The Role of Ventral Tegmental Area Estrogen Receptors in Neuronal Responses to Dopamine and Ethanol

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THESIS

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CONTRIBUTION OF AUTHORS

Portions of the published work “Estradiol increases the sensitivity of ventral tegmental area dopamine neurons to dopamine and ethanol” (*Plos One, 2017*) have been included in this dissertation. The experiments/figures that I have included in this dissertation are (1) the representative images of vaginal smears from mice in estrus and diestrus, (2) the increased sensitivity of VTA DA neurons to inhibition by DA during diestrus, (3) the enhanced inhibition by quinpirole in VTA DA neurons in diestrus mice, (4) the increased sensitivity of VTA DA neurons to ethanol excitation during diestrus, (5) the E2 enhances DA inhibition and ethanol excitation of VTA DA neurons, (6) the GABA antagonists do not alter the ethanol-stimulated increase in firing of DAneurons in OVX mice treated with E2, and (7) the ERs in the VTA acutely regulate the sensitivity of DA neurons to ethanol but not DA. I conducted the experiments and data analysis from (1), (3), (5), (6), and (7) in their entirety, under the guidance of my advisors, Mark Brodie and Amy Lasek, the senior authors of the paper. Dr. Chang You contributed to half of the data collection from (2) and (4). The other contributing author, Dr. Rosalba Satta, trained me and assisted in the initial ovariectomy surgeries and vaginal smears. Dr. Satta also performed an experiment that was published in the original manuscript that I did not include in this thesis (Supplemental Figure 1 of the *Plos One* paper).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE **PAGE**

LIST OF ABBREVIATIONS

ALK	Anaplastic lymphoma kinase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BEC	Blood ethanol concentration
cAMP	Cyclic adenosine monophosphate
CLi	Caudal linear
COMT	Catechol-O-methyl transferase
CPP	Conditioned place preference
CREB	cAMP response element binding protein
D1R	Dopamine D1 receptor
D2L	Dopamine D2 receptor long isoform
D2R	Dopamine D2 receptor
D2S	Dopamine D2 receptor short isoform
D3R	Dopamine D3 receptor
DA	Dopamine
DAergic	Dopaminergic
DAT	Dopamine transporter
DBD	DNA-binding domain
DIR	Dopamine inhibition reversal
E2	17β-estradiol
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta

ERE	Estrogen response elements
FSH	Follicle stimulating hormone
GABA	Gamma-aminobutyric acid
GIRK	G protein-regulated inwardly rectifying potassium
GnRH	Gonadotropin releasing hormone
GPER	G-protein coupled estrogen receptor
HCN	Hyperpolarization-activated cyclic nucleotide–gated
HVA	Homovanillic acid
I_h	Hyperpolarization-activated inward current
IF	Interfasicular
IP3	1,4,5-triphosphate
L-DOPA	L-dihydroxyphenylalanine
LH	Lutenizing hormone
MAPK	mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptor
NMDA	N-methyl-D-aspartate
NTD	N-terminal domain
OVX	Ovariectomy
PBP	Parabrachial pigmented area
PIP2	Phosphatidylinositol 4,5-biphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
PN	Paranigral nucleus

RLi	Rostral linear nucleus of raphe
RMTG	Rostromedial tegmental nucleus
TH	Tyrosine hydroxylase
THIK	Tandem-pore halothane-inhibited potassium
VTA	Ventral tegmental area

SUMMARY

There are considerable gender differences throughout all of the stages of drug addiction, including differences in amount of drug use, age of initiation, reasons for abuse, development of dependence, severity of health problems, and comorbidities (Becker 2016; Becker and Chartoff 2019; Becker and Hu 2008). Women escalate from initial alcohol use to addiction more rapidly compared to men (Moran-Santa Maria, Flanagan, and Brady 2014; Bobzean, DeNobrega, and Perrotti 2014) and sex differences in the midbrain dopaminergic (DAergic) system may partially drive women's vulnerability to drug abuse and relapse. Understanding the biological causes of these gender differences has been limited as the majority of research on drug abuse and the ventral tegmental area (VTA), a brain region responsible for the rewarding and reinforcing effects of drugs of abuse (Koob and Volkow 2010), has been performed with male subjects.

Sex differences in ethanol reward and consumption has been well established in rodents. Female mice drink more than males (Becker and Koob 2016; Hwa et al. 2011; Jury et al. 2017; Middaugh et al. 1999; Priddy et al. 2017; Satta, Hilderbrand, and Lasek 2018) and are more sensitive to the rewarding effects of ethanol (Torres et al. 2014). Many studies in rodents have demonstrated enhancing effects of 17β-estradiol (E2), the main form of estrogen in adult pre-menopausal females, on drinking behaviors (Ford, Eldridge, and Samson 2004; Rajasingh et al. 2007; Reid, Hubbell, and Reid 2003; Ford, Eldridge, and Samson 2002a; Marinelli, Quirion, and Gianoulakis 2003; Quirarte et al. 2007; Satta, Hilderbrand, and Lasek 2018), which may partially mediate sex differences in drinking behaviors.

E2 has profound effects on midbrain dopamine (DA) systems including the VTA. E2 actions on DAergic pathways regulates reproductive behavior (Foreman and Hall 1987; Balthazart, Baillien, and Ball 2002) and drug abuse (Carroll et al. 2004). E2 effects on drinking behaviors

may be partially due to E2 acting on VTA DAergic neurons because of the critical role of the VTA in behavioral responses to ethanol. The mechanisms of action of E2 on VTA neurons with regard to ethanol were not known prior to the studies conducted in this dissertation project. Therefore, the purpose of this dissertation was to examine the role of E2 in modulating VTA physiology in female mice. I determined the effects of E2 on the inhibition by DA and excitation by ethanol on the VTA DAergic neurons in female mice. I began the work of elucidating the mechanisms through which E2 acts in the VTA by determining which estrogen receptors (ERs) and their related actions are responsible for E2 effects on responses to DA and ethanol.

My primary hypothesis is that E2 regulates excitation by ethanol and inhibition by DA of VTA DA neurons in female mice through activation of ERs. The aims of this dissertation are therefore to (1) characterize the effects of high E2 states in the responses of VTA DA neurons to ethanol and DA, (2) determine which ERs mediate E2 effects on the sensitivity of VTA DA neurons to ethanol and DA, (3) investigate possible downstream pathways which may mediate E2 effects on the sensitivity of VTA DA neurons to ethanol and DA, and (4) determine the effects of E2 on the desensitization of dopamine D2 receptor (D2R) in the VTA of female mice.

I have found that ethanol excitation and DA inhibition of VTA DAergic neurons is enhanced in high E2 states. E2-mediated enhancement of ethanol excitation is through activation of ERα and metabotropic glutamate receptor 1 (mGluR1), and this may be through rapid signaling mechanisms. E2-mediated enhancement of DA inhibition, on the other hand, is through activation of both ERα and ERβ and does not appear to be acutely regulated in VTA neurons. E2 also enhances the desensitization of D2R in the VTA.

This body of work is the first to comprehensively explore the role of ERs in regulating the physiology of VTA DAergic neurons, particularly how ERs regulate the sensitivity of VTA

DAergic neurons to DA and ethanol. These changes in VTA functioning may have significant behavioral effects to drive binge drinking and ethanol reward in female and have implications for biased incidence of DA-dependent psychiatric diseases.

1. INTRODUCITON

1.1 Addiction

According to the 2018 World Drug Report, approximately 31 million people suffer from substance abuse or dependence (United Nations Office on Drugs and Crime.). As a result, this places enormous economic and sociological consequences on the health systems and communities burdened by drug abuse. In 2010, alcohol abuse cost the United States $223.5 billion (Sacks et al. 2015). Drug addiction is a disorder in which there is a persistent urge to acquire and use a drug to an uncontrollable degree such that one experiences anxious, irritable, and uneasy emotional affects in the absence of the drug (Koob and Le Moal 1997). Addiction can be conceptualized as a cycle with three stages which interact with each other as a patient spirals from drug use to addiction: "binge/intoxication", "withdrawal/negative affect", and "preoccupation/anticipation" (Koob and Volkow 2010). Positive reinforcement, which is the process of a positive stimulus or state increasing behavior resulting in drug seeking and drug taking, is associated with the "binge/intoxication" phase while negative reinforcement, which is the process of removing the aversive withdrawal state to reinforce drug taking, plays a role in the "withdrawal/negative affect" phase (Edwards 2016). The conditioning of both positive and negative reinforcement contributes to the preoccupation/anticipation phase, which is characterized by craving and seeking the drug. This leads to the next binge/intoxication stage as a patient spirals to the pathophysiological state of addiction (Wise and Koob 2014).

Each stage of the addiction cycle is associated with distinct adaptations in neurocircuitry. Corticotropin-releasing factor, norepinephrine, and dynorphin signaling in the extended amygdala are thought to play a central role during the "withdrawal/negative affect" stage and glutamatergic signaling in brain regions such as the prefrontal cortex and the basolateral

amygdala is thought to play a central role in the "preoccupation/anticipation" stage of addiction (Koob 2008; Koob and Kreek 2007; Koob and Volkow 2010). DAergic systems in the brain are critically involved in the reinforcement of drugs of abuse during the "binge/intoxication stage" (Koob and Volkow 2010).

The mesocorticolimbic DAergic system, in particular, plays a central role in the rewarding and aversive properties of drugs (Wise 1987; Volman et al. 2013). This system includes VTA DAergic neurons and their projections to other brain areas such as the nucleus accumbens, amygdala, and prefrontal cortex (Oades and Halliday 1987; Koob 2003; Swanson 1982). These projection sites are respectively involved in the reward/reinforcement, emotional response, and craving/drug-seeking of drug addiction (Koob 2003). The physiological function of the VTA is to assess the salience of incoming stimuli for the organism. VTA DAergic neurons are activated and release DA to their projection sites in response to stimuli that are rewarding or novel (Berridge 2007; Koob and Volkow 2010). The hijacking of this system during drug addiction is thought to be responsible for the rewarding and reinforcing effects of drugs of abuse (Koob and Volkow 2010; Jones and Bonci 2005). DAergic neurotransmission is also modulated during withdrawal from drugs such as ethanol and nicotine with a decrease in activity of VTA DAergic neurons, as demonstrated by a decrease in the number of spontaneously active neurons or basal firing rate (Liu and Jin 2004; Shen 2003; Diana et al. 1992), and an associated reduction in DA release during ethanol withdrawal which may also contribute to the negative affective state and the craving and relapse that may occur during abstinence (Hunt and Majchrowicz 1974; Darden and Hunt 1977; Diana et al. 1993; Weiss et al. 1996).

1.1.1 Sex differences in addiction

There are considerable gender differences in addiction throughout all the stages of drug addiction, including differences in amount of drug use, age of initiation, reasons for abuse, development of dependence, severity of health problems, and comorbidities (Becker 2016; Becker and Chartoff 2019; Becker and Hu 2008). 'Telescoping', which is a term for the escalation from initial drug use to addiction, is much more rapid in women compared to men (Moran-Santa Maria, Flanagan, and Brady 2014; Bobzean, DeNobrega, and Perrotti 2014). The severity of negative withdrawal symptoms differs between the sexes depending on the drug of abuse. Women report more severe withdrawal symptoms during the abstinence of cocaine, amphetamine, and nicotine use (Kosten et al. 1993; Hudson and Stamp 2011; Hogle and Curtin 2006). However, men report more severe withdrawal symptoms from alcohol (Devaud, Alele, and Ritu 2003).

Although more men drink alcohol than women, recent investigations have concluded that the difference in the incidence of alcohol abuse is narrowing between genders in the United States (White et al. 2015; Grant et al. 2017; Cheng, Cantave, and Anthony 2016). Even though women have in the past drank less alcohol for shorter periods of time, women are more susceptible to devastating health effects associated with alcohol abuse such as liver damage, cardiovascular disease, and cancer (Eagon 2010; Szabo 2018; Agabio et al. 2017; Rehm et al. 2010; Agabio et al. 2016). Women who binge-drink, which is defined as consuming enough alcohol to reach a blood ethanol concentration (BEC) of at least 80 mg/dL (NIAAA Council, 2014), reported having more days described as physically and mentally poor (Wen et al. 2012). Similar to other drugs of abuse such as cocaine, women also exhibit a greater escalation of alcohol use than men and are more likely to relapse in response to stress and anxiety (Becker and

Koob 2016). Understanding of these gender differences has been limited due to the majority of research having been performed on males.

There are species-specific sex differences in alcohol consumption. In nonhuman primates, males drink more than females (Vivian et al. 2001). Sex differences in ethanol consumption have also been well established in rodents. In general, female rodents drink more than males and reach higher BECs compared to males across various drinking paradigms (Becker and Koob 2016; Hwa et al. 2011; Jury et al. 2017; Middaugh et al. 1999; Priddy et al. 2017; Satta, Hilderbrand, and Lasek 2018). However, the increase in drinking observed in female rodents only occurs in adults, and male rats drink more during a brief early postpubertal period suggesting that gonadal hormones may drive sex differences in drinking (Lancaster et al. 1996). Female mice drink more than males during the drinking in the dark (DID) test, which is a model for binge-like drinking in which mice are given limited access to ethanol for 2 to 4 hours during their dark cycle; in this model, females achieved higher BECs than males (Satta, Hilderbrand, and Lasek 2018).

Female rodents are also more sensitive to the rewarding effects of ethanol as measured by the conditioned place preference (CPP) test (Torres et al. 2014). CPP is a model for the rewarding effects of drugs such as ethanol (Prus, James, and Rosecrans 2009). During the CPP protocol, animals are injected with a drug in a specific context, while a neutral substance (such as saline) is injected in a different context, during the conditioning phase. After conditioning, the animal is allowed to move freely between the two distinct contexts in the absence of the drug. Preference for the drug is measured by the time the animal spends in the environment associated with the drug (Huston et al. 2013). Female rats exhibit CPP at doses of ethanol that do not produce CPP in males (Torres et al. 2014), however another group found no sex differences in

ethanol CPP in mice (Cunningham and Shields 2018). Because the VTA plays a critical role in the acquisition of ethanol CPP (Bechtholt and Cunningham 2005), sex differences in VTA physiology may be driving the sex differences in ethanol reward and consumption.

1.2 Ventral tegmental area

The VTA plays an important role in the rewarding and reinforcing effects of drugs of abuse and natural rewards such as food (Ranaldi 2014; Lammel, Lim, and Malenka 2014). The VTA can be subdivided into subregions or nuclei, including paranigral nucleus (PN), parabrachial pigmented area (PBP), caudal linear (CLi), interfasicular (IF) nuclei, and the rostral linear nucleus of raphe (RLi); however, the classification and delineation of VTA subregions varies between different studies (Swanson 1982; Oades and Halliday 1987; McRitchie, Hardman, and Halliday 1996; Ikemoto 2007). These VTA subregions differ in their cellular composition and projections (Ikemoto 2007). The VTA is a heterogeneous structure composed of DAergic, gamma-aminobutyric acid-producing (GABAergic), and glutamatergic neurons. The most abundant type of neuronal cell in the VTA is the DAergic neuron that project to many other brain regions. The regulation of VTA DA neuron firing is summarized in Figure 1. The regulation of DAergic neuronal activity in the VTA is regulated, in part, by inhibitory GABAergic interneurons in the VTA. In addition to the projecting DAergic neurons, the VTA has projecting GABAergic (Van Bockstaele and Pickel 1995), glutamatergic (Yamaguchi, Sheen, and Morales 2007; Yamaguchi et al. 2011; Kawano et al. 2006), and combination neurons that co-release multiple neurotransmitters (Zhang et al. 2015; Root et al. 2014; Berrios et al. 2016). The PN and PBP are highly rich in DA neurons, as measured by tyrosine hydroxylase (TH) expression (Ikemoto 2007).

VTA DAergic neurons can be identified based on anatomical location, molecular, and electrophysiological properties that distinguish them from the other neuronal cell types residing in the VTA. However, because of the heterogeneity in the expression of these characteristics throughout the DAergic neuronal populations in the VTA, using a limited set of criteria to identify neuronal populations may result in the mislabeling of neurons. The biphasic action potentials of VTA neurons are broad with a duration of >2.2 ms (Grace and Bunney 1983). VTA DAergic neurons are also characterized by a hyperpolarization-activated inward current (I_h), however some neurons that have I_h are non-DAergic (Zhang, Placzek, and Dani 2010). Administering DA D2 receptor (D2R) agonists inhibits the firing of VTA DAergic neurons (Chiodo et al. 1984; Lammel et al. 2008), however, some non-DAergic neurons in the VTA are also inhibited by D2R agonists (Margolis et al. 2006). A distinguishing feature of VTA DAergic neurons is inhibition by baclofen, a GABA-B receptor agonist. Baclofen inhibits VTA DAergic neurons but not GABA neurons in the VTA (Margolis et al. 2012).

VTA DAergic neurons have spontaneously occurring action potentials with slow firing rates of < 10 Hz as well as burst firing which are bursts of a series of spikes (2-10) that have decreasing amplitudes and increasing durations. Burst firing is associated with increases in the basal firing rate of DA neurons (Grace and Bunney 1984, 1980). In contrast to the tonic and burst firing patterns of DAergic neurons *in vivo*, DAergic neurons *in vitro* have 'pacemaker'-like firing, (Grace and Bunney 1984; Hyland et al. 2002; Overton and Clark 1997). *In vitro* measurements of VTA DAergic neurons from brain slice preparations may lack burst firing because of severing the glutamatergic afferents from the laterodorsal tegmentum which may be responsible for burst firing during slice preparation (Lodge and Grace 2006). Appetitive and rewarding stimuli will switch the tonic firing of DAergic neurons to transient bursts in certain

neuronal populations of the VTA (Grenhoff, Aston-Jones, and Svensson 1986; Schultz, Apicella, and Ljungberg 1993; Romo and Schultz 1990). Burst firing induces a large release of DA in target regions (Gonon 1988; Chergui, Suaud-Chagny, and Gonon 1994), and this is thought to mediate the rewarding and reinforcing effects of drugs of abuse (Schultz 2002; Phillips et al. 2003).

Acute treatment with ethanol in rodent brains dose-dependently enhances the basal firing rate of VTA DAergic neurons both *in vitro* (Brodie, Shefner, and Dunwiddie 1990) and *in vivo* (Gessa et al. 1985). However, while ethanol increases the basal firing of VTA DAergic neurons in the posterior VTA, DAergic neurons in the anterior VTA are inhibited (Guan et al. 2012). Female rats will self-administer ethanol directly into the posterior VTA, but not the anterior VTA (Rodd-Henricks et al. 2000). These studies indicate that the VTA is functionally heterogeneous, and the posterior VTA may primarily be driving the reinforcing and rewarding effects of alcohol.

The exact mechanism by which ethanol excites VTA DAergic neurons is unclear; however, ethanol may be directly altering a variety of ionic currents or synaptic activity. Ethanol can still excite dissociated VTA neurons isolated from synaptic inputs (Brodie, Pesold, and Appel 1999), indicating that ethanol directly acts on VTA DAergic neurons. A very promising potential target for the actions of ethanol on VTA DAergic neurons are the tandem-pore halothane-inhibited potassium (THIK) channels (You et al. 2019). A reduction of the m-current (I_M) (Koyama, Brodie, and Appel 2007) and an increase in I_h (Brodie and Appel 1998; Okamoto, Harnett, and Morikawa 2006) may also contribute to ethanol excitation of VTA DA neurons. Ethanol may also act on G protein-regulated inwardly rectifying potassium (GIRK) channels (Herman et al. 2015). GIRK mediates the inhibition by D2R and GABA-B receptors (Labouebe

et al. 2007; Lacey, Mercuri, and North 1987). Ethanol can bind to and directly activate GIRK channels, which would lead to an inhibitory effect on neurons (Lewohl et al. 1999; Kobayashi et al. 1999; Aryal et al. 2009). The balance between excitatory and inhibitory effects of ethanol may play an important role in regulating the aversive and rewarding effects of ethanol and the adaptations that occur as acute ethanol exposure transitions to chronic exposure (Pignatelli and Bonci 2015).

Ethanol can also modulate the synaptic input onto the VTA DAergic neurons. The VTA receives glutamatergic inputs from various brain regions including the prelimbic cortex, lateral hypothalamus, periaqueductal and central gray, and the dorsal raphe (Geisler, Derst, Veh, Zahm, 2007). Ethanol can increase the glutamatergic excitatory transmission onto VTA DAergic neurons by indirectly activating presynaptic dopamine D1 receptors (D1R) (Xiao et al. 2009). While ethanol generally increases the firing of VTA DAergic neurons, the activity of GABAergic neurons is inhibited (Xiao et al. 2007; Gallegos et al. 1999; Stobbs et al. 2004). Ethanol can decrease the release of GABA from neurons in the VTA or GABAergic inputs from the rostromedial tegmental nucleus (RMTG) and nucleus accumbens, and this may then disinhibit VTA DAergic neurons (Gallegos et al. 1999). Interestingly, GABAergic input on DAergic neurons is differentially regulated in the anterior and posterior VTA. Ethanol inhibits GABA release in the posterior VTA and increases GABA release in the anterior VTA. This is consistent with the opposite effects of ethanol on DAergic neurons in the posterior and anterior VTA (Guan et al. 2012). In addition to GABA and glutamate, many neurotransmitters such as glycine (Ye et al. 2001; Jiang and Ye 2003; Tao and Ye 2002), serotonin (Brodie, Trifunovic, and Shefner 1995; Trifunovic and Brodie 1996; Theile et al. 2009), acetylcholine (Blomqvist et al. 1993; Tizabi et al. 2002), opioids (Margolis, Fields, et al. 2008), and cannabinoids (Perra et

al. 2005) have been suggested to be involved in the actions of ethanol in the VTA. However, note that ethanol still activates dissociated VTA DAergic neurons (Brodie, Pesold, and Appel 1999), and the effects of ethanol on VTA DAergic neurons are not blocked in the presence of combinations of antagonists for a variety of receptors including GABA, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), metabotropic glutamate, and cholinergic receptors in males (Nimitvilai et al. 2016). These observations indicate that while synaptic inputs may play a role in modulating the effects of ethanol on the VTA DAergic neurons, these are not the primary targets of ethanol action in the VTA.

VTA DAergic neurons have dopamine autoreceptors (D2R) on their soma and dendrites that mediate inhibitory regulation of their excitability (Adell 2004; Aghajanian 1977). In addition to releasing DA onto their projection sites, DAergic neurons are also locally regulated by DA (Kita et al. 2009). This D2R-mediated autoregulation has an inhibitory effect on the activity of VTA DAergic neurons (Ackerman et al. 1993; Brodie and Dunwiddie 1990), and may decrease the release and synthesis of DA (Benoit-Marand, Borrelli, and Gonon 2001; Wolf and Roth 1990; Phillips, Hancock, and Stamford 2002).

