were conducted in 2D or 3D. Taken together with our results demonstrating that mounting medium and immersion oil can affect the ability to resolve individual spines in both the lateral and axial planes, immersion oil is a crucial variable, especially for 3D confocal analysis. As changes in dendritic spine density reflect changes in neuroanatomical circuitry, the inconsistency in data trends originating from imaging and analytical methods have lead to

ambiguous conclusions regarding relationships among synaptic connectivity, neuronal function and behavior. Generating more accurate 3D representations with confocal imaging can improve the ability clarify these neural structure/function relationships.

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