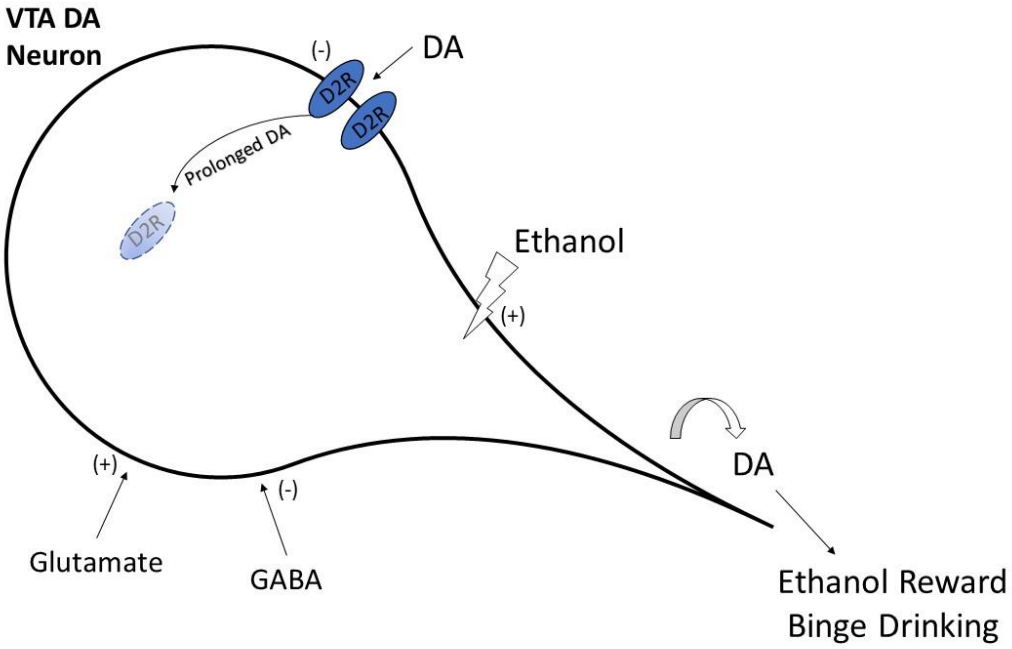

Figure 1: Regulation of VTA neuron firing.

VTA DAergic neuron firing is regulated by many neurotransmitters and drugs, including ethanol, DA, glutamate, and GABA. Ethanol excites (lightning bolt) VTA DAergic neurons, which leads to the release of DA in target regions and is thought to mediate the rewarding effects of ethanol. DA is also locally released and inhibits the firing of DAergic neurons through the autoinhibitory D2R. It has been well established in male rodents that prolonged exposures to DA which may occur during drug abuse causes D2R desensitization. Glutamate and GABA increase and reduce VTA DA neuron firing, respectively.

1.2.1 Sex differences in the ventral tegmental area

Sex differences in reward-seeking behaviors may partially be due to sex differences in the structure and cellular composition of the VTA and projecting neurons. In females, the VTA is a larger volume and contains a larger number of DAergic neurons (McArthur, McHale, and Gillies 2007). In addition to sex differences in the overall shape of the VTA, the distribution of neurons throughout the VTA are sex specific. In females, DAergic neurons are more concentrated in specific regions of the VTA whereas in males there is a comparatively more even distribution of DAergic neurons throughout the VTA (McArthur, McHale, and Gillies 2007). The ratio of DAergic neurons to non-DAergic neurons projecting to the prefrontal cortex is higher in females than in males (Kritzer and Creutz 2008).

1.3 Dopaminergic neurotransmission

DA synthesis, which occurs in several brain regions including the VTA and the substantia nigra, depends on the rate-limiting enzyme TH to convert tyrosine to L-dihydroxyphenylalanine (L-DOPA) which is then converted to DA by the DOPA decarboxylase enzyme (Blaschko 1942; Meiser, Weindl, and Hiller 2013). DA is also a precursor for norepinephrine and epinephrine (Goldstein, Fuxe, and Hokfelt 1972; Blaschko 1942). DA is stored in synaptic vesicles and released upon excitation of DAergic neurons through exocytosis, and DA can subsequently act on postsynaptic or presynaptic DA receptors (Zhang and Sulzer 2012; Werkman et al. 2006).

Termination of the DA signal initiated by DA release can be achieved by reuptake of DA into the neurons by the dopamine transporter (DAT). Cocaine and amphetamine block reuptake of DA by DAT and increases synaptic DA concentrations (Kuhar, Ritz, and Boja 1991). DA is degraded to 3,4-dihydroxyphenylacetic acid (DOPAC) through the enzyme monoamine oxidase (MOA) and eventually from DOPAC to homovanillic acid (HVA) through the enzyme catechol-

O-methyl transferase (COMT) (Meiser, Weindl, and Hiller 2013). Alternatively, DA can be recycled for reuse into vesicles through vesicular monoamine transporter 2 (VMAT2) (Hoffman, Hansson, Mezey, Palkotis, 1998).

DA receptors are G-protein coupled receptors (GPCRs) and are divided into five subtypes which are further classified according to biochemical and pharmacological properties into D_1-like (D1 and D5) and D_2-like (D2, D3, and D4) receptors (Missale et al. 1998). D_1-like DA receptors activate adenylyl cyclase and couple to the G protein G_s. D_2-like DA receptors, on the other hand, are coupled to G_i, inhibit adenylyl cyclase, and through an action of the $G\beta\gamma$ subunit of the G protein can also activate K^+ channels (Beaulieu and Gainetdinov 2011). Both D_1- and D_2-like DA receptors in the VTA play a role in drug seeking and relapse. Injecting antagonists for D_1- and D_2-like DA receptors systemically and directly into the VTA decreases relapse to morphine and heroin in rats (Farahimanesh et al. 2018; Shaham and Stewart 1996).

VTA DAergic neurons express two isoforms of D2R which result from alternative splicing of the sixth exon of the same gene. The long isoform (D2L) is 29 amino acids longer than the short isoform (D2S) (Dal Toso et al. 1989; Giros et al. 1989). This results in differences in an intracellular region which functions in the regulation of G_i coupling (Senogles 1994; Guiramand et al. 1995). The ratio of D2S to D2L varies by brain region (Mack, Todd, and O'Malley 1991; Neve et al. 1991; Montmayeur et al. 1991), and changes in the D2S/D2L ratio are associated with alterations in drug-related behaviors in mice (Radl et al. 2018; Bulwa et al. 2011). For example, knockdown of D2L increases ethanol intake in both sexes, which is thought to be due to a compensatory increase in the D2S isoform (Bulwa et al. 2011).

During drug addiction D2R levels and activity are decreased (Volkow et al. 1990; Martinez et al. 2004; Volkow et al. 1997; Hietala et al. 1994). Trafficking, endocytosis, and

desensitization play important roles in the regulation of D2R. G protein-coupled receptor kinases (GRKs) and protein kinase C (PKC) phosphorylate D2R, and D2R is internalized in a clathrin-mediated mechanism through β-arrestin and dynamin (Namkung and Sibley 2004; Namkung et al. 2009; Paspalas, Rakic, and Goldman-Rakic 2006). A prolonged activation of the D2-like receptors by DA results in the decreased responsiveness to DA inhibition in a time- and concentration-dependent manner through a mechanism involving PLC and PKC (Nimitvilai, Arora, and Brodie 2012) This process is termed DA inhibition reversal (DIR) (Nimitvilai and Brodie 2010). Anaplastic lymphoma kinase (ALK), a receptor tyrosine kinase, regulates DIR in VTA DAergic neurons. Inhibiting ALK reduces DIR without affecting acute responses to DA (Dutton et al. 2017).

DAergic neurotransmission plays an important role in alcohol drinking. Blocking D2R in the nucleus accumbens either reduces (Myers and Robinson 1999; Rassnick, Pulvirenti, and Koob 1992; Samson et al. 1993) or increases (Levy et al. 1991) ethanol intake. These discrepancies may be due to strain, drinking protocol, or treatment differences. Injecting a D2R antagonist in the nucleus accumbens of female alcohol preferring (P) rats with access to ethanol for 60 minutes a day increased drinking (Levy et al. 1991). Additionally, inhibition of VTA DA neurons by injecting the D2R agonist quinpirole into the VTA decreases ethanol consumption (Hodge et al. 1993). Infusion of baclofen, which inhibits VTA DA neurons, decreases ethanol CPP in mice (Bechtholt and Cunningham 2005). Rodents will also self-administer ethanol into the VTA, and this is blocked by D2R agonists (Gatto et al. 1994; Rodd et al. 2005; Rodd et al. 2004).

1.3.1 Sex differences in dopaminergic neurotransmission

Previous studies have demonstrated sex differences in DAergic systems which may drive sex differences in drug abuse (Becker 1999). Clinical studies have identified sex differences in DAergic systems of the brain. In the putamen, women have higher DOPAC/DA ratios compared to males, which suggests women have higher turnover rates for DA (Konradi et al. 1992). Striatal DA synthesis is higher in females compared to men (Laakso et al. 2002). Women also have higher levels of cortical D2R (Kaasinen et al. 2001) and DAT in the striatum, diencephalon, and brainstem compared to men (Staley et al. 2001; Lavalaye et al. 2000). Additionally, women exhibit increased cortical and striatal amphetamine-induced DA release (Riccardi et al. 2006).

There are sex differences in D2R ligand binding, with decreased D2R binding in the rostral lateral striatum in castrated males compared to OVX females, and there is differential regulation of D2R binding in a brain region-, sex-, and time-specific manner after E2 treatment in rats (Bazzett and Becker 1994). E2 increases D2R in the striatum and nucleus accumbens of female rats through activation of ERβ (Le Saux, Morissette, and Di Paolo 2006). These studies show that E2 can modulate the density and ligand binding of D2R in a sex specific manner.

1.4 Female reproductive cycle

1.4.1 Menstrual and estrous cycles

In a mature female adult, the menstrual cycle lasts for about 28 days and is divided into the follicular/proliferative (prior to ovulation) and luteal/secretory (after ovulation) phases (Treloar et al. 1967). A high frequency pulsatile release of gonadotropin releasing hormone (GNRH) from the hypothalamus stimulates the release of follicle stimulating hormone (FSH) and lutenizing hormone (LH) from the anterior pituitary (Maeda et al. 2010). FSH and LH then act on the ovaries to stimulate the release of estrogen. Follicles in the ovary develop as a single

oocyte is prepared for ovulation. A critical level of estrogen then stimulates a surge of GnRH release and subsequent LH and FSH secretion by a positive feedback mechanism (Karsch et al. 1973). Ovulation then occurs in response to this surge, and an egg is released to the uterus by way of the fallopian tube. The remaining follicular cells form the corpus luteum which secretes progesterone. Estrogen levels also rise again during the luteal phase (Reed and Carr 2000). If fertilization fails to occur, then menstruation and the resumption of the cycle occur. Regulation of hormone secretion throughout the menstrual cycle is complex and involves positive and negative feedback mechanisms as well as autocrine and paracrine factors (Hawkins and Matzuk 2008).

The female hormonal cycle of rodents, the estrous cycle, differs remarkably from humans. Mice do not have a luteal phase and the estrous cycle of mice is around four to five days, which are divided into proestrus, estrus, metestrus, and diestrus (Byers et al. 2012; Nelson et al. 1982). E2 begins to be released from the ovaries during metestrus and diestrus phases, which are equivalent to the follicular phase in humans. E2 levels peak during the proestrus phase which induces a LH surge and the secretion of progesterone. Ovulation occurs during the estrus phase. If fertilization does not occur, the cycle continues through metestrus and so forth (Becker 2016). Nilsson et al. used gas chromatography in tandem with mass spectrometry to measure circulating levels of hormones produced by the ovaries in freely cycling female mice. Using their sensitive approach, E2 levels were found to be highest in diestrus and proestrus (around 6 pg/ml and 8 pg/ml, respectively) and lowest during estrus and metestrus (<0.3 pg/ml) (Nilsson et al. 2015).

In females, estrogen can be synthesized in gonadal tissues including the ovaries and placenta (Robic et al. 2016; Knight 1994). Additionally, estrogen can be synthesized in other

organs including the brain, skin, fat, and liver (Robic et al. 2016; Berkovitz, Brown, and Fujimoto 1987; Roselli and Resko 2001; Cleland, Mendelson, and Simpson 1983). The main forms of estrogens are estrone, estriol, and E2, which is the major form of estrogen in premenopausal women (Cui, Shen, and Li 2013). The final step in estrogen synthesis is mediated by the cytochrome P450 enzyme, aromatase (Simpson et al. 1997). The brain is a major site for estrogen synthesis, and aromatase is expressed in many brain regions including the hypothalamus, hippocampus, cerebral cortex, cerebellum, and the limbic system (Naftolin, Ryan, and Petro 1971b, 1971a; Azcoitia, Yague, and Garcia-Segura 2011). Aromatase activity is detected in the rodent midbrain of from birth to adulthood (MacLusky et al. 1994), and aromatase protein as measured by immunofluorescence is present in the VTA of adult male mice (MacLusky et al. 1994).

1.4.2 The influence of ovarian hormones on drug abuse

Hormones, including E2, may play a key role in the gender differences of addiction and alcoholism. The rewarding effects of psychostimulants such as cocaine and amphetamine vary along the human menstrual cycle with increased effects during the follicular phase (Evans, Haney, and Foltin 2002; White, Justice, and de Wit 2002), when estrogen levels are high and progesterone levels are low (Vollman 1977). The role of ovarian hormones on the effects of psychostimulants has also been established in rodents. Cocaine self-administration is highest during estrus (Hecht, Spear, and Spear 1999). Ovariectomized (OVX) rats exhibit a decrease in self-administration of cocaine, and supplementation with E2 increases cocaine intake (Lynch et al. 2001). Treatment with progesterone and E2 reverses the enhancing effects of E2 on cocaine administration in OVX rats (Jackson, Robinson, and Becker 2006). This suggests opposing effects of progesterone and estrogen on the reinforcing effects of cocaine.

Studies investigating differences in alcohol consumption during the normal human menstrual cycle are conflicting and indeterminate; thus, the precise role that the hormonal cycle has on alcohol intake in females is unknown (Becker and Koob 2016). While one study reported the anxiolytic effects of alcohol occurring only during the follicular phase of the menstrual cycle (Logue et al. 1981), another found anxiolytic effects only during the luteal phase (Sutker et al. 1987). Studies on the effects of the menstrual cycle on the subjective effects of alcohol are also conflicting and have been reported to either not differ across the menstrual cycle (Evans and Levin 2011) or were greater in the luteal phase (Holdstock and de Wit 2000). Studies measuring alcohol consumption along the menstrual cycle have either reported no changes (Charette, Tate, and Wilson 1990; Christensen, Oei, and Callan 1989; Griffin et al. 1987; Pomerleau et al. 1994; Tate and Charette 1991) or mixed differences by cycle phase (Harvey and Beckman 1985; Marks et al. 1994; McLeod et al. 1994; Mello, Mendelson, and Lex 1990; Svikis et al. 2006). However, there is a positive correlation between blood E2 levels and alcohol consumption in women, and women who drink were found to have higher blood E2 levels, suggesting that high E2 may be a risk factor or a marker for increased alcohol intake in women (Muti et al. 1998).

There are several studies in rats that have not found any effects of the estrous cycle on ethanol consumption (Priddy et al. 2017; Ford, Eldridge, and Samson 2002b; Satta, Hilderbrand, and Lasek 2018). However, one study found that while there was no estrous cycle effect on self-administration of ethanol in freely-cycling female rats, rats with synchronized estrous cycles exhibited decreased administration of ethanol in estrus and proestrus (Roberts et al. 1998). Another group found decreased ethanol intake during proestrus (Forger and Morin 1982), when circulating E2 serum levels are the highest (Nilsson et al. 2015). These discrepancies in both clinical and preclinical studies may be due to methodological differences and ultimately

highlights the importance of a more thorough investigation on the mechanisms of hormones in alcohol abuse.

Female rodents generally drink more ethanol than males (Priddy et al. 2017; Satta, Hilderbrand, and Lasek 2018). Because E2-treated and gonadally intact female mice drink more than OVX and male mice, increased drinking by females may be due, in part, to the actions of E2 (Ford, Eldridge, and Samson 2004, 2002a). Work in Dr. Amy Lasek's lab has shown that E2 augments binge-drinking in C57BL/6J mice. OVX mice treated with E2 showed increased amounts of ethanol consumption when compared to those treated with vehicle. The increase in binge-like drinking was complimented with an increase in BEC in the OVX mice treated with E2 (Satta, Hilderbrand, and Lasek 2018). Additionally, E2 injections in OVX female mice also increase preference for ethanol in a CPP experiment (Hilderbrand and Lasek 2018). Because E2 affects ethanol CPP and binge drinking, E2 may play a role in the rewarding and reinforcing properties of alcohol.

1.5 Mechanisms of estrogen action

The effects of estrogen are mediated by binding to estrogen receptors (ER), and estrogen can signal through ERα, ERβ, and G protein-coupled ER (GPER). ERs can signal through genomic or non-genomic (rapid) signaling mechanisms (Vrtacnik et al. 2014). ERα and ERβ are receptors that are a part of the steroid hormone superfamily (Osz et al. 2012). Upon ligand binding, ERα and ERβ can form homo- and heterodimers which regulate gene transcription in the nucleus (Cowley et al. 1997; Paulmurugan et al. 2011; Kuntz and Shapiro 1997). ERα and ERβ are composed of functional domains which include the N-terminal domain (NTD), DNA-binding domain (DBD), hinge region, and ligand-binding domain (Beato, Herrlich, and Schutz 1995). There are also two activation function (AF) domains, AF1 and AF2, which respectively

mediate the hormone-independent and -dependent transcriptional activities (Beato 1989). The DBD domain forms a complex with estrogen response elements (ERE) on promoter or enhancer regions in the DNA in order to regulate transcription of target genes through the recruitment of co-activators or co-repressors (Tsai and O'Malley 1994). It is thought that the genomic actions of ERs take hours for the transcriptional and translational effects to manifest (O'Lone et al. 2004).

GPER, which was originally classified as the orphan receptor GPR30 due to the unknown ligand (Carmeci et al. 1997), is coupled to G_s and stimulates the cAMP synthesis through activation of adenylyl cyclase (Thomas et al. 2005). The Gβγ subunit coupled to GPER activates a signaling pathway that releases intracellular calcium and activates epidermal growth factor receptor (EGFR) (Thomas et al. 2005). In addition to the GPER, membrane associated ERα and ERβ can also signal from the membrane and activate many rapid signaling pathways including calcium, adenylate cyclase, phosphoinositol kinase, mitogen-activated protein kinase (MAPK), tyrosine kinase signaling, and cAMP response element binding protein (CREB) (Wu, Chen, and Brinton 2011; Titolo et al. 2008; Qiu et al. 2003; Cavalcanti et al. 2015). The non-genomic signaling pathways of membrane-bound ERs can also converge with genomic signaling to alter transcription (Marino, Galluzzo, and Ascenzi 2006).

Estrogen receptors can also transactivate GPCRs, such as mGluRs, on the membrane in a GPCR ligand-independent manner (Seredynski et al. 2015; Boulware, Heisler, and Frick 2013). mGluRs are activated by E2 in hippocampal (Huang and Woolley 2012) and striatal neurons (Grove-Strawser, Boulware, and Mermelstein 2010). ER/mGluR signaling has sex-specific and brain region-specific effects on CREB phosphorylation, L-type calcium channel currents, and MAPK signaling (Boulware et al. 2005; Grove-Strawser, Boulware, and Mermelstein 2010). In the hippocampus of female rats, ERα/mGluR1 activation suppresses the inhibition of CA1

pyramidal neurons by GABA, through retrograde endocannabinoid action through cannabinoid 1 receptor (CB1R) on inhibitory inputs (Huang and Woolley 2012).

ER/mGluR signaling mechanisms play distinct roles in natural and drug rewards in male and female animals (Dewing et al. 2007; Tonn Eisinger et al. 2018). Interactions between ERα and mGluR1 in the arcuate nucleus of the hypothalamus enhances sexual receptivity in female rodents (Dewing et al. 2007), and interactions between ERβ and mGluR1 enhance sexual behaviors in male Japanese quail (Seredynski et al. 2015). In rodents, E2-induced enhancement of cocaine intake in OVX females is through mGluR5 activation because an antagonist of mGluR5 reverses the E2 enhancement of cocaine self-administration (Martinez et al. 2016). E2-induced enhancement of cocaine stimulated DA release in the nucleus accumbens is through ERβ activation (Yoest, Cummings, and Becker 2018). Amphetamine-induced DA release in the striatum is also enhanced by E2, and mGluR5 is required for this effect (Song et al. 2019).

mGluRs play important roles in alcohol abuse, for example mGluR5 antagonists reduce alcohol consumption and relapse (Backstrom et al. 2004; Schroeder, Overstreet, and Hodge 2005; Schroeder et al. 2008). The type I mGluRs, mGluR1 and mGluR5, are coupled to $G_{q/11}$. mGluR1 activation leads to increased phospholipase C (PLC) activity (Niswender and Conn 2010). Acamprosate, which is clinically effective in treating patients with alcohol dependence (Mason 2001), may decrease alcohol relapse partially through its action on mGluR5 (Harris et al. 2002). Treatment with mGluR1 and mGluR5 antagonists decreases ethanol reward and consumption in male rodents (Backstrom et al. 2004; Lominac et al. 2006), but it is not known if ER/mGluR signaling is involved in these effects.

The actions of ERs can be investigated using ER modulators. The ER agonists and antagonists used in this dissertation project are summarized in Table 1.

Table 1: List of Estrogen Receptor Modulators			
Compound Name	**ERα Action**	**ERβ Action**	**GPER Action**
7α,17β-[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780)	Antagonist	Antagonist	Agonist
4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT)	Agonist (410-fold selectivity over ERβ)	-	-
2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN)	-	Agonist (70-fold selectivity over ERα)	-
1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP)	Antagonist (200-fold selectivity over ERβ)	-	-
4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP)	-	Antagonist (36-fold selectivity over ERα)	-

1.6 Effects of estrogen on ventral tegmental area and dopamine

E2 actions on DAergic pathways regulates reproductive behavior (Foreman and Hall 1987; Balthazart, Baillien, and Ball 2002) and drug abuse (Carroll et al. 2004). Unpublished work by the Lasek lab has shown that ERα and ERβ are both expressed in the VTA, but it is not yet known what the role these receptors have in the VTA. Various groups have identified nuclear ERβ in VTA neurons using a specific antibody (Mitra et al. 2003) and in a transgenic mouse line which expresses the fluorescent protein GFP driven from the ERβ promoter (Milner et al. 2010). Although some groups were not able to identify ERα or GPER in VTA neurons (Milner et al. 2010; Almey et al. 2012), others have identified sparse expression of ERα in a subpopulation of DA VTA neurons (Kritzer 1997). E2 may affect the function of the VTA in both physiological conditions as well as in diseased states such as addiction and alcoholism.

E2 increases the density of D2R on the plasma membrane in the nucleus accumbens and striatum (Chavez et al. 2010; Roy, Buyer, and Licari 1990). However, E2 either decreases or has no change on the expression of the D2R gene in the striatum (Lammers et al. 1999; Le Saux, Morissette, and Di Paolo 2006). Activation of ER has been shown to have cellular effects in the VTA of female rodents. For instance, twenty-seven days of E2 treatment via implant resulted in changes in increased expression of the D3R gene in the VTA (Zhou, Cunningham, and Thomas 2002).

Striatal DA concentrations and DA release are regulated by E2 (Xiao and Becker 1994; Becker 1990; Dazzi et al. 2007), and E2 may be driving some of the sex differences in DA neurotransmission and ultimately drug abuse. E2 increases the synthesis and release of DA (Pasqualini et al. 1995, 1996; Xiao and Becker 1998) and decreases the re-uptake of DA (Watson et al. 2006; Disshon, Boja, and Dluzen 1998). In rats, females will acquire cocaine self-

administration and CPP more rapidly and readily than males (Lynch and Carroll 1999; Carroll et al. 2002; Russo et al. 2003; Hu et al. 2004; Zakharova, Wade, and Izenwasser 2009). E2 supplementation in OVX rats increases cocaine- and amphetamine-stimulated DA release in the nucleus accumbens, cocaine self-administration, and cocaine CPP (Lynch et al. 2001; Hu et al. 2004; Segarra et al. 2010; Segarra et al. 2014; Tobiansky et al. 2016; Thompson and Moss 1994). E2 administration directly into the medial preoptic area enhances cocaine-induced DA release in the nucleus accumbens (Tobiansky et al. 2016). Administration of E2 enhances amphetamine-stimulated DA release in OVX females but not in castrated or intact male mice (Becker 1990) , and this is dependent upon ERs and mGluR5 in the striatum (Song et al. 2019). Treatment with ERα and ERβ agonists in OVX rats enhanced the activation of the VTA by amphetamines (Sarvari 2014).

E2 effects on drinking behaviors may be partially due to E2 acting on VTA DAergic neuron excitation. Many studies in rodents have demonstrated enhancing effects of E2 on drinking behaviors (Ford, Eldridge, and Samson 2004; Rajasingh et al. 2007; Reid, Hubbell, and Reid 2003; Ford, Eldridge, and Samson 2002a; Marinelli, Quirion, and Gianoulakis 2003; Quirarte et al. 2007; Satta, Hilderbrand, and Lasek 2018), and E2 injections in female mice increases preference for ethanol in a CPP experiment through activation of ERα and ERβ (Hilderbrand and Lasek 2018). Ethanol-induced DA release in the prefrontal cortex is prevented in OVX rats and is recovered by E2 supplementation (Dazzi et al. 2007).

Because E2 has been shown to upregulate the rewarding and reinforcing properties of alcohol and other drugs of abuse and the release of DA, E2 may modulate the excitability of the VTA. E2 modulates the excitability of other neuronal cell types, such as visceral ganglion

neurons, which also express the channel responsible for I_h currents, HCN. In female rats, OVX reduced HCN expression and I_h currents and was recovered by E2 (He et al. 2014).

1.7 Summary and project overview

In summary, previous work suggests that E2 plays a role in regulating ethanol reward and DAergic neurotransmission in female mice. The VTA is a brain region critical for the rewarding and reinforcing effects of alcohol and other rewards (Ranaldi 2014; Lammel, Lim, and Malenka 2014), yet few studies have directly investigated the actions of E2 in the VTA in females. This is increasingly important to examine since the incidence of alcohol abuse in women is rising (White et al. 2015; Grant et al. 2017; Cheng, Cantave, and Anthony 2016). The ethanol-induced increase in firing rate of VTA DAergic neurons is thought to mediate the rewarding and reinforcing effects of ethanol, and autoinhibition of VTA DAergic neurons by DA is an important regulatory mechanism for inhibiting the firing of these cells (Ackerman et al. 1993; Brodie and Dunwiddie 1990). Because of this, the responses of VTA DA neurons to ethanol and DA are important to investigate in females and specifically determine whether hormones such as E2 are involved in responses in females. Both ERα and ERβ are expressed in the VTA and activation of these receptors may alter the response of VTA DA neurons to ethanol and DA. Determining the effects of ER activation in VTA DA neurons could provide insight into the molecular mechanisms of drug and ethanol reward in women.

This dissertation focuses on the role of ERs in modulating the sensitivity of VTA DAergic neurons to ethanol and DA in female mice. The primary hypothesis is that E2 regulates the excitation by ethanol and inhibition by DA of VTA DAergic neurons in female mice through activation of ERs. The aims of this dissertation are therefore to (1) characterize the effects of high E2 states in the responses of VTA DAergic neurons to ethanol and DA, (2) determine which

ERs mediate the E2 effects on the sensitivity of VTA DAergic neurons to ethanol and DA, (3) investigate possible downstream pathways which may mediate the E2 effects on the sensitivity of VTA DAergic neurons to ethanol and DA, and (4) determine the effects of E2 on the desensitization of D2R in the VTA of female mice. The second chapter will therefore focus on the effects of high E2 states in regulating the inhibition by DA and excitation by ethanol of VTA DAergic neurons from female mice. The extracellular electrophysiological responses of brain slices containing VTA DAergic neurons from female mice in high E2 states, both from OVX mice supplemented with E2 and gonadally-intact mice, were used to initially characterize the effects of high E2 states on the sensitivity of VTA DAergic neurons to ethanol and DA. Then, VTA DA neurons from freely-cycling female mice were acutely treated with an ERα/ERβ antagonist to further investigate the role of ERs in ethanol and DA sensitivity. The third chapter then investigates which ER and downstream pathways mediate the enhanced sensitivity of VTA DAergic neurons to ethanol. Specific agonists and antagonists for ERα and ERβ were used to determine whether ERα or ERβ mediates the increased sensitivity of VTA DAergic neurons to ethanol in female mice. Then, mGluR1 antagonists were used to determine if the enhanced sensitivity to ethanol in high E2 states is dependent upon mGluR1 activity. In the fourth chapter, specific agonists for ERα and ERβ were used alone and in combination to determine whether ERα and ERβ mediate the enhancement of DA sensitivity of VTA DAergic neurons in high E2 states. Finally, the fifth chapter will characterize the effects of E2-supplementation in OVX mice on the desensitization of D2R and gene expression analyses.

2. ESTRADIOL INCREASES THE SENSITIVITY OF VTA NEURONS TO DA AND ETHANOL

(ADAPTED FROM VANDEGRIFT ET AL, PLOS ONE 2017)

2.1 Introduction

Dysfunctions of DAergic neurotransmission contribute to many psychiatric disorders including mood disorders, schizophrenia, Parkinson's disease, Tourette's syndrome, and addiction (Russo and Nestler 2013; Beaulieu and Gainetdinov 2011; Polter and Kauer 2014; Dichter, Damiano, and Allen 2012). The role of the DAergic system in psychiatric disorders has been established in animal models of these conditions and using human brain imaging and post-mortem tissue analysis (Russo and Nestler 2013; Dichter, Damiano, and Allen 2012). Interestingly, several psychiatric disorders also exhibit a gender bias in prevalence and severity (Gobinath, Choleris, and Galea 2017). Gender differences in the age of onset, prevalence, progression, and severity of symptoms associated with psychiatric disorders may be due to inherent sex differences in the organization of the DAergic system and/or modulated by hormones (Riccardi et al. 2011; Zagni, Simoni, and Colombo 2016; Gogos et al. 2015). It is therefore important to understand the biological factors that underlie sex differences in DAergic neurotransmission to help guide the development of future treatments for psychiatric disorders that will be effective in both sexes.

The mesocorticolimbic DAergic system is critically involved in the development of addiction. This system encompasses the VTA DAergic neurons and their projections to the nucleus accumbens, amygdala, and prefrontal cortex (Oades and Halliday 1987; Koob 2003; Oliva and Wanat 2016). VTA neurons are activated and release DA in their target areas in response to stimuli that are rewarding or novel (Berridge 2007), and release of DA by VTA DA

neurons underlies the rewarding and reinforcing effects of drugs of abuse (Koob and Volkow 2010; Jones and Bonci 2005). The VTA is important for promoting alcohol consumption and reward (Bechtholt and Cunningham 2005; Rodd et al. 2004). Rodents will self-administer ethanol directly into the VTA, suggesting that at least some of the reinforcing effects of ethanol are mediated through the VTA (Rodd et al. 2004; Gatto et al. 1994; Ding et al. 2015). Acute administration of ethanol dose-dependently enhances the basal firing rate of rodent VTA DAergic neurons both *in vitro* (Brodie, Shefner, and Dunwiddie 1990; Brodie and Appel 1998) and *in vivo* (Gessa et al. 1985).

There are considerable gender differences in addiction, including differences in the amount of use, age of initiation, reasons for abuse, development of comorbidities, and severity of health problems (Becker, Perry, and Westenbroek 2012). Although more men abuse alcohol than women, the difference in the incidence of alcohol abuse is narrowing between genders (Cheng, Cantave, and Anthony 2016; Keyes, Grant, and Hasin 2008). Women are more susceptible to the devastating health effects associated with alcohol abuse such as liver disease and neurotoxicity (Eagon 2010; Mann et al. 1992). Similar to other drugs of abuse such as cocaine, women also exhibit a greater escalation of alcohol use and are more likely to relapse in response to stress and anxiety (Becker and Koob 2016). Studies investigating differences in alcohol consumption during the normal human menstrual cycle are conflicting and indeterminate; thus, the precise role that the hormonal cycle has on alcohol intake in women is unknown (Becker and Koob 2016). However, steroid hormones, including estrogen, may play a role in sex differences in addiction and alcoholism (Becker, Perry, and Westenbroek 2012; Carroll et al. 2004; Carroll and Anker 2010; Anker and Carroll 2011).

Estrogen, specifically E2, may affect the normal physiology of the VTA and the pathophysiology of disease states such as addiction. E2 modulates DAergic responses to psychostimulants (Segarra et al. 2010; Zhou, Cunningham, and Thomas 2002; Madularu et al. 2015; Madularu et al. 2016) and increases the rewarding and reinforcing properties of cocaine and amphetamine (Bobzean, Dennis, and Perrotti 2014; Galankin, Shekunova, and Zvartau 2010; Segarra et al. 2010; Silverman and Koenig 2007; Chen et al. 2003; Lynch et al. 2001; Hu et al. 2004; Hu and Becker 2008). E2 alters mesolimbic DAergic transmission pre- and postsynaptically by increasing DA synthesis, reuptake, and release and changing receptor expression and activity (Febo et al. 2003; Gordon and Fields 1989; Joyce, Smith, and van Hartesveldt 1982; Thompson, Bridges, and Weirs 2001; Thompson and Moss 1994). Although much is known regarding the effects of E2 on behavioral and neurochemical responses to psychostimulants, the potential effects of E2 on behaviors related to alcohol abuse and the physiological responses of VTA DAergic neurons to ethanol are less well studied. In premenopausal women, alcohol consumption is positively correlated with serum E2 levels (Muti et al. 1998) and E2 supplementation in OVX mice increases ethanol-stimulated DA release in the prefrontal cortex (Dazzi et al. 2007). ERs are expressed in VTA neurons (Milner et al. 2010; Creutz and Kritzer 2002). However, the effects of E2 specifically on the DA- and ethanol-regulated responses of VTA neurons have not been examined. Here, gonadally intact female mice at different stages of the estrous cycle characterized by different circulating hormone levels and OVX mice supplemented with E2 were examined for the effects of these different E2 states on the firing of DAergic neurons in response to DA and ethanol. We demonstrate that E2 increases the sensitivity of VTA DA neurons to both ethanol and DA, indicating that sex

differences in the DA system may be, in part, regulated hormonally. These results have implications for alcohol use disorder and other psychiatric diseases in women.

2.2 Materials and methods

2.2.1 Animals

Female C57BL/6J mice were used for all experiments. Mice were 8 weeks old when purchased from The Jackson Laboratories (Bar Harbor, ME, USA) and were tested between 10-14 weeks of age. Mice were group-housed under a 12 hour light-dark cycle in a temperature-and-humidity-controlled facility. Mice were treated in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Chicago.

2.2.2 Vaginal cytology

Vaginal cytology was performed for at least two weeks in freely cycling females prior to performing experiments. A cotton swab was moistened with sterile water and gently inserted less than 1 mm into the vaginal opening and rotated. Immediately after removal from the vaginal opening, the swab was wiped on a microscope slide and the smear analyzed by bright field microscopy using an EVOS® FL inverted microscope (Thermo Fisher Scientific). Estrous phase was determined based on vaginal cellular composition. For freely cycling mice, estrus vaginal composition was identified by an abundance of cornified epithelial cells, while diestrus II (herein referred to diestrus) was identified by the predominance of leukocytes in the smears (Nelson et al. 1982) (Figure 2). These two phases show large differences in circulating estrogen levels in mice, and are of substantially longer duration than either proestrus or metestrus/diestrus I

(Nilsson et al. 2015). For electrophysiology, the final smears were obtained 1-2 hours prior to euthanasia.

2.2.3 Ovariectomy and estradiol treatments

Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (8 mg/kg). After shaving the hair on the back of the mouse, a small incision was made on the dorsal side, the uterine horn was pulled out of the abdominal cavity, and the ovary and associated fat pad were dissected away from the uterine horn with a cauterizer. The uterine horn was pushed back into the abdominal cavity. The incisions were closed with sterile sutures and wound clips. This procedure was performed bilaterally. Mice received an injection of meloxicam (2 mg/kg, subcutaneous) for analgesia immediately after surgery and 24 hours later. To confirm ovary removal, vaginal smears were taken daily from mice for 4-5 days and analyzed using bright field microscopy as described above. All OVX mice used in these experiments were no longer cycling through estrous, demonstrating successful ovary removal. After 10 days of recovery from surgery, the mice were treated for three days with 0.05 mL of sesame oil plus 10% ethanol vehicle (VEH) or E2 benzoate in VEH subcutaneously. For the first two days of treatment, mice were injected once daily with 0.2 µg E2 or VEH, which resulted in serum E2 levels 4 hours after injection that were comparable to E2 levels in proestrus (Vandegrift et al. 2017). On the final day of treatment, the mice were injected with 1 µg E2 or VEH one hour before sacrifice, which resulted in serum E2 levels 30 min after injection that were comparable to E2 levels in proestrus (Vandegrift et al. 2017).

2.2.4 Preparation of brain slices

Brain slices containing the VTA were prepared for electrophysiology as previously described (Brodie, Pesold, and Appel 1999; Dutton et al. 2016). Mice were briefly anesthetized

with isoflurane prior to rapid removal of the brain. Using a vibratome and cold cutting solution, a tissue block containing the VTA and substantia nigra was cut into coronal sections of 400-µm thickness. Slices containing the VTA were placed in a chamber containing artificial cerebrospinal fluid (aCSF) flowing at a rate of 2 ml/min at 35°C. A small chamber (~0.5 ml) was used in these studies to allow the rapid application and washout of drug solutions in the bath. The slices were incubated for one hour in the aCSF before experiments were conducted. The composition of the aCSF was (in mM): NaCl 126, KCl 2.5, NaH2PO4 1.24, CaCl2 2.4, MgSO4 1.3, NaHCO3 26, glucose 11. The cutting solution composition was (in mM): KCl 2.5, CaCl2 2.4, MgSO4 1.3, NaHCO3 26, glucose 11, and sucrose 220. Both solutions were saturated with 95% O_2/ 5% CO_2 (pH=7.4).

2.2.5 Cell identification for electrophysiology

Putative DA cells (herein referred to as VTA DA neurons) were identified by anatomical location and electrophysiological characteristics. The VTA was visible in the slices as a grey area medial to the substantia nigra. White matter separated the VTA from the substanta nigra. DA neurons have been shown to have distinctive electrophysiological characteristics including broad action potentials (at least 2.5 msec), slow spontaneous firing rate (0.5 – 5 Hz), and a regular inter-spike interval. Only neurons located within the lateral VTA that conformed to previously established criteria for DA neurons were included (Mueller and Brodie 1989). The criteria used for the recorded VTA neurons are associated with DA-containing neurons projecting to the nucleus accumbens (Margolis, Mitchell, et al. 2008), although it should be noted that there is some controversy regarding the neurotransmitter identity of neurons with this electrophysiological profile (Margolis, Mitchell, et al. 2008; Margolis et al. 2010; Chieng et al. 2011; Margolis et al. 2006). We also tested baclofen on each of the neurons for the quinpirole

studies, and 100% of them were inhibited by 1 μM baclofen. Baclofen has been shown to inhibit VTA DA neurons and not GABA neurons in the VTA (Margolis et al. 2012).

2.2.6 Drug administration

With the exception of the E2 and VEH treatment of OVX mice, all drugs were administered during electrophysiological experiments. When ethanol and DA were added to the aCSF, a calibrated infusion pump from stock solutions was used. The final concentrations were calculated from the flow rate and stock solution concentrations (100 to 1000 times the desired final concentrations). The addition of the concentrated drugs to the flowing aCSF using the pumps was performed in such a way as to allow the drug solution to mix completely and reach the final desired concentration in the aCSF before reaching the slice. In some experiments, 10 μM ICI 182,780 was added to the microelectrode filling solution for pipette delivery, a technique that has been used in the past by our lab and others (Nimitvilai, Arora, and Brodie 2012; Nimitvilai et al. 2013).

2.2.7 Extracellular recording

Spontaneous spike frequency of the putative DA neurons was measured using extracellular recordings; spike frequency (firing rate) was recorded and averaged over one-minute intervals. Recordings were made using micropipettes filled with 0.9% NaCl with tip resistances of 2 to 4 MΩ. Changes in firing rate were determined as peak percentage change relative to the baseline prior to drug administration.

2.2.8 Statistical analysis

Averaged numerical values are presented as the mean ± SEM. Statistical comparisons were made using two-way ANOVA or two-way repeated measures ANOVA followed by Sidak's

multiple comparisons tests as appropriate (Origin, Originlab, Northampton, MA or Prism, Graphpad Software, Inc., La Jolla, CA). A *P* value of < 0.05 was considered significant.

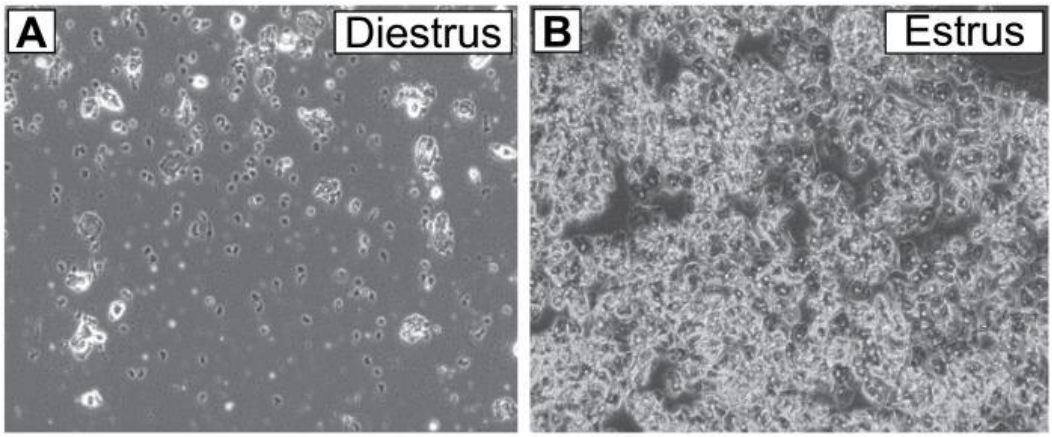

Figure 2. Representative images of vaginal smears from mice in estrus and diestrus.

(A) Image showing a predominance of leukocytes in diestrus. (B) Image showing a predominance of anucleated epithelial cells in estrus.

2.3 Results

2.3.1 VTA neuron characteristics

A total of 121 VTA neurons from 67 mice were recorded in this study. Their initial basal firing rates ranged from 0.55 to 3.92 Hz. The mean firing rate was 2.00 ± 0.80 Hz. There were no significant differences in basal firing rate between estrus and diestrus of freely cycling mice (Estrus: 2.05 ± 0.15 Hz, Diestrus: 1.89 ± 0.16 Hz, $t_{53} = 0.72$, $P = 0.48$) or between E2- and vehicle-treated OVX mice (E2-treated: 1.95 ± 0.19 Hz, VEH-treated: 2.18 ± 0.13 Hz, $t_{50} = 1.00$, $P = 0.09$). All neurons conformed to the rate and patterns of DA VTA neurons as established in the literature and in this laboratory.

2.3.2 Increased sensitivity of VTA DA neurons to inhibition by DA in diestrus

In addition to releasing DA onto their projection sites, DAergic neurons are also locally regulated by DA (Kita et al. 2009). This DA-mediated autoregulation inhibits the firing of DA VTA neurons through actions at the D2R (Ackerman et al. 1993; Brodie and Dunwiddie 1990). In order to determine whether natural fluctuations of hormones affect responses to DA, I tested DA inhibition of VTA DAergic neurons from mice in diestrus and estrus. Representative vaginal smears of the estrus and diestrus phases of the cycle are shown in Figure 2. VTA DAergic neurons from mice in diestrus exhibited a significantly enhanced inhibition by DA (0.5-10 μM) compared with VTA DAergic neurons from mice in estrus (Figure 3, n=9 per group, two-way ANOVA, concentration: $F_{4,79} = 11.05$, $P < 0.0001$; phase: $F_{1,79} = 4.75$, $P = 0.032$; concentration x phase interaction: $F_{4, 79} = 0.51$, $P = 0.73$). For example, 5 μM DA decreased the firing rate by 48.0 ± 12.7% in neurons from mice in diestrus, while that same concentration of DA decreased the firing rate of neurons from mice in estrus by 29.6 ± 5.7%. To confirm that the enhanced inhibition observed during diestrus is mediated by D2/D3R stimulation, I tested the effect of

quinpirole, a selective D2/D3R agonist. Quinpirole more potently inhibited DAergic neurons from mice in diestrus compared with estrus, similar to what I observed with DA (Figure 4, n = 7 per group, two-way RM ANOVA, concentration: $F_{3, 36} = 112.2$, $P < 0.0001$; phase: $F_{1, 12} = 6.39$, $P = 0.027$; interaction: $F_{3, 36} = 3.94$, $P = 0.016$). Post-hoc multiple comparisons tests indicated a significant effect of 50 nM quinpirole ($P = 0.001$). In estrus, 50 nM quinpirole inhibited DA neurons by 36.6 ± 4.2%, while in diestrus, 50 nM quinpirole inhibited DA neurons by 72.9 ± 13.6%. These results indicate that DA autoinhibition of VTA DAergic neurons fluctuates during the estrous cycle and may be modulated by ovarian hormones.

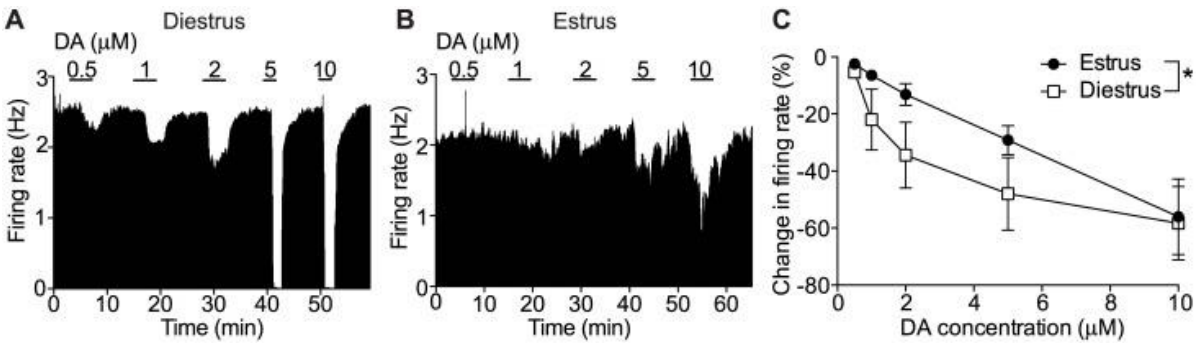

Figure 3. Increased sensitivity of VTA DA neurons to inhibition by DA during diestrus.

Extracellular recordings of VTA DA neurons were obtained from mice in diestrus and estrus. The DA response was measured in putative VTA DA neurons with 0.5, 1, 2, 5, and 10 μM DA. (A-B) Representative rate meter graphs show the effects of DA during (A) diestrus and (B) estrus. (C) Concentration-response graph showing enhanced inhibition to DA in mice during diestrus (n = 9) compared with estrus (n = 9). **P* < 0.05 by two-way ANOVA.

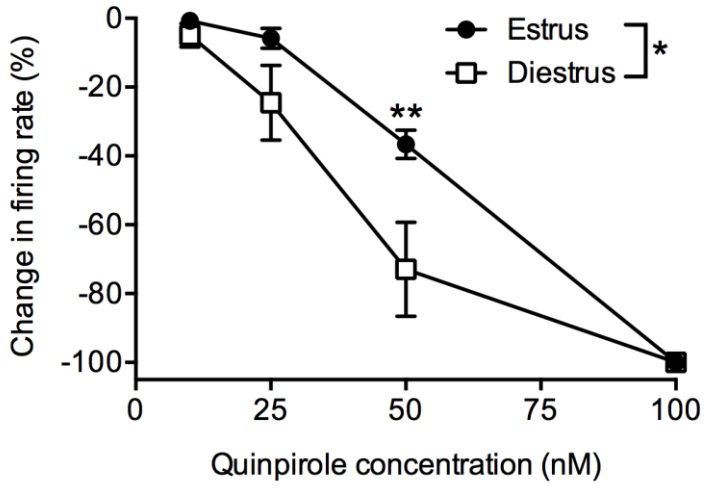

Figure 4. Enhanced inhibition by quinpirole in VTA DA neurons in diestrus mice.

Extracellular recordings were made from VTA slices from mice in estrus or diestrus. Shown are the concentration-response curves showing quinpirole inhibition of VTA DA neurons (n = 7 per group). There was a significant main effect of concentration ($F_{3, 36}$ = 112.2, *P* < 0.0001), estrous cycle phase ($F_{1, 12}$ = 6.39, **P* = 0.027), and a significant interaction ($F_{3, 36}$ = 3.94, *P* = 0.016) by two-way RM ANOVA. Post-hoc Sidak's multiple comparisons tests indicated a significant difference between estrus and diestrus at 50 nM quinpirole, indicated by two asterisks (***P* < 0.01).

2.3.3 Increased sensitivity of VTA DA neurons to ethanol excitation in diestrus

The Brodie lab has established that ethanol directly increases the firing rate of VTA neurons of male rodents (Brodie, Pesold, and Appel 1999). To determine if natural hormonal fluctuations in female mice affect the response of VTA DAergic neurons to ethanol, I tested excitation of VTA DA neurons from mice in diestrus and estrus by 40, 80, and 120 mM ethanol. VTA DAergic neurons from mice in diestrus showed significantly greater excitation by ethanol compared with neurons from mice in estrus (Figure 5, n=7-9 per group, two-way ANOVA, concentration: $F_{2, 42} = 27.74$, $P < 0.0001$; phase: $F_{1, 42} = 11.76$, $P = 0.0014$; concentration x phase interaction: $F_{2, 42} = 2.15$, $P = 0.13$). For example, neurons from mice in estrus responded to 80 mM ethanol with a 12.4 ± 2.6% increase in firing rate, whereas in neurons from mice in diestrus, the same concentration of ethanol produced a 23.7 ± 4.7% increase in firing rate. These results indicate that ethanol more potently excites VTA DA neurons from female mice during diestrus compared with estrus, demonstrating that different hormonal states affect the response to ethanol.

Because circulating E2 levels are slightly higher during proestrus (~8 pg/mL) than in diestrus (~ 6 pg/mL) (Nilsson et al. 2015), I tested excitation of VTA DAergic neurons from mice in proestrus by 40, 80, and 120 mM ethanol. VTA DA neurons from mice in proestrus showed no significant difference in excitation by ethanol compared with neurons from mice in diestrus (Figure 6, n=6-9 per group, two-way ANOVA, concentration: $F_{2, 20} = 27.39$, $P < 0.0001$; phase: $F_{1, 13} = 0.032$, $P = 0.8613$; concentration x phase interaction: $F_{2, 26} = 1.85$, $P = 0.17$). For example, neurons from mice in proestrus responded to 80 mM ethanol with a 22.5 ± 8.8% increase in firing rate, and in neurons from mice in diestrus, the same concentration of ethanol produced a 23.7 ± 4.7% increase in firing rate. These results indicate that ethanol excitation of VTA DA neurons from female mice in diestrus is comparable with proestrus.

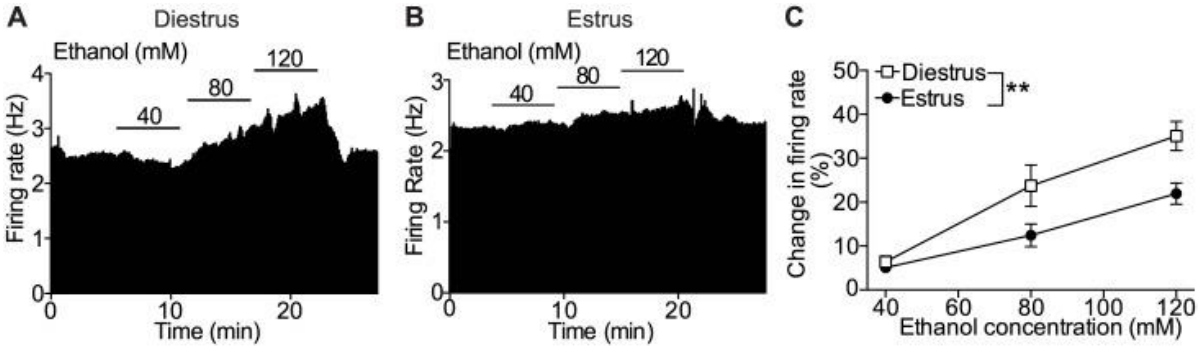

Figure 5. Increased sensitivity of VTA DA neurons to ethanol excitation during diestrus.

Extracellular recordings of VTA DA neurons were obtained from mice in diestrus and estrus. The ethanol response was measured in VTA DA neurons with 40, 80, and 120 mM ethanol. Representative rate meter graphs show the effects of ethanol during (A) diestrus and (B) estrus. (C) Pooled concentration-response graph showing excitation by ethanol in mice during diestrus (n = 9) compared with estrus (n = 7). $^{**}P < 0.01$ by two-way ANOVA.

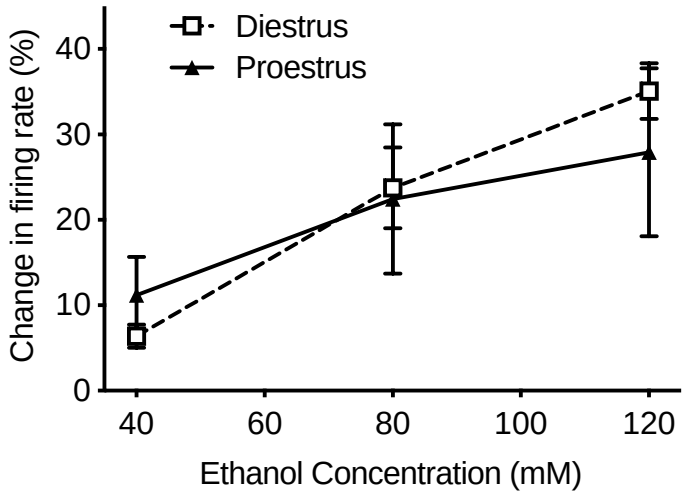

Figure 6. Ethanol excitation in VTA DA neurons in proestrus is similar to neurons in diestrus.

Extracellular recordings were obtained from VTA DA neurons in proestrus and diestrus. The ethanol response was measured in VTA DA neurons with 40, 80, and 120 mM ethanol. Pooled concentration-response graph shows no significant difference in excitation by ethanol in mice during diestrus (n = 9) compared with proestrus (n =6). The ethanol response from neurons in mice in diestrus is the same data as shown in Figure 5, and is shown here for reference.

2.3.4 Estradiol enhances responses of VTA DA neurons to DA and ethanol

Since E2 levels are higher in diestrus compared with estrus (Nilsson et al. 2015), I hypothesized that the increased responses of VTA neurons to DA and ethanol during diestrus may be due to an effect of elevated E2. To more directly test this hypothesis, mice were OVX to remove circulating hormones produced by the ovaries and treated systemically with daily E2 or VEH injections for three days prior to sacrifice. Brain slices were prepared one hour after the last E2 or VEH injection and VTA neurons tested for electrophysiological responses to DA and ethanol. VTA DAergic neurons in OVX mice treated with E2 demonstrated a significantly increased sensitivity to DA inhibition (0.5-10 μM) compared with neurons from OVX VEH-treated mice (Figure 7, n = 9 per group, concentration: $F_{4, 80} = 19.05$, $P < 0.0001$; treatment: $F_{1, 80} = 6.41$, $P = 0.013$; concentration x treatment interaction: $F_{4, 80} = 0.88$, $P = 0.48$). For example, 5 μM DA decreased the firing rate by 65.6 ± 13.4% in neurons from E2-treated mice, while the decrease in firing rate in neurons from VEH-treated mice was 40.2 ± 11.2%. This result indicates that E2 pretreatment increases the sensitivity of VTA DAergic neurons to inhibition by DA.

I next tested for the effect of E2 treatment on the excitation of VTA DAergic neurons by ethanol. Neurons from OVX mice treated with E2 showed a significantly enhanced ethanol-induced (40-120 mM) excitation compared with neurons from OVX VEH-treated mice (Figure 7, n = 12-13, two-way ANOVA, concentration: $F_{2, 69} = 24.83$, $P < 0.0001$; treatment: $F_{1, 69} = 7.81$, $P = 0.0067$; concentration x treatment interaction: $F_{2, 69} = 0.71$, $P = 0.49$). As an example, neurons from E2-treated mice responded to 80 mM ethanol with an increase in firing rate of 26.5 ± 4.2%, whereas neurons from VEH-treated mice responded to ethanol with an increase in firing rate of 14.5 ± 1.6%. Because GABAergic neurotransmission in the VTA may regulate the firing of VTA DAergic neurons (Theile et al. 2011), I also tested whether the enhanced ethanol

excitation in neurons from E2-treated OVX mice was affected by GABA antagonists (concurrent administration of bicuculline and CGP35348, 10 μM each). In the presence of GABA antagonists, the response of VTA DA neurons to ethanol in OVX VEH- or E2-treated mice was similar to what I observed in the absence of these antagonists, suggesting that the increase in ethanol excitation elicited by E2 may be independent of GABA neurotransmission in the VTA (Figure 8, n = 4–5, two-way ANOVA, For vehicle treated: ethanol concentration: $F_{2, 20}$ = 10.50, $P< 0.0001$; treatment: $F_{1,20} = 0.02$, $P = 0.90$; For E2 treated: ethanol concentration: $F_{2, 26} = 16.05$, $P< 0.0001$; treatment: $F_{1,26} = 0.11$, $P = 0.75$).

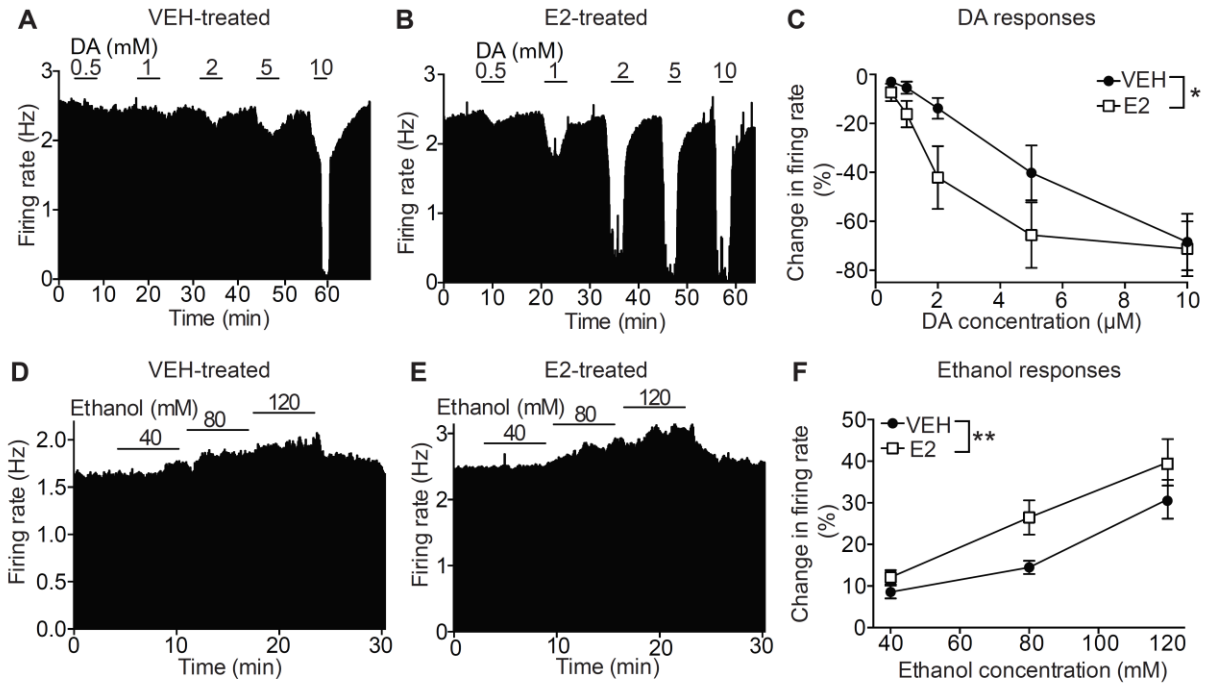

Figure 7. E2 enhances DA inhibition and ethanol excitation of VTA DA neurons. Extracellular recordings were obtained from VTA DA neurons in ovariectomized OVX mice previously treated with E2 or VEH (A-B) Representative rate meter graphs showing response to 0.5-10 μM DA of a VTA DA neuron from a mouse treated with VEH (A) or E2 (B). (C) DA responses in VTA DA neurons treated with 0.5-10 μM DA. Concentration-response graph shows enhanced inhibition by DA in OVX mice treated with E2 compared with VEH (n = 9 per group). (D-E) Representative rate meter graphs showing response to 40-120 mM ethanol of a VTA DA neuron from a mouse treated with VEH (D) or E2 (E). (F) Ethanol responses in VTA DA neurons treated with 40, 80, and 120 mM ethanol. Pooled concentration-response graph shows enhanced excitation to ethanol in OVX mice treated with E2 (n = 13) compared with VEH (n = 12). *$P < 0.05$, **$P < 0.01$ by two-way ANOVA.

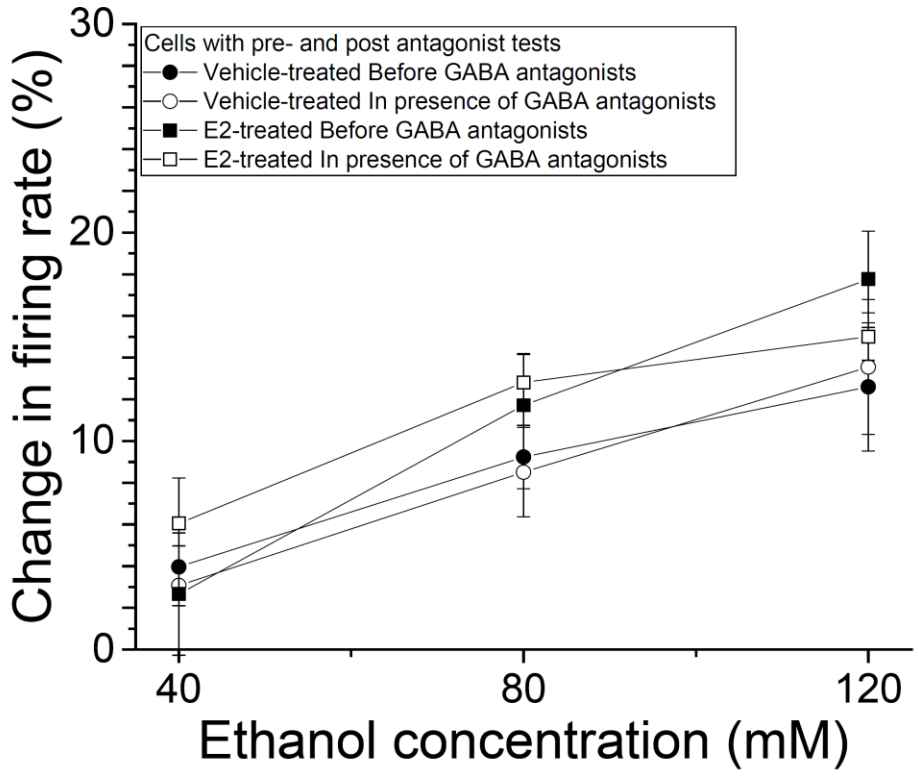

Figure 8. GABA antagonists do not alter the ethanol-stimulated increase in firing of DA neurons in OVX mice treated with E2.

Extracellular recordings were made from VTA slices from OVX mice treated with E2 (n = 5) or VEH (n = 4). The GABA antagonists bicuculline and CGP35348 (10 μM each) were administered concurrently to the slices. Shown is the ethanol concentration-response graph in the presence or absence of the GABA antagonists. Pooled concentration-response graph shows excitation to ethanol in OVX mice treated with E2 or VEH is unchanged by the presence or absence of GABA antagonists.

2.3.5 Estrogen Receptors in the VTA acutely regulate the sensitivity of DA neurons to ethanol but not DA

ERs are present in the VTA and are expressed in DAergic neurons (Creutz and Kritzer 2002; Milner et al. 2010; Mitra et al. 2003; Shughrue, Lane, and Merchenthaler 1997). To determine if ERs in the VTA regulate the increased sensitivity of VTA DAergic neurons to ethanol and DA, I treated brain slices containing the VTA with the ER (ERα and ERβ) antagonist ICI 182,780 and measured the electrophysiological responses to DA and ethanol in neurons from mice in estrus and diestrus. ICI 182,780 was delivered via the recording pipette, a method that the Brodie laboratory has used successfully in the past (Nimitvilai, Arora, and Brodie 2012). ICI 182,780 did not affect inhibition by DA in either estrus or diestrus. However, similar to the effect shown above in Figure 3, there was a significant main effect of estrous cycle phase, indicating enhanced DA inhibition in neurons from mice in diestrus (Figure 9, n = 6 per group, two-way RM ANOVA, treatment: $F_{1,10} = 1.75$, $P = 0.22$; phase: $F_{1,10} = 6.45$, $P = 0.029$; treatment x phase interaction: $F_{1,10} = 1.62$, $P = 0.23$), consistent with the results depicted in Figure 3. The lack of effect of ICI 182,780 on inhibition of VTA DAergic neurons by DA suggests that ERs do not acutely affect DA inhibition, or function in a different brain region to modulate this effect.

In contrast, ethanol excitation of VTA DAergic neurons from mice in diestrus was decreased after treatment with ICI 182,780 (Figure 9, n = 6 per group, two-way RM ANOVA, treatment: $F_{1, 10} = 7.29$, $P = 0.022$; phase: $F_{1, 10} = 1.83$, $P = 0.21$; treatment x phase interaction: $F_{1, 10} = 8.79$, $P = 0.014$). Post-hoc multiple comparisons testing demonstrated a significant difference in ethanol-stimulated increase in firing in VTA DAergic neurons from mice in diestrus compared with estrus before treatment with ICI 182,780 ($P = 0.018$), consistent with results shown in Figure 5. This difference in ethanol-stimulated increase in firing between these

two phases of the estrous cycle was abolished after allowing 90 minutes for ICI 182,780 to diffuse from the recording pipette onto the slice. Neurons from mice in the diestrus phase responded to 80 mM ethanol with an 18.8 ± 7.1% increase in firing rate at the beginning of the experiment, but after the 90 minute of pipette delivery of ICI 182,780, the response to 80 mM ethanol was increased by only 8.4 ± 1.9%. As a control, I also tested if inclusion of DMSO vehicle in the recording pipette would change the response to ethanol of VTA DA neurons from diestrus mice over the same 90-minute period. There was no change in ethanol-stimulated increase in firing over time with DMSO vehicle in the pipette ($t_4 = 0.94$, $P = 0.4$), indicating that the decrease in ethanol response from mice in diestrus after incubation with ICI 182,780 is not consequence of time, DMSO, or repetition of ethanol administration. In addition, the response to ethanol of VTA DA neurons from mice in estrus was not altered by ICI 182,780. At the beginning of the experiment, neurons from mice in the estrus phase responded to 80 mM ethanol with an increase of firing rate of 10.1 ± 4.2%; after 90 minutes of the pipette delivery of ICI 182,780, neurons from the estrus phase responded with an 11.5 ± 5.6% increase in firing rate. These results demonstrate that ERs in the VTA acutely regulate the enhancement of ethanol stimulation in the diestrus phase, when E2 levels are higher than in estrus.

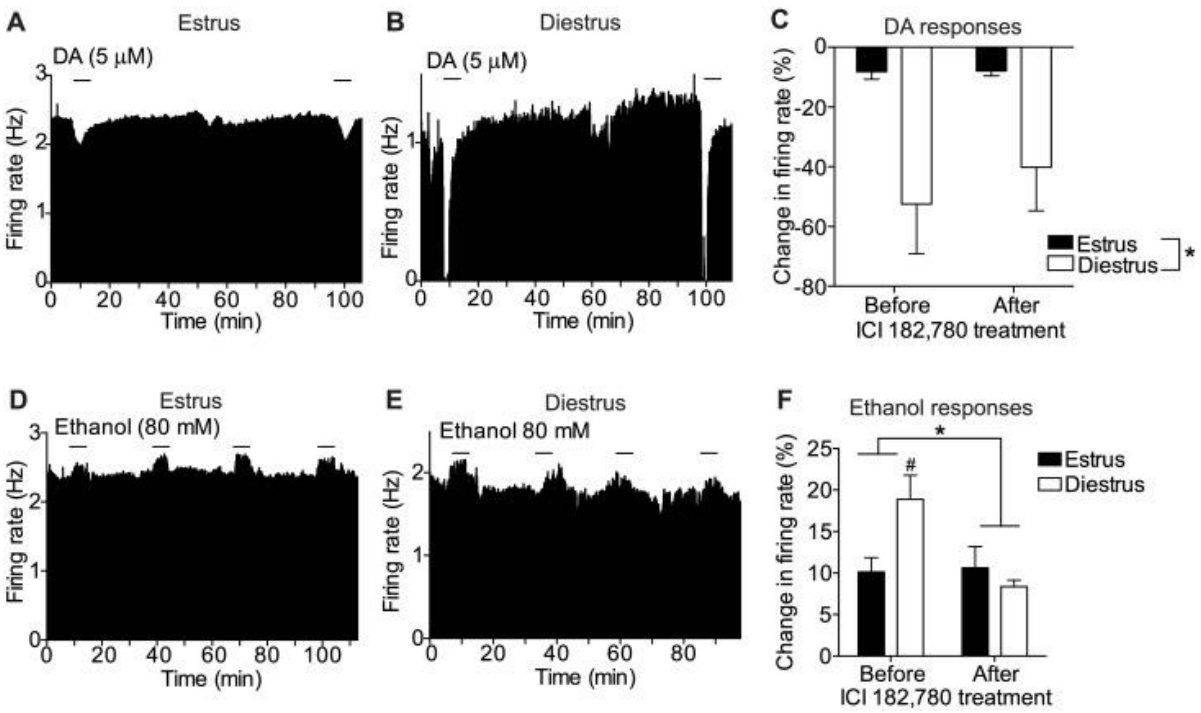

Figure 9. ERs in the VTA acutely regulate the sensitivity of DA neurons to ethanol but not DA.

(A-B) Representative rate meter graphs showing response to 5 μM DA of a VTA DA neuron from a mouse in estrus (A) or diestrus (B) recorded before and during treatment with ICI 182,780. (C) Response to 5 μM DA of VTA DA neurons from mice in estrus (n = 6) or diestrus (n = 6) before and after treatment of slices containing the VTA with ICI 182,780. There was no change in DA inhibition with ICI 182,780 treatment, but there was a significant effect of cycle phase (*$P < 0.05$). (D-E) Representative rate meter graphs showing response to 80 mM ethanol of a VTA DA neuron from a mouse in estrus (D) or diestrus (E) recorded before and during treatment with ICI 182,780. (F) Response to 80 mM ethanol of VTA DA neurons from mice in estrus (n = 6) or diestrus (n = 6) before and after treatment of slices containing the VTA with ICI 182,780. The enhanced VTA DA neuron excitation by ethanol that was observed in mice in diestrus was decreased after treatment with ICI 182,780, whereas the ethanol response of VTA DA neurons from mice in estrus was not altered by ICI 182,780. *$P < 0.05$ by two-way RM ANOVA. $^{\#}P < 0.05$ between mice in estrus and diestrus before ICI 182,780 treatment by post-hoc Sidak's multiple comparisons test.

2.4 Discussion

These results show that E2 modulates the response of VTA DAergic neurons to ethanol and DA. The ethanol and DA responses of VTA neurons were tested in both OVX and gonadally intact female mice. Elevated E2 corresponded with enhanced responses in VTA neurons. This was observed in neurons in OVX mice treated with E2 and in mice in diestrus, where serum E2 levels have been reported to reach ~6 pg/ml (Nilsson et al. 2015) compared with neurons from OVX mice and mice in estrus, where serum E2 levels have been estimated to be less than 0.3 pg/ml (Nilsson et al. 2015). The responses to ethanol and DA of VTA DAergic neurons in mice in estrus (when circulating E2 levels are low) are comparable to responses that the Brodie laboratory has observed previously in male C57BL6/J mice, providing additional evidence that E2 increases the sensitivity of VTA DAergic neurons to ethanol and DA (Brodie and Appel 2000; Dutton et al. 2017). I have also demonstrated that the enhanced ethanol excitation, but not the enhanced DA inhibition, observed in VTA neurons from mice in diestrus is reversed after acute treatment with ICI 182,780, an antagonist of ERα and ERβ.

DA inhibition was enhanced in VTA neurons from gonadally intact mice in diestrus and in OVX mice treated with E2. Inhibition of firing by D2R activation is mediated by potassium channels activated by the βγ subunit of G proteins (Kim, Nakajima, and Nakajima 1995). The alterations of responses to DA by E2 that I observed might be due to a change in D2R expression, trafficking, G protein coupling, or other downstream signaling components. Future studies should address which of these possibilities contribute to the E2 enhancement of DA inhibition.

Acute in vitro treatment with the ER antagonist, ICI 182,780, did not alter the inhibitory DA response of VTA DAergic neurons from mice in diestrus or estrus. While it is possible that

the increased DA inhibition observed in VTA neurons from mice in diestrus is not due to increased E2 levels, this seems unlikely. I found that treatment of OVX mice with E2 resulted in increased inhibition of VTA DA neurons by DA when compared with VEH-treated mice, similar to differences in DA responses of VTA DAergic neurons from mice in diestrus (higher E2 levels) compared with estrus (lower E2 levels); these observations support the argument that E2 is responsible for increasing the sensitivity of VTA DA neurons to inhibition by DA in intact females. It seems more likely that the alteration of D2 sensitivity is caused by E2-induced changes in DA responsiveness in the VTA elicited prior to performing the electrophysiology. For instance, acute ICI 182,780 treatment would not be expected to reverse the increased DA sensitivity in high estrogen states if E2 changed the expression of genes that regulate D2R signaling or trafficking prior to slice preparation (i.e. the classical or genomic action of ERs). The timing of E2 injections in our experiments is sufficiently long to permit genomic changes to occur, since I pre-treated the mice for three days prior to performing the experiment. Presumably the rise in E2 levels in mice in diestrus before the slices were prepared is also long enough to elicit genomic changes that could increase the response to DA. Alternatively, the effect of E2 in vivo might indirectly increase the response to DA by employing a polysynaptic mechanism, for example. Finally, it is possible that the lack of effect of acute ICI 182,780 on DA inhibition is merely the result of E2 acting on ERs outside of the VTA to change responses to DA in the VTA. Future experiments need to be performed to distinguish among these possibilities.

Ethanol excitation was enhanced in VTA neurons from gonadally intact mice in diestrus and OVX mice treated with E2. Increased ethanol-induced excitation of DA neurons during elevated E2 states would lead to increased DA release, and is predicted to enhance ethanol reward (Di Chiara and Imperato 1988; Weiss et al. 1993). Ethanol excites VTA neurons *in vivo*

(Gessa et al. 1985) and directly increases the spontaneous firing rate of dissociated VTA DA neurons (Brodie, Pesold, and Appel 1999). Ethanol excitation is retained under conditions that block synaptic transmission in brain slices containing the VTA (Brodie, Shefner, and Dunwiddie 1990) and in dissociated VTA DA neurons (Brodie, Pesold, and Appel 1999). The response to ethanol of VTA neurons in mice in proestrus, when circulating E2 levels are slightly higher (~8 pg/mL) than in diestrus, are comparable to responses in neurons in mice in diestrus. Although I did not measure responses of VTA DA neurons to DA during proestrus, when serum E2 levels in mice are slightly higher than in diestrus, I would expect that VTA DA neurons from mice in proestrus would behave in a similar manner to neurons from mice in diestrus in response to DA.

The increased sensitivity to ethanol of VTA DAergic neurons in mice in diestrus and in OVX mice treated with E2 suggests that behaviors related to the rewarding and reinforcing properties of ethanol will be enhanced when circulating E2 levels are high. Indeed, several studies have demonstrated that OVX mice and rats treated with E2 consume more ethanol compared with vehicle-treated OVX mice (Ford, Eldridge, and Samson 2004; Quirarte et al. 2007; Ford, Eldridge, and Samson 2002a; Marinelli, Quirion, and Gianoulakis 2003; Rajasingh et al. 2007; Reid et al. 2002; Reid, Hubbell, and Reid 2003). In addition, female rodents tend to consume more ethanol than male rodents (Becker and Koob 2016; Jury et al. 2017; Priddy et al. 2017; Rhodes et al. 2007). By re-examining the Brodie and Lasek laboratories' published studies of the ethanol responses of VTA DA neurons in male C57BL/6J mice (Brodie and Appel 2000; Dutton et al. 2017), I observed that the concentration-response to ethanol is similar between males and estrus females (when serum E2 concentrations are low). This might suggest that mice in diestrus or proestrus would consume more ethanol than male or estrus mice. However, several studies have found that ethanol consumption does not change throughout the estrous cycle in rats

(Ford, Eldridge, and Samson 2002b; Priddy et al. 2017; Roberts et al. 1998) and the Lasek lab has also not observed differences in ethanol consumption throughout the estrous cycle in freely cycling mice (Satta, Hilderbrand, and Lasek 2018). One reason for this may be that timing is critical when testing for behavioral differences. The estrous cycle in mice and rats is very short (~4 days) and within a specific cycle phase, hormone levels can change rapidly. Indeed, Roberts *et al* did find cycle phase differences in ethanol self-administration in rats when their cycles were synchronized using an agonist to the gonadotropin-releasing hormone receptor (Roberts et al. 1998). The sex difference in ethanol consumption observed in mice could be due to higher levels of E2 in females contributing to increased sensitivity of VTA DAergic neurons to excitation by ethanol and perhaps higher levels of drinking. This remains to be determined but is an important area for future investigation.

The ethanol-induced excitation of VTA DAergic neurons was assessed using high concentrations (40 -120 mM). Although the concentrations used here were relatively high, the Lasek laboratory and others have found that 4 hours of access to 20% ethanol in the "drinking in the dark" protocol routinely leads to blood ethanol concentrations of ~35-43 mM, with females at the high end of that range (Dutton et al. 2016; Rhodes et al. 2007; Satta, Hilderbrand, and Lasek 2018). In addition, the *in vitro* brain slice preparation may eliminate innervation from other brain regions that may contribute to the VTA response to ethanol, such as the serotonergic innervation from the Raphe nuclei (Brodie, Trifunovic, and Shefner 1995). Without these synaptic inputs, the VTA DA sensitivity to ethanol may be suppressed.

The Brodie laboratory has demonstrated previously that ethanol-induced excitation of VTA DAergic neurons from male mice is unaltered by GABAergic or glutamatergic antagonists (Nimitvilai et al. 2016). In the present study, I also found that the enhancement of ethanol-

stimulated increase in firing of VTA DA neurons by E2 is not altered by GABA receptor antagonists. Although E2 has been shown to inhibit GABA release in the striatum and hippocampus and alter expression of GABA-A receptor subunits (Huang and Woolley 2012; Schultz et al. 2009; Locci et al. 2017), these data suggest that the ability of E2 to increase ethanol-induced excitation of VTA DA neurons is likely not due to changes in GABA neurotransmission elicited by E2.

In addition to finding enhanced ethanol-induced excitation of VTA DAergic neurons from mice in diestrus compared with estrus, I observed that treatment of slices acutely with ICI 182,780 reduced ethanol excitation during diestrus, but not estrus. This suggests that ongoing signaling by membrane-bound ERs in the VTA is responsible for the enhanced excitation by ethanol, rather than the effects of ERs on transcription. These observations from mice in diestrus are consistent with the finding that treatment of mice with E2 increases excitation of VTA DA neurons by ethanol. ICI 182,780 is considered to be a "pure" ERα/β antagonist (Howell et al. 2000), but was recently found to act as an agonist at the G protein coupled ER, GPER1 (Thomas et al. 2005). I believe that the ability of ICI 182,780 to block ethanol-induced excitation of VTA DAergic neurons during diestrus is likely due to its inhibition of ERα and/or ERβ because activation of GPER1 by ICI 182,780 would have an estrogenic action rather than reversal of the estrogen effect on ethanol potency that we observed. The mechanism of action of ICI 182,780 on inhibiting ERα activity has been described on multiple levels including interfering with the formation of ERα dimers, inhibiting nuclear translocation, increasing protein degradation, and blocking rapid signaling by membrane-bound ERα (Howell et al. 2000; Dauvois, White, and Parker 1993; Parker 1993; Wade et al. 2001). ICI 182,780 also blocks ERβ-mediated transcriptional activity (Tremblay et al. 1997) and inhibits E2-mediated changes in

amphetamine-stimulated striatal DA release in female rats (Xiao, Jackson, and Becker 2003). The acute reduction of ethanol excitation by ICI 182,780 observed in VTA neurons from mice in the diestrus phase suggests that the increased sensitivity to ethanol during diestrus may be due to active engagement of ERs by E2 in the slice preparation. The E2-mediated enhancement of ethanol-stimulated increase in firing of VTA DA neurons might therefore be due to rapid signaling effects mediated by membrane-bound ERs in the VTA, rather than genomic or transcriptional actions of these receptors, although there is cross-talk between genomic and membrane E2 action observed in regions such as the hippocampus and hypothalamus that could be responsible for the enhancement of ethanol excitation of VTA DA neurons (Roepke 2009; Zhao et al. 2005).

In the next two chapters, I investigated which ER subtypes are involved in modulating DA and ethanol sensitivity of VTA DA neurons. I hypothesized that E2 is acting through ERα or ERβ. ERβ expression has been investigated using a transgenic reporter mouse (Milner et al. 2010) and specific antibodies (Kritzer and Creutz 2008) and is expressed in VTA DA and non-DA neurons. The Lasek laboratory has also performed fluorescent immunostaining for ERα in mouse VTA and have found that ERα is expressed in both VTA DA and non-DA neurons (R. Satta and A.W. Lasek, unpublished results). It is possible that the ability of E2 to affect responses of VTA DA neurons to ethanol and DA are through actions on either ERα and/or ERβ. Overall the data presented here indicate that elevated estrogen states, in both gonadally intact and E2-supplemented OVX mice, enhance ethanol excitation and DA inhibition.

2.5 Acknowledgements

Dr. Chang You performed some of the electrophysiological experiments. Thank you.

3. ESTRADIOL ENHANCES VTA DA SENSITIVITY TO ETHANOL THROUGH AN ERα AND MGLUR1-DEPENDENT MECHANISM

3.1 Introduction

Alcohol abuse places an enormous global economic and social burden on our society, and 5.1% of injury and disease is due to alcohol abuse (World Health Organization. Management of Substance Abuse Team). While less women abuse alcohol and begin drinking at an older age compared to men, this gender difference in the incidence of alcohol abuse is decreasing and women progress to dependence more rapidly (Cheng, Cantave, and Anthony 2016; Keyes, Grant, and Hasin 2008; Diehl et al. 2007; Ehlers et al. 2010). Despite their lower drinking levels compared to men, women are more vulnerable to the development of alcohol-related health problems such as liver damage and neurological dysfunction (Rehm et al. 2010; Flannery et al. 2007; Nixon, Tivis, and Parsons 1995; Eagon 2010). Because of this, women need more targeted sex-specific alcohol abuse prevention and treatments.

The factors underlying the gender differences in alcohol abuse may be sociological or biological. Hormones such as estrogen play a critical role in brain development, mood, cognition, pain sensitivity, and drug abuse (Vigil et al. 2016; Luine 2014; Henry et al. 2014; Wharton et al. 2012; Almey, Milner, and Brake 2015; Moran-Santa Maria, Flanagan, and Brady 2014). Female rodents drink more than males, and removing gonadal hormones by OVX reduces drinking in females, suggesting that hormones produced by the ovaries may drive sex differences in alcohol use (Barker et al. 2010; Ford, Eldridge, and Samson 2002a; Forger and Morin 1982; Satta, Hilderbrand, and Lasek 2018). Supplementation with E2 in OVX rodents increases ethanol intake (Satta, Hilderbrand, and Lasek 2018), while supplementation with progesterone does not

(Ford, Eldridge, and Samson 2002a), indicating the important role of E2 in regulating ethanol consumption in females.

The mesolimbic DAergic system, which consists of VTA DAergic neurons and their projections, plays an important role in mediating the rewarding and reinforcing effects of drugs of abuse (Wise 1987; Weiss et al. 1993). Ethanol increases the basal firing rate of VTA DAergic neurons *in vivo* (Gessa et al. 1985) and *in vitro* (Brodie, Shefner, and Dunwiddie 1990) and induces the release of DA from VTA neurons into their target regions such as the nucleus accumbens, which is also involved in alcohol abuse (Weiss et al. 1993). In Chapter 2, I showed that high E2 states in gonadally intact and OVX female mice increase the ethanol-stimulated increase in firing of VTA DAergic neurons. An acute treatment with an ERα/ERβ antagonist reverses this enhancement in neurons from females in the diestrus phase, suggesting that E2 actions on ethanol sensitivity in the VTA may be due to rapid signaling mechanisms (Vandegrift et al. 2017).

ERs can signal through genomic or non-genomic (rapid) signaling mechanisms (Vrtacnik et al. 2014). In addition to the GPER, ERα and ERβ are also associated with and signal from the membrane. Estrogen binding to membrane-bound ERs activates many rapid signaling pathways including those controlled by calcium, adenylate cyclase, phosphoinositide 3 kinase, mitogen-activated protein kinases (MAPK), tyrosine kinase, and CREB (Wu, Chen, and Brinton 2011; Titolo et al. 2008; Qiu et al. 2003; Cavalcanti et al. 2015). Activation of membrane-bound ERs can also converge with genomic (nuclear) ER signaling to alter transcription (Marino, Galluzzo, and Ascenzi 2006). Estrogen receptors can also transactivate GPCRs, such as mGluRs, on the membrane in a glutamate-independent manner (Seredynski et al. 2015; Boulware, Heisler, and Frick 2013). The type I mGluRs, mGluR1 and mGluR5, are coupled to $G_{q/11}$, and activation of

mGluR1 leads to increased phospholipase C (PLC) activity (Niswender and Conn 2010). Acamprosate, a compound that is clinically effective in decreasing relapse in patients with alcohol dependence (Mason 2001), may mediate these effects partially through its action on mGluR5 (Harris et al. 2002). Treatment with mGluR1 and mGluR5 antagonists decreases ethanol reward and consumption in male rodents (Backstrom et al. 2004; Lominac et al. 2006), but it is not known if this is mediated in part through mGluRs coupled to ER.

ERα and ERβ are both expressed in the VTA (Lasek Lab, unpublished), but it is not yet known which of these receptors and downstream signaling pathways might mediate E2 modulation of ethanol-induced excitation of VTA DAergic neurons. To identify which ER is involved, I used specific agonists and antagonists of ERα and ERβ in OVX and gonadally intact female mice. In order to determine if mGluR1 is required for the E2 enhancement of VTA DA sensitivity to ethanol, an mGluR1 antagonist was used in either OVX female mice supplemented with E2 or in gonadally-intact female mice in estrus or diestrus. I found that E2 enhances VTA DA sensitivity to ethanol through an ERα and mGluR1-depenedent mechanism in female mice.

3.2 Methods

3.2.1 Animals

Female C57BL/6J mice (8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were 10-14 weeks old at the time of experiments. Animal care adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and all procedures were approved by the UIC Animal Care Committee.

3.2.2 Vaginal Cytology

The estrous cycles of gonadally-intact female mice were assessed by vaginal cytology as previously described in Chapter 2. A large quantity of cornified epithelial cells in the smear is

present during estrus, while the cellular composition of smears from mice in diestrus II (herein referred to diestrus) is predominately leukocytes (Nelson et al. 1982). These phases differ in circulating E2 levels and are characterized by a longer duration compared to proestrus and metestrus/diestrus I (Nilsson et al. 2015).

3.2.3 Ovariectomy and drug treatments

The technique for mouse OVX has been described previously in Chapter 2 (Vandegrift et al. 2017; Hilderbrand and Lasek 2018). DPN, PPT, and E2-benzoate were prepared in solutions of sesame oil with 10% ethanol (VEH). DPN and PPT were prepared to a final concentration of 0.5 mg/ml, and mice were injected subcutaneously with ~1 mg/kg at volumes of 50 μL. Mice were treated once per day for three days, and mice were injected one hour prior to euthanization on the final day. For E2-treated mice, 50 μl of 0.2 μg E2 was injected subcutaneously on the first two days and 50 μl of 1 μg E2 on the third day. This dosage of E2 was chosen because it results in E2 plasma levels similar to proestrus levels (Vandegrift et al. 2017).

3.2.4 Extracellular recording & drug administration

VTA-containing brain slices were prepared for electrophysiology as previously described in Chapter 2 (Dutton et al. 2017; Brodie, Pesold, and Appel 1999). Putative DA VTA neurons were identified both anatomically and based on electrophysiological characteristics that have been well established in the literature and in the Brodie lab (Brodie, Shefner, and Dunwiddie 1990). Spike frequency (firing rate) was recorded and changes in firing rate were determined as previously described. A calibrated infusion pump was used to apply drugs to the aCSF from stock solutions prepared at 100-1000 times final concentration. In some of the experiments, the selective ERβ and ERα antagonists, 4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP) and 1,3-*Bis*(4-hydroxyphenyl)-4-methyl-5-[4-(2-

piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride (MPP; Tocris Biosciences, Minneapolis, MN) were administered via the micropipette by adding the drug to the microelectrode filling solution. In these cases, drugs were added to the 0.9% NaCl microelectrode filling solution and time was permitted for the antagonists to diffuse from the recording pipette into the extracellular space around the neuron being recorded.

3.2.5 Statistical analysis

Averaged numerical values are presented as the mean ± SEM. Statistical comparisons were made using one-way ANOVA, two-way ANOVA or two-way repeated measures ANOVA followed by Sidak's multiple comparisons tests as appropriate (Origin, Originlab, Northampton, MA or Prism, Graphpad Software, Inc., La Jolla, CA). A *P* value of < 0.05 was considered significant.

3.3 Results

3.3.1 VTA neuron characteristics

In this study, a total of 74 VTA neurons from 48 mice were recorded. The initial firing rates of these neurons ranged from 0.54 to 4.66 Hz, and the mean firing rate was 1.92 ± 0.11 Hz. There was a significant difference in baseline firing rate in the freely cycling mice with a mean firing rate of 2.28 ± 0.25 Hz during estrus and 1.71 ± 0.18 Hz during diestrus (t_{33}=1.89, P = 0.0338). There were no significant differences in baseline firing rate between the treated OVX mice with a mean firing rate of 2.34 ± 0.38 Hz in VEH-treated, 1.57 ± 0.23 Hz in PPT-treated, and 1.97 ±0.33 Hz in DPN-treated OVX mice (one-way ANOVA, $F_{2,25}$ = 1.452, P = 0.2532). All of the neurons in this study conformed to the rate and patterns of VTA DAergic neurons as previously described in Chapter 2.

3.3.2 Activation of estrogen receptor α enhances sensitivity of VTA DAergic neurons to ethanol in ovariectomized mice

I demonstrated in Chapter 2 that E2 enhances the ethanol-induced excitation of VTA DA neuronal firing in OVX female mice (Vandegrift et al. 2017). The Lasek lab has shown that both ERα and ERβ are expressed in VTA DAergic neurons (Lasek Lab, unpublished). In order to determine which ER regulates the sensitivity of VTA DAergic neurons to ethanol, I tested ethanol-induced excitation (40-120 mM) of VTA DAergic neurons from OVX female mice treated with PPT (an ERα-specific agonist), DPN (an ERβ-specific agonist), or VEH. VTA neurons from OVX mice treated with PPT showed significantly higher ethanol-induced excitation compared with neurons from DPN-treated and VEH-treated mice. (Figure 10, n= 10-11 per group, two-way RM ANOVA, concentration: $F_{2,56} = 57.02$, $P < 0.0001$; treatment: $F_{2,28} = 4.05$, $P = 0.0284$; concentration x treatment interaction: $F_{4,56} = 0.0237$, $P = 0.0237$). In response to bath application of 80 mM ethanol, neurons from PPT-treated mice had an increase in firing rate of 18.5 ± 6.2% whereas neurons from DPN- and VEH-treated mice had an increase in firing rate 7.7 ± 5.7% and 11.1 ± 9.3%, respectively. Post-hoc Holm-Sidak's multiple comparisons test demonstrated a significant increase in ethanol-stimulated increase in firing in VTA DAergic neurons from mice treated with PPT compared with VEH in response to 80 mM ($P < 0.05$) and 120 mM ($P < 0.05$) ethanol. This finding indicates that activation of ERα enhances the response of VTA DA neurons to ethanol in OVX mice. These results suggest that the E2-induced enhancement of ethanol sensitivity I previously reported is likely due to activation of ERα.

3.3.3 Estrogen receptor α in the VTA acutely regulates ethanol sensitivity in freely cycling female mice

In order to determine whether ERα in the VTA acutely regulates ethanol sensitivity in gonadally intact female mice at different estrous cycle phases, I administered MPP, an ERα-specific antagonist, and PHTPP, an ERβ-specific antagonist, to neurons using the recording pipette during the recordings. This pipette delivery method has been successfully used by the Brodie lab and was described in Chapter 2 (Nimitvilai, Arora, and Brodie 2012; Vandegrift et al. 2017). In this protocol, 80 mM ethanol was tested initially during the recording and 60 minutes later, which allowed time for the antagonist to diffuse from the recording pipette onto the cell of interest. A 60-minute treatment with MPP had no effect on the excitatory response to 80 mM ethanol of VTA DAergic neurons from mice in estrus, but decreased the excitatory response of ethanol of VTA DAergic neurons from mice in diestrus (Figure 11 A-C, n = 5-6 per group, two-way RM ANOVA, treatment: $F_{1,9} = 7.455$, $P = 0.0232$; phase: $F_{1,9} = 3.504$, $P = 0.0940$; treatment x phase interaction: $F_{1,9} = 10.50$, $P = 0.0102$). Post-hoc Holm-Sidak's multiple comparisons test demonstrated a significant difference in ethanol-induced excitation in estrus and diestrus before treatment with MPP, consistent with my previous findings (Vandegrift et al. 2017). After allowing 60 min for the diffusion of MPP from the pipette, there was no longer a difference in ethanol-induced excitation in diestrus compared to estrus ($P = 0.6014$). Neurons from mice in the diestrus phase initially responded to 80 mM ethanol with a 15.9 ± 0.8% increase in firing rate, but after MPP delivery, the response to 80 mM ethanol was only 9.6 ± 1.3%. In contrast, neurons from mice in the estrus phase initially responded to 80 mM ethanol with a 9.9 ± 1.4% increase in firing rate, and after MPP delivery, the response to 80 mM ethanol was similar (10.5 ± 1.1% increase). These results suggest that ERα acutely regulates ethanol sensitivity in the VTA in an estrous cycle phase-dependent manner.

In contrast, PHTPP did not affect ethanol sensitivity of VTA DA neurons from mice in estrus or diestrus. Consistent with my previous results in Chapter 2 (Vandegrift et al. 2017), there was a significant main effect of estrous phase, indicating enhanced ethanol excitation in neurons from mice in diestrus compared to estrus (Figure 11 D-F, n = 5-6 per group, two-way RM ANOVA, treatment: $F_{1,9} = 0.1836$, $P = 0.6784$; phase: $F_{1,9} = 7.174$, $P = 0.0253$; treatment x phase interaction: $F_{1,9} = 0.0879$, $P = 0.7735$). Neurons from mice in diestrus initially responded to 80 mM ethanol with an 18.1 ± 7.5% increase in firing rate, and after 60 minutes PHTPP delivery, the response to 80 mM ethanol was a 17.4 ± 10.0% increase in firing rate. This finding suggests that ERβ does not acutely regulate ethanol sensitivity in the VTA. Together, these results indicate that ERα, but not ERβ, enhances the ethanol-induced excitation of VTA DA neurons in female mice, but only during a specific phase of the estrous cycle when E2 levels are rising.

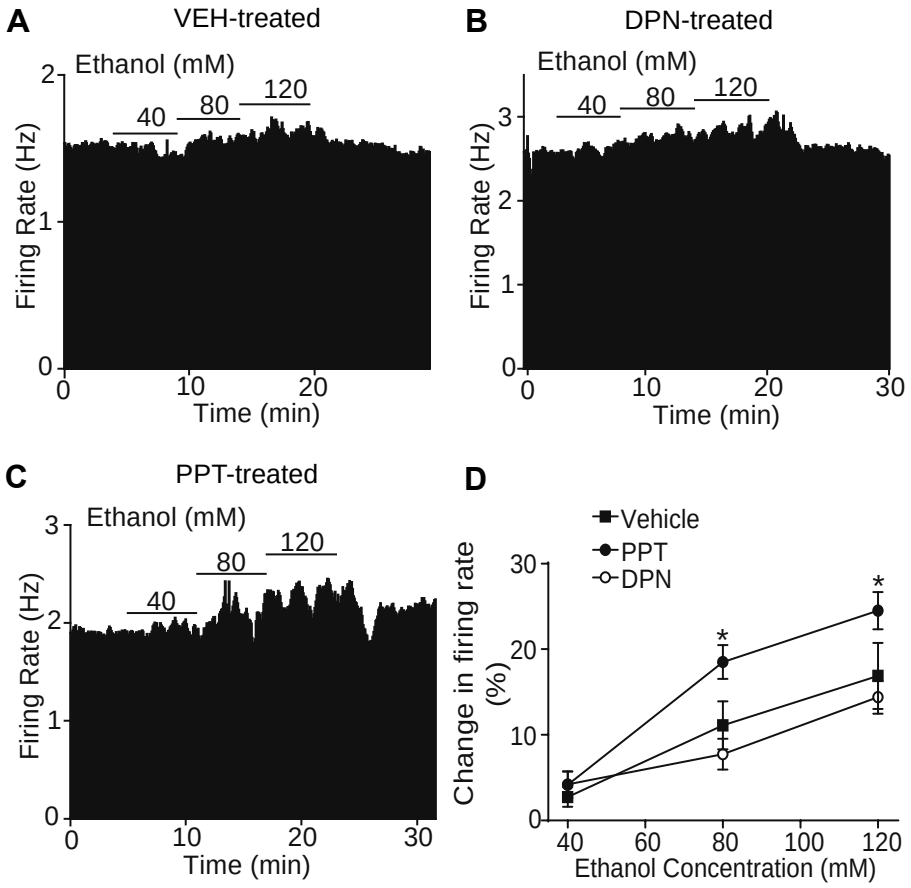

Figure 10: The ERα-specific agonist PPT enhances ethanol excitation of VTA DA neurons.

Extracellular recordings were obtained from VTA DA neurons in OVX mice previously treated systemically with DPN, PPT, or VEH (A-C) Representative rate meter graphs showing responses to 40-120 mM ethanol of a representative VTA DA neuron from an OVX mouse treated with VEH (A), DPN (B), or PPT (C). (D) Pooled concentration-response graphs shows ethanol (40, 80, and 120 mM) responses in VTA DA neurons. OVX mice treated with PPT (n=10), but not DPN (n=10), show enhanced excitation to ethanol compared with VEH (n=10). *P < 0.05 by post hoc Holm-Sidak's multiple comparisons test.

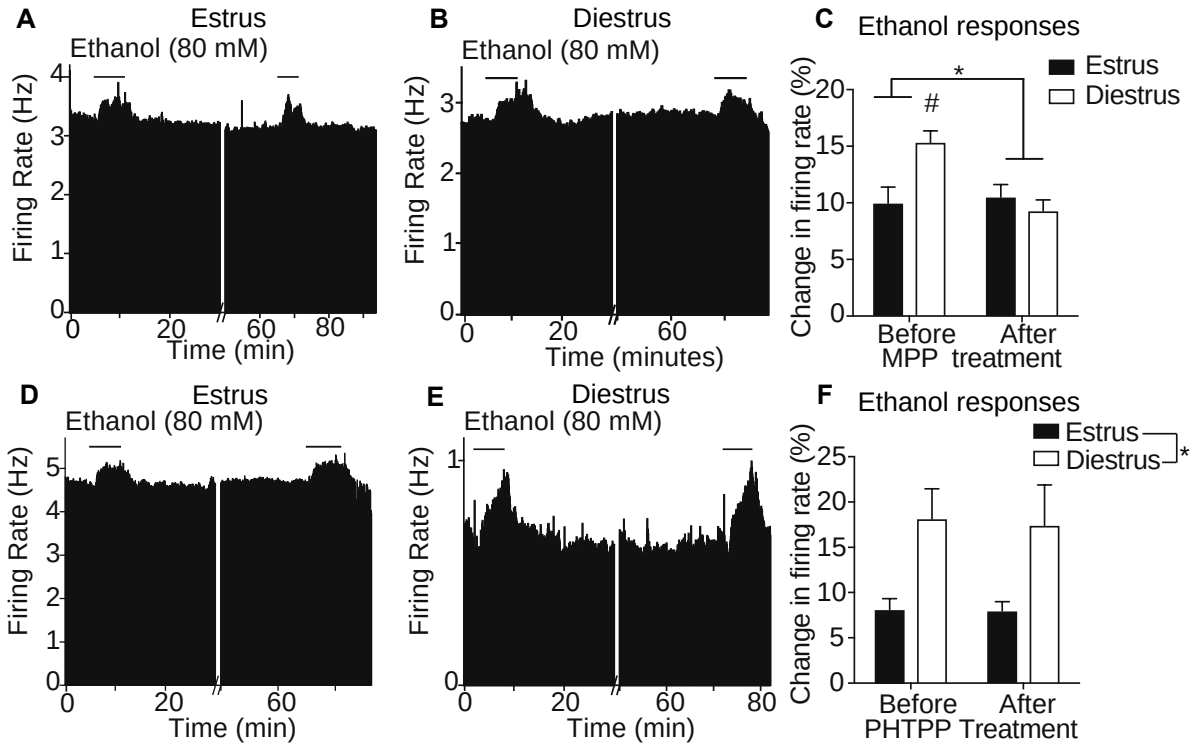

Figure 11: ER α antagonist in the VTA acutely reduces the sensitivity of DA neurons to ethanol from mice in diestrus.

(A-B) Representative rate meter graphs showing response to 80 mM ethanol before and during treatment with MPP in slices containing the VTA from mice in estrus (A) and diestrus (B). (C) Pooled responses to 80 mM ethanol of VTA DA neurons from mice in estrus (n=5) and diestrus (n=6) before and after treatment of slices with MPP. The enhanced VTA DA neuron excitation to ethanol that was observed in mice from diestrus was decreased after treatment with MPP, whereas the ethanol responses of DA VTA neurons from mice in estrus are unchanged by MPP. *$P < 0.05$ by two-way RM ANOVA, $^{\#}P < 0.05$ between mice and estrus and diestrus before MPP treatment by post-hoc Sidak's multiple comparisons test. (D-E) Representative rate meter graphs showing responses to 80 mM ethanol before and during treatment with PHTPP in slices containing the VTA from mice in estrus (D) and diestrus (E). (F) Pooled responses to 80 mM ethanol of VTA DA neurons from mice in estrus (n=6) and diestrus (n=5) before and after treatment of slices with PHTPP. There was no change in ethanol excitation with PHTPP, but there was a significant effect of cycle phase. *$P < 0.05$ by two-way ANOVA.

3.3.4 Metabotropic glutamate receptor 1 is required for the estradiol-induced enhancement of ethanol excitation

E2 has been shown to activate mGluRs in hippocampal (Huang and Woolley 2012) and striatal neurons (Grove-Strawser, Boulware, and Mermelstein 2010). In order to determine if a similar mGluR activation occurs in the E2-mediated modulation of ethanol-simulated firing, the response to 40-120 mM ethanol was measured in DA neurons from gonadally-intact females in either diestrus or estrus before and after 45 minutes of bath application of the mGluR1 antagonist, JNJ 16259685 (0.2 μM) or DMSO vehicle. JNJ16259685 did not have an effect on ethanol-stimulated increase in firing in VTA DAergic neurons from females in estrus (Figure 12 A, C, n = 5 per group, two-way RM ANOVA, treatment: $F_{1,4} = 0.1755$, $P = 0.6968$; concentration: $F_{2,8} = 60.66$, $P < 0.0001$; treatment x concentration interaction: $F_{2,8} = 1.512$, $P = 0.2773$), but did decrease excitation of DA neurons to ethanol from females in diestrus (Figure 12 B, D, n = 6 per group, two-way RM ANOVA, treatment: $F_{1,5} = 7.037$, $P = 0.0453$; concentration: $F_{2,10} = 7.290$, $P = 0.0111$; treatment x concentration interaction: $F_{2,10} = 2.173$, $P = 0.1634$). As an example, neurons from mice in the diestrus phase initially responded to 80 mM ethanol with an increase in firing rate of 12.9 ± 1.4%, and after 45 minutes of incubation with JNJ16259685, the neurons responded to ethanol with an increase in firing rate of 6.2 ± 2.4%. This finding suggests that mGluR1 is required for the enhancement of ethanol excitation in gonadally-intact females during diestrus.

In order to further investigate the role of E2 in the mGluR1-dependent enhancement of ethanol excitation, OVX female mice were supplemented with E2 or VEH for 3 days prior to electrophysiological experiments. I measured the response to 40-120 mM ethanol in VTA DAergic neurons from OVX mice treated with E2 or VEH before and after 45 minutes of bath

application of the mGluR1 antagonist, JNJ16259685 (0.2 µM) or DMSO vehicle. Similar to my findings in mice in estrus, mGluR1 inhibition did not have an effect on ethanol-stimulated increase in firing of VTA DAergic neurons from OVX mice treated with VEH (Figure 13 A, C, n = 5 per group, two-way RM ANOVA, treatment: $F_{1,4} = 0.5215$, P = 0.5102; concentration: $F_{2,8} = 21.47$, P = 0.0006; treatment x concentration interaction: $F_{2,8} = 1.386$, P = 0.3401), whereas JNJ16259685 decreased excitation of VTA DAeric neurons to ethanol from OVX mice treated with E2 (Figure 13 B, D, n = 6 per group, two-way RM ANOVA, treatment: $F_{1,5} = 10.44$, P = 0.0232; concentration: $F_{2,10} = 23.51$, P = 0.0002; treatment x concentration interaction: $F_{2,10} = 2.463$, P = 0.1350). For example, neurons from E2-treated OVX mice initially responded to 80 mM ethanol with an increase in firing rate of 18.6 ± 2.8%, and after 45 minutes of JNJ16259685 exposure, the neurons responded to ethanol with an increase in firing rate of 9.9 ± 3.1%. These results suggest that E2-mediated enhancement of excitation by ethanol is through a mGluR1-dependent mechanism.

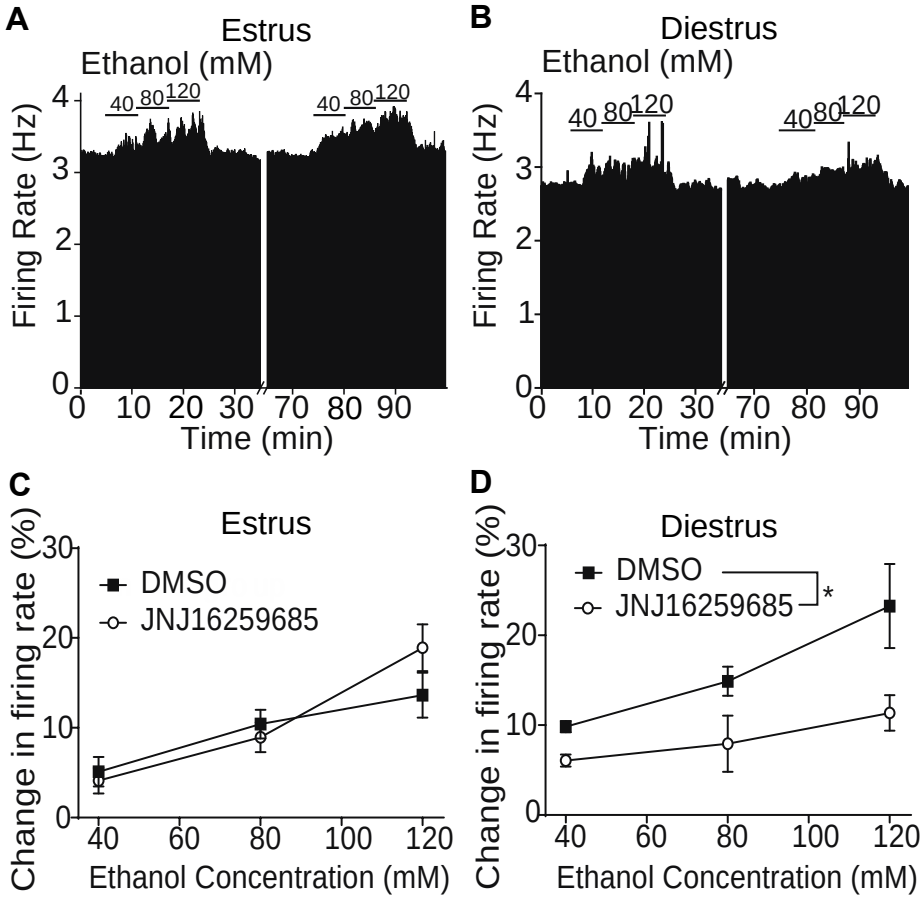

Figure 12: An mGluR1 antagonist blocks enhancement of ethanol excitation of VTA DA neurons from mice in diestrus.

(A-B) Representative rate meter graphs showing response to 40-120 mM ethanol before and after JNJ in slices containing the VTA from mice in estrus (A) and diestrus (B). (C) Pooled responses to 40-120 mM ethanol of VTA DA neurons from mice in estrus (n=5) before and after treatment of slices with JNJ shows no change in ethanol-stimulated increase in firing (D) Pooled responses to 40-120 mM ethanol of VTA DA neurons from mice in diestrus (n=6) before and after treatment of slices with 0.2 µM JNJ. The ethanol excitation of neurons from females in diestrus was significantly reduced after JNJ. *$P < 0.05$ by two-way RM ANOVA.

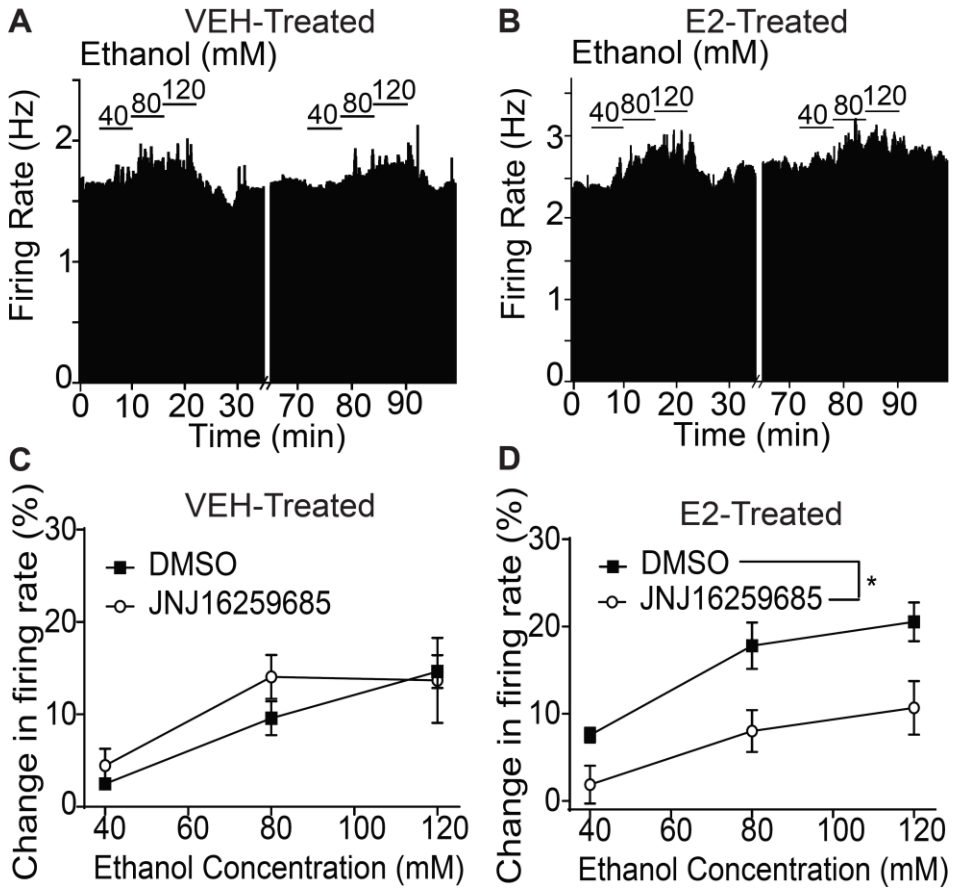

Figure 13: An mGluR1 antagonist blocks enhancement of ethanol excitation of VTA DA neurons in E2-treated OVX mice.

(A-B) Representative rate meter graphs showing response to 40-120 mM ethanol before and after JNJ16259685 in slices containing the VTA from OVX mice treated with VEH (A) or E2 (B). (C) Pooled responses to 40-120 mM ethanol of VTA DA neurons from VEH-treated OVX mice (n=5) before and after treatment of slices with JNJ16259685 shows no change in ethanol-stimulated increase in firing. (D) Pooled responses to 40-120 mM ethanol of VTA DA neurons from E2-treated OVX mice (n=6) before and after treatment of slices with 0.2 µM JNJ16259685. The ethanol excitation of neurons from E2-treated OVX mice was significantly reduced after JNJ16259685. *$P < 0.05$ by two-way RM ANOVA.

3.4 Discussion

My results show that E2 enhances the ethanol-induced excitation of VTA DAergic neurons through activation of ERα and mGluR1. Here I tested the ethanol responses of VTA DAergic neurons using specific ER agonists in OVX mice and ER antagonists in freely-cycling female mice. I also tested the ethanol responses of VTA DAergic neurons from OVX mice and freely-cycling female mice using a specific antagonist for mGluR1.

Ethanol excitation was enhanced in VTA neurons from OVX mice treated with PPT (ERα agonist) compared to DPN (ERβ agonist) and VEH. The concentrations of PPT and DPN were chosen to balance receptor selectivity with occupancy (Sepehr et al. 2012; Hilderbrand and Lasek 2018). The increased ethanol-stimulated increase in firing of neurons from OVX mice treated with an ERα agonist predicts that activation of ERα by E2 would lead to increased DA release, which may contribute to increased rewarding and reinforcing effects of ethanol (Di Chiara and Imperato 1988; Weiss et al. 1993).

Consistent with my results with the ERα/ERβ antagonist ICI 182780 that I showed in Chapter 2, an acute treatment with MPP, a selective ERα antagonist, reduced ethanol excitation during diestrus, but not estrus. An acute treatment with PHTPP, an ERβ antagonist, did not change ethanol excitation in estrus or diestrus. These results indicate that activation of ERα is required to mediate the enhancement of ethanol-stimulated increase in firing during diestrus. Together, the agonist and antagonist experiments indicate that activation of ERα, but not ERβ, increases the sensitivity of VTA DAergic neurons to ethanol in female mice.

The enhancement of ethanol sensitivity in the VTA by ERα may affect alcohol drinking by females (Di Chiara and Imperato 1988; Weiss et al. 1993). Female rodents generally drink more ethanol than males (Priddy et al. 2017; Satta, Hilderbrand, and Lasek 2018). Because E2-

treated and gonadally intact female mice drink more than OVX and male mice, increased drinking by females may be due, in part, to the actions of E2 through activation of ERα in the VTA (Ford, Eldridge, and Samson 2004, 2002a). Although alcohol consumption does not appear to change along the estrous cycle of rodents, the rapid nature of the 4-day estrous cycle and the timing of drinking protocols may mask E2 effects on alcohol consumption in females (Satta, Hilderbrand, and Lasek 2018; Ford, Eldridge, and Samson 2002b).

The VTA also plays a critical role in ethanol reward, as measured by the CPP test. ER activity could also could also increase ethanol CPP (Bechtholt and Cunningham 2005). While E2 enhances ethanol CPP in OVX female mice, this requires activation of both ERα and ERβ (Hilderbrand and Lasek 2018). Although my results indicate that activation of only ERα is required for the E2-enhancement of ethanol stimulated firing of VTA neurons, ERβ may also play a role in ethanol reward and reinforcement. The exact roles of ERα and ERβ on ethanol reward needs to be further investigated.

Treatment of slices with the mGluR1 antagonist JNJ16259685 significantly reduced ethanol-induced excitation of VTA DAergic neurons from mice in diestrus and in E2-treated OVX mice, but not from mice in estrus and in VEH-treated OVX mice. These results indicate that mGluR1is required for E2-mediated enhancement of ethanol-stimulated increase in firing of VTA DAergic neurons. It is unknown, however, what role mGluR1 plays in enhancing ethanol sensitivity and whether ERα directly or indirectly interacts with mGluR1. Membrane bound ERs co-immunoprecipitate with mGluRs in experiments with cultured cells, and ERs are functionally coupled to mGluRs in the hippocampus, striatum, and preoptic nucleus (Dewing et al. 2007; Grove-Strawser, Boulware, and Mermelstein 2010; Oberlander and Woolley 2016). In addition

to the ‘rapid’ non-genomic effects, ER/mGluR signaling may also have downstream effects on gene transcription (Meitzen and Mermelstein 2011).

ERα/mGluR1 signaling can affect VTA DA firing through direct or indirect mechanisms. There are sex-specific and brain region-specific effects of ER/mGluR on CREB phosphorylation, L-type calcium channel currents, and mitogen-activated protein kinase (MAPK) signaling (Boulware et al. 2005; Grove-Strawser, Boulware, and Mermelstein 2010). In the hippocampus, E2 suppresses the inhibition of CA1 pyramidal neurons by GABA, and this is through ERα/mGluR1 activation and retrograde endocannabinoid action through CB1R on inhibitory inputs (Huang and Woolley 2012). Interestingly, this was observed in females, but not males. A similar mechanism could be occurring in VTA DA neurons where ERα activation of mGluR1 could lead to the suppression of inhibitory inputs through endocannabinoids acting on the CB1Rs of GABAergic neurons. In VTA neurons from male mice, JNJ16259685 induced LTD of inhibitory currents through CB1R and reduced cocaine CPP (Yu et al. 2013). Future studies should investigate the mechanism of E2-mediated enhancement of ethanol sensitivity through mGluR1 activation.

ER/mGluR signaling plays a role in sexual behaviors in both male and female animals. ERα activation of mGluR1 in the arcuate nucleus of the hypothalamus enhances sexual receptivity in female rodents (Dewing et al. 2007). Interactions between ERβ and mGluR1 also enhance sexual behaviors in male Japanese quail (Seredynski et al. 2015). In addition to the role in natural rewards, there is evidence for ER/mGluR signaling to play important roles in drug reward (Tonn Eisinger et al. 2018). In rodents, E2-induced enhancement of cocaine intake in OVX females is through mGluR5 activation, and an antagonist of mGluR5 reverse the E2 enhancement of cocaine self-administration (Martinez et al. 2016). Amphetamine-induced DA

release in the striatum is enhanced by E2, and mGluR5 is required for this effect (Song et al. 2019). mGluRs play important roles in alcohol abuse, for example mGlur5 antagonists reduce alcohol consumption and relapse (Backstrom et al. 2004; Schroeder, Overstreet, and Hodge 2005; Schroeder et al. 2008). Future studies should therefore investigate how the interactions between ERs and mGluRs may modulate ethanol reward and abuse in females.

4. ESTRADIOL ENHANCES VTA SENSITIVITY TO DA THROUGH ACTIVATION OF ERα AND ERβ

4.1 Introduction

Hormones such as E2 have been implicated in natural and drug reward processes (Yoest, Cummings, and Becker 2014). The VTA and the mesolimbic DAergic pathway play a central role in motivation and rewarding effects of natural and drug rewards, and E2 actions on DAergic pathways regulates reproductive behavior (Foreman and Hall 1987; Balthazart, Baillien, and Ball 2002) and drug abuse (Calipari et al. 2017; Carroll et al. 2004). More broadly, this interplay between estrogen and DA systems may also play important roles in the influence of gender on the incidence of many psychiatric diseases such as schizophrenia, Alzheimer's, and Parkinson's disease (Watson et al. 2010). Understanding the mechanisms of hormonal effects on DA neurotransmission is important for developing therapeutics that will be effective in both sexes.

Previous studies have demonstrated sex differences in DAergic systems which may drive sex differences in drug abuse (Becker 1999). In humans, females exhibit increased cortical and striatal AMPH-induced DA release (Riccardi et al. 2006) and ratios of DA metabolite DOPAC/DA in the putamen, which suggests females have higher turnover rates for DA (Konradi et al. 1992). Females also have higher levels of cortical D2R (Kaasinen et al. 2001) and DAT in the striatum, diencephalon, and brainstem compared to males (Staley et al. 2001).

Rodent studies have also shown sex differences in DAergic neurotransmission, and hormones may underlie some of these differences (Becker 1999). Striatal DA concentrations vary throughout the estrous cycle of rats, with higher levels in proestrus and estrus compared with diestrus and after OVX. Importantly, decreased DA concentrations after OVX are partially recovered by E2 supplementation (Xiao and Becker 1994). Administration of E2 enhances

amphetamine-stimulated DA release in OVX females but not in castrated or intact male mice (Becker 1990). These studies indicate that striatal DA concentrations and DA release are regulated by E2, and E2 may be driving some of the sex differences in DA neurotransmission and ultimately drug abuse.

There are sex differences in D2R ligand binding, with decreased D2R binding in the rostral lateral striatum in castrated males compared to OVX females, and there is differential regulation of D2R binding in a brain region-specific and sex-specific manner 30 minutes and 4 hours after E2 treatment (Bazzett and Becker 1994). E2 increases D2R in the striatum and nucleus accumbens of female rats through activation of ERβ (Le Saux, Morissette, and Di Paolo 2006). These studies show that E2 can modulate the density and ligand binding of D2R in a sex specific manner. Activation of ER has been shown to have cellular effects in the VTA of female rodents. For instance, 27 days of E2 treatment via implant in rats resulted in increased expression of the D3R gene in the VTA (Zhou, Cunningham, and Thomas 2002). The exact mechanisms of E2 effects on DAergic neurotransmission are not fully known, thus investigating these mechanisms may elucidate potential therapeutic targets for dysregulated DAergic systems in conditions such as Parkinson's disease and substance use disorders.

I demonstrated in Chapter 2 that high E2 states in female mice enhance the inhibitory effects of DA on VTA DA neurons in both gonadally-intact and OVX female mice (Vandegrift et al. 2017). It was unknown whether activation of ERα or ERβ modulates DA sensitivity in female VTA DAergic neurons. Here I determined whether ERα or ERβ mediate the enhanced inhibition of VTA DAergic neurons by DA. I used OVX mice treated with DPN and PPT and performed electrophysiology experiments. I found that activation of ERα or ERβ alone does not

modulate DA inhibition, however, activation of both ERα and ERβ mimics the enhancement of DA inhibition of VTA DAergic neurons by E2 (Vandegrift et al. 2017).

4.2 Methods

4.2.1 Animals

Female C57Bl/6J mice (8 weeks old) were ordered from the Jackson Laboratory (Bar Harbor, ME, USA), and electrophysiological experiments were conducted between 10-14 weeks of age. Animal care adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and all procedures were approved by the UIC the Animal Care Committee.

4.2.2 Ovariectomy and in vivo drug treatments

OVX and *in vivo* drug treatments were performed as described in Chapter 2 (Hilderbrand and Lasek 2018; Vandegrift et al. 2017). Briefly, the ERβ agonist (DPN), and the ERα agonist (PPT) were prepared in a sesame oil with 10% ethanol vehicle (VEH). Mice were treated with PPT or DPN SC for three days, and the last injection was done 1 hour prior to sacrifice on the last day of treatment. Mice that were treated with either PPT or DPN, received one 50 µl injection per day of 1 mg/kg DPN or PPT (Hilderbrand and Lasek 2018; Sepehr et al. 2012). Mice that were treated with both PPT (1 mg/kg) and DPN (1 mg/kg) received two sequential 50 µl injections per day, and the control mice were treated with two sequential 50 µl VEH injections a day for a total of three days.

4.2.3 Brain slice preparation and extracellular recording

For the electrophysiological studies, mice were sacrificed 1 hour after the last injection. VTA-containing brain slices were prepared as described in Chapter 2 (Brodie et al. 1999). DA was administered during the electrophysiological experiments by adding stock solutions (100 to 1000 times the final concentrations) into the aCSF via a calibrated infusion pump, which allowed

the DA to fully mix with the aCSF prior to reaching the chamber. The final concentration was determined according to the flow rate and concentration of the stock solution.

4.2.4 Statistical Analysis

Averaged numerical values are presented as the mean ± SEM. All other statistical comparisons were made using two-way repeated measures ANOVA followed by Sidak's multiple comparisons tests as appropriate (Prism, Graphpad Software, Inc., La Jolla, CA). A ***P*** value of < 0.05 was considered significant.

4.3 Results

4.3.1 VTA neuron characteristics

A total of 31 neurons from 20 mice were used for electrophysiological recordings. The baseline firing rates ranged from 0.79 to 3.76 Hz with an average firing rate of 1.82 ± 0.13 Hz. There were no significant differences in firing rates between the VEH-, DPN-, and PPT-treated groups (VEH: 1.26 ± 0.31 Hz, DPN: 1.8 ± 0.25 Hz, PPT: 1.55 ± 0.33 Hz, one-way ANOVA, $F_{2,17} = 0.09$, $P = 0.9346$) or between the VEH+VEH- and DPN+PPT-treated groups (VEH+VEH: 2.12 ± 0.37 Hz, DPN+PPT: 1.39 ± 0.15 Hz, $t_5 = 7.29$, $P = 0.077$).

4.3.2 DPN and PPT alone do not affect DA inhibition of VTA DA neurons

I have previously shown that high levels of E2, in both gonadally-intact and OVX female mice treated with E2, enhances the inhibitory effects of DA on VTA DAergic neurons (Vandegrift et al. 2017). In order to determine whether activation of ERα or ERβ mediates this increase in DA sensitivity, OVX mice were treated subcutaneously for three days with selective agonists prior to electrophysiology experiments. Because our group has shown ERα and ERβ are expressed in the VTA (Rosalba Satta, Rex Tai, and Amy Lasek, unpublished), OVX mice were treated with agonists for ERα (PPT; 1 mg/kg), ERβ (DPN; 1 mg/kg) or vehicle (VEH). There

were no differences in DA inhibition (0.5-10 μM) among the VEH-, DPN-, and PPT-treated mice (Figure 14, n = 5-8/group, two-way RM ANOVA, concentration: $F_{4,68} = 52.89$, $P < 0.0001$; treatment: $F_{2,17} = 0.2084$, $P = 0.8139$; concentration x treatment interaction: $F_{8,68} = 1.328$, $P = 0.2446$). For example, neurons from OVX mice treated with VEH responded to 5 μM DA with a -62.0 ± 14.8% decrease in firing rate, and mice treated with DPN or PPT responded to the same concentration of DA with -68.7 ± 11.9% and -57.5 ± 13.0% decreases in firing rates, respectively. These results suggest that activation of ERα or ERβ alone does not affect the sensitivity of VTA DA neurons to DA.

4.3.3 Combined treatment with DPN and PPT enhance DA inhibition of VTA DA neurons from ovariectomized mice

Because treatment with individual agonists selective for ERα or ERβ did not affect the sensitivity of VTA DAergic neurons to DA, I hypothesized that activation of both ERα and ERβ may be required to enhance DA sensitivity. To test this hypothesis, OVX mice were treated for three days with 50 μl of 1 mg/kg DPN and 50 μl of 1 mg/kg PPT (DPN+PPT) or two injections of 50 μl VEH (VEH+VEH) each day. Neurons from OVX mice treated with DPN+PPT showed an enhanced sensitivity to DA compared to OVX mice treated with VEH+VEH (Figure 15, n = 5-6/group, two-way RM ANOVA, concentration: $F_{4,36} = 55.06$, $P < 0.0001$; treatment: $F_{1,9} = 6.352$, $P = 0.0327$; concentration x treatment interaction: $F_{4,36} = 5.051$, $P = 0.0025$). Sidak's post-hoc multiple comparisons test demonstrated a significant difference between the response to 2 μM ($P = 0.0018$) and 5 μM ($P = 0.0063$) DA between DPN+PPT and VEH+VEH treated OVX mice. OVX mice treated with VEH+VEH responded to 5 μM DA with a 55.8 ± 14.0% reduction in firing rate while DPN+PPT treated OVX mice responded to 5 μM DA with a 100 ± 0%

reduction in firing rate. These results suggest that activation of both ERα and ERβ are required to enhance the DA sensitivity of VTA DA neurons.

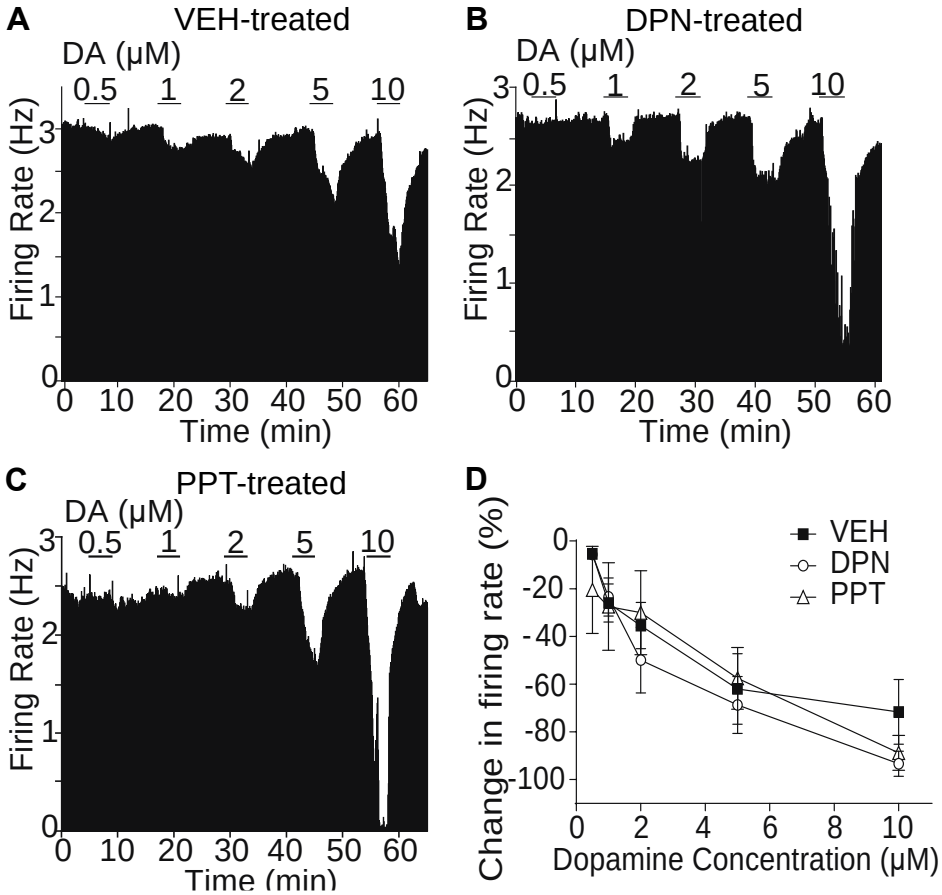

Figure 14: DPN and PPT alone do not affect DA inhibition of VTA DA neurons from OVX mice.

Extracellular recordings were performed in the VTA from OVX mice that were previously treated with DPN, PPT, or VEH for three days. Representative ratemeter graphs showing the effects of (a) VEH (n = 8), (b) DPN (n = 8), and (c) PPT (n = 5) treatment in OVX females on DA inhibition of VTA DA neurons. (d) Pooled DA-concentration response curves, showing that inhibition by DA is unaffected by DPN or PPT treatment alone compared to VEH.

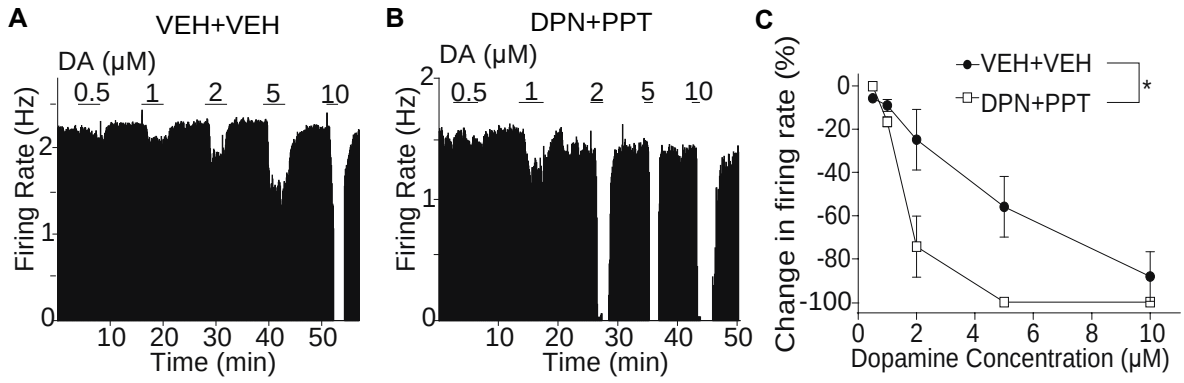

Figure 15: Combined treatment with DPN and PPT enhance DA inhibition of VTA DA neurons from OVX mice.

Extracellular recordings were performed in the VTA from OVX mice that were previously treated with two treatments of VEH per day or with both DPN and PPT for three days. Representative ratemeter graphs showing the effects of (a) VEH+VEH (n = 6) and (b) DPN+PPT (n = 5) treatment. (c) Pooled DA-concentration response curves, showing that DPN+PPT treatment enhances inhibition by DA compared to VEH-VEH treatment in OVX female mice. *P < 0.5 by two-way RM ANOVA; **P < 0.005 by Sidak's post hoc multiple comparisons

4.4 Discussion

I demonstrated in Chapter 2 that high E2 states enhance the inhibition by DA in VTA neurons in female mice (Vandegrift et al. 2017). The goal of the experiments in this chapter were to determine which ERs modulate the DA sensitivity of VTA DA neurons. Here I have shown that activation of both ERα and ERβ is required to enhance VTA DA inhibition in OVX female mice. I found that separate treatment with an ERα (PPT, 1 mg/kg) or ERβ (DPN, 1 mg/kg) agonists did not alter VTA DA responses. OVX mice treated with both ERα and ERβ agonists, however, showed increased sensitivity to DA. These results suggest that the ability of E2 to increase the inhibitory effect of DA on VTA neurons is through activation of both ERα and ERβ.

The dosage of DPN+PPT treatments may have a stronger effect on the DA inhibition compared to the E2 dosage from Chapter 2. In Chapter 2 I showed that OVX mice treated with E2 responded to 5 μM DA with a 65.6 ± 13.4% reduction in firing rate while DPN+PPT treated OVX mice responded to 5 μM DA with a 100 ± 0% reduction in firing rate. This may be due to stronger receptor occupancy at the doses of PPT and DPN that I used. PPT has a higher affinity to full length ERα and a truncated isoform of ERα compared to E2 (Lin et al. 2013; Stauffer et al. 2000).

I demonstrated in Chapter 2 that an acute treatment of VTA slices with an antagonist of ERα and ERβ (ICI 182,780) did not alter the DA sensitivity of VTA neurons (Vandegrift et al. 2017), suggesting that the effects of E2 on DA inhibition are not through rapid ER signaling in the VTA DA neurons. My results instead suggest that E2 alters VTA DA sensitivity through a process requiring more time to take effect. This might be due to transcriptional changes induced by E2. Upon ligand binding, ERs can form homo- and heterodimers which regulate gene transcription in the nucleus (Cowley et al. 1997; Paulmurugan et al. 2011; Kuntz and Shapiro

1997). It is unknown if simultaneous activation of ERα/ERα and ERβ/ERβ homodimers or activation of an ERα/ERβ heterodimer is required to modulate DA sensitivity in the VTA. In order to investigate the possible role of heterodimer activation in DA sensitivity of VTA neurons, agonists specific for heterodimers, but not homodimers, could potentially be used. Compounds that specifically promote ER heterodimer formation have been identified and could potentially be used in future experiments (Coriano et al. 2018). Alternatively, activation of ERα and ERβ in separate brain regions may also mediate these effects, and further studies will be needed to investigate the mechanism of ERα and ERβ activation in DA sensitivity.

The VTA is integral in the development of ethanol reward (Bechtholt and Cunningham 2005). CPP is a test used for the rewarding effects of drugs such as ethanol (Prus, James, and Rosecrans 2009), and the VTA plays a critical role in the acquisition of ethanol CPP (Bechtholt and Cunningham 2005). Interestingly, E2 enhances CPP in OVX mice, and co-activation of both ERα and ERβ is required (Hilderbrand and Lasek 2018). D2R also plays a role in the acquisition of ethanol CPP. Ethanol CPP is decreased in D2R knockout mice (Cunningham et al. 2000). It is possible that the mechanism for E2 enhancement of DA inhibition in the VTA may also play a role in the enhancing effects that E2 has on ethanol CPP. Future studies are required to explore this relationship.

Activation of ERs may modulate the autoinhibitory effects of DA on VTA DA neurons by modulating the expression, trafficking, and activity of proteins involved in DA neurotransmission. In general, E2 strengthens DA transmission by increasing the synthesis and release of DA (Pasqualini et al. 1995, 1996; Xiao and Becker 1998) and decreasing the re-uptake of DA (Watson et al. 2006; Disshon, Boja, and Dluzen 1998). Twenty-seven days of E2 treatment via implant in OVX female rats increased the mRNA expression for D3R in the VTA

(Zhou, Cunningham, and Thomas 2002). Estrogen increases the density of D2R on the plasma membrane in the nucleus accumbens and striatum (Chavez et al. 2010; Roy, Buyer, and Licari 1990). Because studies have either shown a decrease or no change in the mRNA expression of the D2R gene in the striatum in response to E2 supplementation in OVX rodents (Le Saux, Morissette, and Di Paolo 2006; Lammers et al. 1999), it is possible that estrogen increases the density of D2R by increasing the trafficking of D2R to the membrane. Increased plasma membrane-associated D2R on VTA DA neurons would be predicted to augment the autoinhibitory effect of DA.

These studies are limited by the use of one dose of DPN and PPT which were chosen to ensure receptor selectivity while also maintaining high receptor occupancy (Hilderbrand and Lasek 2018; Sepehr et al. 2012). Different doses and treatment protocols may reveal that activation of either ERα or ERβ alone may alter DA inhibition in VTA DAergic neurons, however, higher doses of these agonists would be non-selective for specific isoforms of ERs. Future studies should therefore focus on other methods such as genetic modification of ERα and ERβ expression in the VTA. Future studies will also investigate the mechanisms downstream of ERα and ERβ activation.

5. ESTRADIOL ENHANCES DOPAMINE D2 RECEPTOR DESENSITIZATION IN THE VTA

5.1 Introduction

E2 plays an important role in modulating DAergic systems and reward-related behaviors (Yoest, Cummings, and Becker 2014). VTA DAergic neurons express the rate-limiting enzyme involved in DA synthesis, TH (Molinoff and Axelrod 1971), and they have D2R autoreceptors on their soma and dendrites that mediate inhibitory regulation of their excitability (Adell and Artigas 2004; Aghajanian and Bunney 1977). D2R levels and activity are generally decreased in addiction (Volkow et al. 1990; Martinez et al. 2004; Volkow et al. 1997; Hietala et al. 1994), and are regulated in part by trafficking, endocytosis, and desensitization. GRKs and PKC phosphorylate D2R, resulting in clathrin-mediated internalization of D2R through β-arrestin and dynamin and subsequent endosomal sorting (Namkung and Sibley 2004; Namkung et al. 2009; Paspalas, Rakic, and Goldman-Rakic 2006). Prolonged stimulation of the D2-like receptors by DA results in the decreased responsiveness to DA inhibition in a time- and concentration-dependent manner through a mechanism involving PLC and PKC (Nimitvilai, Arora, and Brodie 2012), a process termed DA inhibition reversal (DIR) (Nimitvilai and Brodie 2010). Previous work from the Brodie and Lasek laboratories demonstrated that ALK, a receptor tyrosine kinase, regulates DIR in VTA DAergic neurons, but not acute responses to DA (Dutton et al. 2017). E2 regulates ALK gene expression via ERα (Lasek et al. 2011).

E2 can regulate D2R density, desensitization, and activity. E2 increases the density of D2R without changes in gene expression in the striatum, suggesting that E2 may regulate the trafficking of D2R to or from the plasma membrane or internalization and degradation of the receptor (Le Saux, Morissette, and Di Paolo 2006; Bosse and DiPaolo 1996). In the nucleus

accumbens and caudate nucleus, E2 decreases the density of D2R (Chavez et al. 2010). E2 increases the desensitization of D2R in the anterior pituitary in OVX rats (Pasqualini, Bojda, and Kerdelhue 1986). The role of E2 in modulating the density, desensitization, internalization, and trafficking of D2R in the VTA, however, is unknown.

In this Chapter, I investigated the role of E2 in regulating D2R desensitization in the VTA of female mice. I determined that E2 promotes the desensitization of D2R in the VTA of OVX female mice. Additionally, I measured the expression of genes involved in DA neurotransmission and found that *Alk* expression was increased in E2-treated OVX mice, providing a potential mechanism by which DIR is increased in mice treated with E2.

5.2 Methods

5.2.1 Animals

Female C57Bl/6J mice (8 weeks) were ordered from The Jackson Laboratory (Bar Harbor, ME, USA). Electrophysiological and molecular experiments were conducted at 10-14 weeks of age. Animal care adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and all procedures were approved by the UIC the Animal Care Committee.

5.2.2 Ovariectomy and in vivo drug treatments

OVX and *in vivo* drug treatments were performed as described in Chapter 2 (Hilderbrand and Lasek 2018; Vandegrift et al. 2017). Briefly E2 benzoate was prepared in a sesame oil with 10% ethanol vehicle (VEH). Mice were treated SC for three days with 50 µl of 0.2 µg E2 on the first two days and 50 µl of 1 µg E2 on the third day. This dosage of E2 was chosen because it results in E2 plasma levels similar to proestrus levels (Vandegrift et al. 2017).

5.2.3 Brain slice preparation and extracellular recording

For the electrophysiological studies, mice were sacrificed one hour after the last injection. VTA-containing brain slices were prepared as described in Chapter 2 (Brodie et al. 1999). DA was administered during the electrophysiological experiments by adding stock solutions (100 to 1000 times the final concentrations) into the aCSF via a calibrated infusion pump, which allowed the DA to fully mix with the aCSF prior to reaching the chamber. The final concentration was determined according to the flow rate and concentration of the stock solution.

5.2.4 Quantitative real-time polymerase chain reaction (qPCR)

VTA tissue was collected from OVX mice one hour after the final injection on the third day for qPCR experiments. The total RNA from the VTA tissue was isolated using the GeneJet RNA Purification Kit (ThermoFisher Scientific, Schaumburg, IL, USA). Following reverse transcription of the RNA to cDNA using the Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific), Maxima qPCR Master Mix (Thermo Scientific) was used to perform qPCR. All procedures were performed according to the manufacturer's protocols. The sequences of primers used were: tyrosine hydroxylase (*Th*) forward primer: 5'-TCTTGAAGGAACGGACTGGC -3', *Th* reverse primer:5'- GAGTGCATAGGTGAGGAGGC-3'; DA D2 receptor short isoform (*Drd2s*) forward primer: (5'-CACCACTCAAGGATGCTGCCCG3', *Drd2s* and *Drd2l* reverse primer: 5'-GTTGCTATGTAGACCGTG -3', DA D2 receptor long isoform (*Drd2l*) forward primer: 5'-AACTGTACCCACCCTGAGGA-3'; DA D3 receptor (*Drd3*) forward primer: 5'-CCTCTGAGCCAGATAAGCAGC-3'; *Drd3* reverse primer, 5'-AGACCGTTGCCAAAGATGATG-3'; and anaplastic lymphoma kinase (*Alk*) forward primer: 5'- TGCCGCTCTCATTGATCCTC -3', *Alk* reverse primer: 5'-

TGCTTCCGACGGTACACAAT - 3'. Relative expression of *Th*, *Drd2s*, *Drd2l*, and *Drd3*, were calculated using the dCq method with *Rpl13a* as a normalization control (*Rpl13a* forward primer: 5'-TACCAGAAAGTTTGCTTCCTGGG-3'; *Rpl13a* reverse primer: 5'-TGCCTGTTTCCGTAACCTCAAG).

5.2.5 Statistical Analysis

Averaged numerical values are presented as the mean ± SEM. qPCR data were analyzed by Student's t-test. All other statistical comparisons were made using two-way repeated measures ANOVA followed by Sidak's multiple comparisons tests as appropriate (Prism, Graphpad Software, Inc., La Jolla, CA). A ***P*** value of < 0.05 was considered significant.

5.3 Results

5.3.1 VTA neuron characteristics

A total of 10 neurons from 10 mice were recorded in electrophysiological experiments. The baseline firing rates ranged from 0.73 to 3.03 Hz with an average firing rate of 2.16 ± 0.28 Hz. There were no significant differences between firing rates between the groups (VEH: 2.42 ± 0.38 Hz, E2: 1.91 ± 0.43 Hz, $t_4 = 1.23$, $P = 0.8442$).

5.3.2 Ovariectomy disrupts dopamine inhibition reversal and supplementation with estradiol restores dopamine inhibition reversal

Because of the role of E2 in regulating the desensitization of D2R in the anterior pituitary (Pasqualini, Bojda, and Kerdelhue 1986), I investigated if E2 treatment affected DIR in OVX female mice. DIR is a process by which DA inhibition diminishes during prolonged administration of DA, and this phenomenon reflects the desensitization of D2R (Nimitvilai, Arora, and Brodie 2012; Nimitvilai and Brodie 2010; Nimitvilai, McElvain, et al. 2012). I measured the response of VTA DA neurons to a concentration of DA that inhibited each neuron

by at least 50%; this concentration was administered over 40 minutes onto the slices from OVX mice that were treated with E2 or VEH (Figure 16). The concentration of DA used to establish initial inhibition was 6.9 ± 1.4 μM and 6.0 ± 3.5 μM in slices from OVX mice treated with VEH and E2, respectively. After five minutes of DA administration, the initial inhibition to DA was -58.8 ± 6.0% in neurons from OVX mice treated with VEH. Interestingly, after 40 minutes of DA administration, the inhibition to DA was -73.8% ± 16.3%, indicating that DIR did not occur in neurons from the VEH-treated OVX mice. In contrast, the initial inhibition to DA in neurons from E2-treated OVX mice was -74.6% ± 8.6%, and after 40 minutes the inhibition to DA was -23.7 ± 7.1% (n = 5/group, two-way RM ANOVA, time: $F_{7,56} = 2.856$, $P = 0.0128$; treatment: $F_{1,8} = 11.07$, $P = 0.0104$; time x treatment interaction: $F_{7,56} = 7.285$, $P < 0.0001$). Sidak's post-hoc multiple comparisons test demonstrated a significant reduction in the inhibitory response to DA at the 15 (P = 0.0219), 20 (P = 0.0028), 25 (P = 0.0010), 30 (P = 0.0021) 35 (P < 0.0001) and 40 (P < 0.0001) minute time points compared to the initial 5-minute time point in E2-treated OVX mice. On the other hand, Sidak's post-hoc multiple comparisons test demonstrated a significant increase in the inhibitory response to DA at the 10 (P = 0.0450), 15 (P = 0.0293), 20 (P = 0.0143), 25 (0.0332), 30 (P = 0.0275) and 35 (P = 0.0188) minute time point compared to the initial 5-minute time point in neurons from VEH-treated OVX mice. These results indicate that, similar to what has been established in VTA slices from male mice (Nimitvilai, Arora, and Brodie 2012; Nimitvilai and Brodie 2010; Nimitvilai, McElvain, et al. 2012), OVX mice treated with E2 exhibit DIR in response to prolonged administration of DA. In contrast, OVX mice treated with VEH do not exhibit DIR. This suggests that E2 is required for desensitization of D2R in the VTA of female mice.

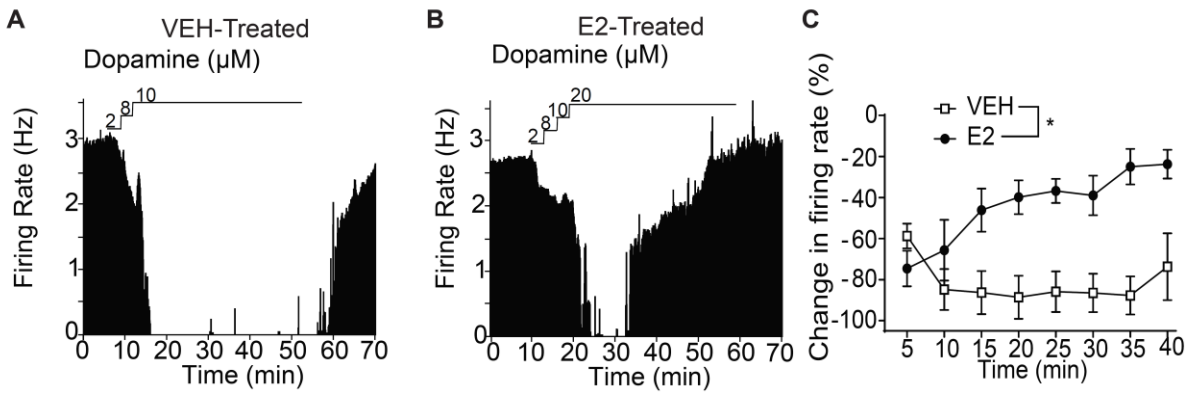

Figure 16. OVX prevents DIR and supplementation with E2 restores DIR.

Extracellular recordings were performed in OVX female mice treated with E2 or VEH for three days. Representative ratemeter graphs showing the effects of (a) VEH (n = 5) and (b) E2 (n = 5) on a 40-minute DA application. (c) Pooled DA-concentration response curves, showing inhibition to a concentration of DA that initially decreased the firing rate by at least 50% in each neuron as a function of time. DIR was abolished in VTA DA neurons from the control-treated OVX mice, and was restored in E2-treated OVX mice. *P < 0.05 by two-way RM ANOVA;

5.3.3 Increased expression of Alk in the VTA in ovariectomized mice treated with estradiol compared to vehicle

To test if the expression of key proteins involved in DA synthesis, signaling, and desensitization are altered by E2 in the VTA, we measured gene expression of VTA tissue from OVX mice treated with E2 (0.2 μg on the first 2 days and 1 μg on the final day) or VEH for 3 days. Because ALK regulates desensitization of D2R in the VTA of male mice (Dutton et al. 2017) and the fact that ALK expression in the striatum is regulated by E2 (Lasek et al. 2011), I measured *Alk* expression in the VTA after E2 treatment. Additionally, in order to investigate if E2 regulates the expression of D2-like receptors and the rate-limiting enzyme for DA synthesis, I measured the expression of tyrosine hydroxylase (*Th)*, DA D2 receptor short isoform (*Drd2s),* DA D2 receptor long isoform (*Drd2l),* DA D3 receptor (*Drd3),* and anaplastic lymphoma kinase (*Alk)*. There were no differences in expression of *Th*, *Drd2s*, *Drd2l,* and *Drd3* in OVX mice treated with E2 compared to VEH-treated mice (Figure 17A-D). Interestingly, *Alk* expression was significantly increased by 111% in the VTA of OVX mice treated with E2 compared to VEH (Figure 17 e, n = 7/group, $t_{12} = 2.5$, $P = 0.0257$). ALK has been shown to be involved in the desensitization of D2R in the VTA (Dutton et al. 2017), and the Lasek lab has shown that ALK regulates the internalization of D2R (Donghong He, Hu Chen, Amy W. Lasek, unpublished results). These results suggest that E2 may be modulating desensitization and trafficking of D2R via increased ALK levels.

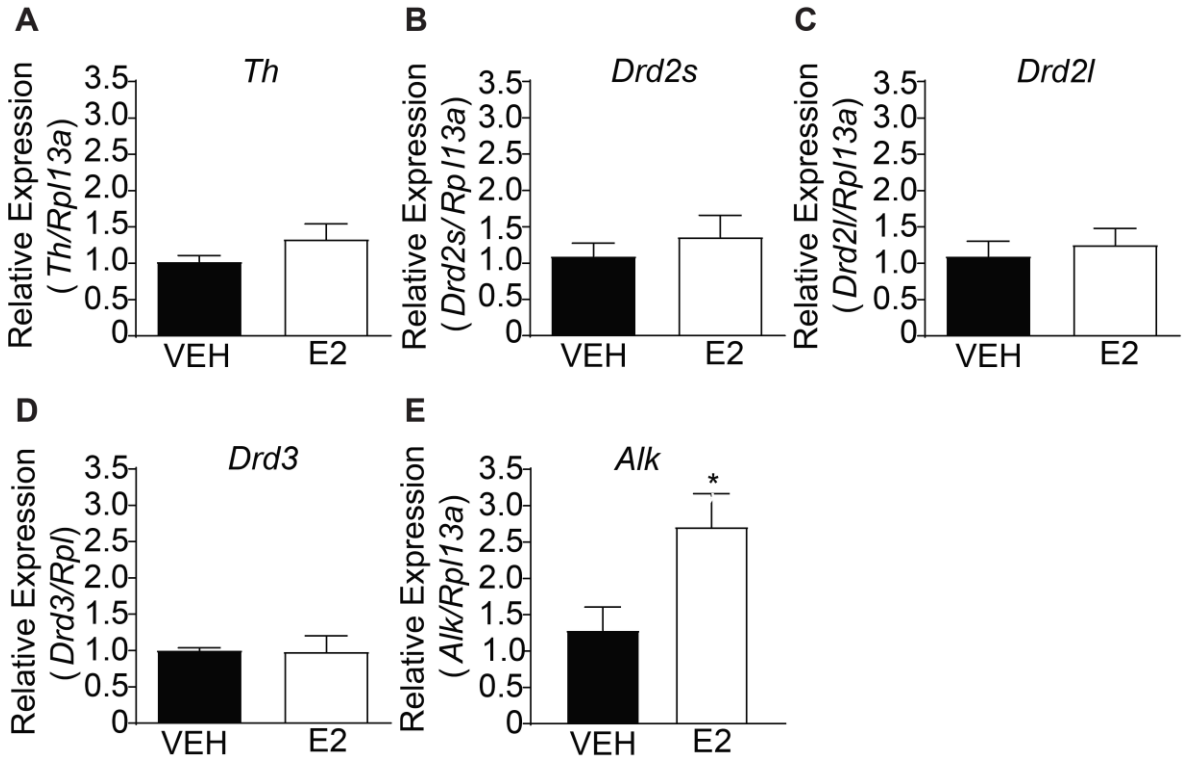

Figure 17. Increased expression of *Alk* in the VTA in OVX mice treated with E2 compared to VEH.

VTA RNA samples from OVX mice treated with VEH (n = 7) or E2 (n = 7) in the VTA were analyzed by qPCR for expression of (a) *Th*, (b) *Drd2s*, (c) *Drd2l*, (d) *Drd3*, and (e) *Alk*. There was a significant increase in *Alk* expression in VTA samples from OVX mice treated with E2 compared with VEH. *P < 0.05 by student's t-test.

5.4 Discussion

OVX mice treated with E2 exhibit desensitization of D2R in response to prolonged administration of DA. In contrast, OVX mice treated with VEH do not exhibit desensitization. The desensitization by DA in the VTA has been well established in male rodents (Nimitvilai, Arora, et al. 2012; Nimitvilai, Arora, and Brodie 2012; Nimitvilai and Brodie 2010). I found that similar to what is observed in male mice, OVX female mice supplemented with E2 exhibited DIR, whereas DIR was abolished in VEH-treated OVX mice. I did not fully investigate DIR in freely-cycling female mice in a similar manner, but I would predict that DIR would be enhanced during the higher E2 phases diestrus and proestrus, and DIR would be decreased in the lower E2 phases estrus and metestrus.

Because of the role ALK plays in the desensitization of D2R in the VTA, this led me to investigate the expression of *Alk* in the VTA from OVX mice treated with E2. These results show that while there were no changes in the mRNA expression of several genes involved DA neurotransmission, including *Th, Drd2s*, *Drd2l*, and *Drd3*, there was a significant increase in the expression of *Alk* in OVX mice supplemented with E2 compared to VEH-treated. This increase in *Alk* expression is consistent with an E2-mediated increase in *Alk* in the striatum of male and female mice (Lasek et al. 2011). E2 activation of ERα may increase *Alk* expression since ERα binds to the *Alk* promotor in cultured cells (Lasek et al. 2011). Increased ALK expression/activity in OVX mice treated with E2 would be expected to promote desensitization of the D2R upon prolonged agonist stimulation, since ALK inhibition blocks D2R desensitization in male mice (Dutton et al). Future experiments could examine if an ALK inhibitor can block E2-mediated DIR in females, thus linking the increase in ALK to DIR in

OVX mice treated with E2. Additionally, future studies should determine if increasing/activating ALK can reproduce the enhancement of DIR in females in a low E2 state.

Inhibiting ALK in the VTA decreases D2R desensitization without changing the initial inhibition or excitation to acute applications of DA or ethanol (Dutton et al. 2017). Prolonged activation of D2R may transactivate ALK, since GPCRs are known to transactivate receptor tyrosine kinases such as ALK (Cattaneo et al. 2014; Delcourt, Bockaert, and Marin 2007) and lead to the internalization and degradation of D2R and ALK. Both ALK and D2R are internalized by agonist stimulation (Mazot et al. 2012; Bartlett et al. 2005). Because D2R desensitization in the VTA is through a PKC-mediated pathway (Nimitvilai, Arora, and Brodie 2012), and ALK can activate PKC signaling through phospholipase C-γ (Bai et al. 1998), ALK may enhance D2R desensitization through increased PKC signaling.

The expression of *Th*, *Drd2s*, *Drd2l*, and *Drd3* was also measured in OVX mice supplemented with E2, and there were no changes. However, other studies have shown that E2 can alter the expression of these genes. Twenty-seven days of E2 treatment via implant resulted in increased expression of the D3R gene in the VTA of female rats (Zhou, Cunningham, and Thomas 2002). The effects of E2 on *Th* expression vary depending on species, brain region, dose, and duration of E2 treatment, and discrepancies remain on the precise role of E2 on changing TH levels (Liaw et al. 1992; Serova et al. 2002; Curran-Rauhut and Petersen 2003; Pau et al. 2000). In a pituitary tumor cell line and in the pituitary of rats, E2 increases D2L expression (Guivarc'h, Vincent, and Vernier 1998; Oomizu, Boyadjieva, and Sarkar 2003), and ERα mRNA expression is positively correlated with D2L in human prolactinomas and gonadotrope tumors. The ratio of D2S and D2L varies by brain region (Mack, Todd, and O'Malley 1991; Neve et al. 1991; Montmayeur et al. 1991). Changes in the ratio of the D2S and

D2L have been implicated in altered drug-related behaviors in mice (Radl et al. 2018; Bulwa et al. 2011). Knockdown of D2L increases ethanol intake in both sexes (Bulwa et al. 2011; Wu et al. 2009).

After a 60 minute incubation with a fluorescent agonist in the CHO cell line, a higher percentage of D2S was internalization compared to D2L (Tabor et al. 2017). The two isoforms of D2R result from alternative splicing of the sixth exon of the same gene. The long isoform, D2L, is 29 amino acids longer than the short isoform, D2S (Dal Toso et al. 1989; Giros et al. 1989). This results in differences in an intracellular region which regulates $G_{i\alpha}$ coupling (Senogles 1994; Guiramand et al. 1995), and this region could also be responsible for the differences in internalization (Clayton et al. 2014). Although there were no changes in mRNA expression of D2S and D2L, there may be differences in protein levels or densities of each isoform on the plasma membrane. Future studies should further investigate the mechanisms of E2 enhancement of D2R desensitization.

6. CONCLUSIONS

6.1 Introduction

There are considerable gender differences in alcoholism, including differences in the amount of use, age of initiation, reasons for abuse, development of comorbidities, and severity of health problems (Becker, Perry, and Westenbroek 2012). Although generally more men drink than women, this gender gap is narrowing (Cheng, Cantave, and Anthony 2016; Keyes, Grant, and Hasin 2008). Gender differences may be due to a combination of sociocultural, economic, developmental, and biological factors (Becker and Koob 2016; Holmila and Raitasalo 2005; Kuhn 2015). Hormones may play a key role in gender differences in addiction and alcoholism, and evidence suggests that estrogen might contribute to higher levels of alcohol drinking in females (Becker, Perry, and Westenbroek 2012; Carroll et al. 2004; Carroll and Anker 2010; Anker and Carroll 2011). Studies investigating the differences in alcohol consumption during the normal human menstrual cycle are conflicting and indeterminate; thus, the precise role that the hormonal cycle has on alcohol intake on females is unknown (Becker and Koob 2016). Much of the research on the VTA, a brain region responsible for the rewarding and reinforcing effects of drugs of abuse (Koob and Volkow 2010), has been established in male subjects. Therefore, the purpose of this dissertation was to examine the role of E2 in modulating VTA physiology in female mice. I demonstrated that E2 enhances the ethanol-induced excitation of VTA DA neurons through activation of ERα and mGluR1. I also demonstrated that E2 enhances the sensitivity of VTA DAergic neurons to DA through co-activation of ERα and ERβ. Finally, I showed that E2 promotes the desensitization of D2R in VTA DAergic neurons. Together, the results from my work indicate that E2 regulates DAergic neurotransmission in the VTA and increases the sensitivity of VTA DAergic neurons to ethanol and DA.

6.2 Estradiol increases the sensitivity of VTA DAergic neurons to ethanol and DA

Using both gonadally intact and OVX female mice, I demonstrated that high E2 states increase VTA DAergic neuron sensitivity to ethanol; this alteration in the neurophysiological response to alcohol may affect alcohol drinking in females. Increases in the basal firing rate of DAergic neurons is associated with burst firing (Grace and Bunney 1984, 1980). Burst firing induces a large release of DA in target regions (Gonon 1988; Chergui, Suaud-Chagny, and Gonon 1994), and this is thought to mediate the rewarding and reinforcing effects of drugs of abuse (Schultz 2002; Phillips et al. 2003). Ethanol-induced DA release in the prefrontal cortex is prevented in OVX rats and is recovered by E2 supplementation (Dazzi et al. 2007). The E2 effects on VTA DAergic neuron excitation and DA release may modulate drinking behaviors.

A study by the Lasek lab investigating binge-like drinking throughout the estrous cycle of female mice found no difference in ethanol intake in any of the phases (Satta, Hilderbrand, and Lasek 2018). This finding is consistent with other studies in rats that did not observe any effects of the estrous cycle on ethanol consumption (Priddy et al. 2017; Ford, Eldridge, and Samson 2002b). One study found that while there was no estrous cycle effect on self-administration of ethanol in freely-cycling female rats, rats with synchronized estrous cycles exhibited decreased administration of ethanol in estrus and proestrus (Roberts et al. 1998). Another group found decreased ethanol intake during proestrus (Forger and Morin 1982), when circulating E2 serum levels are the highest (Nilsson et al. 2015). Because of the inconsistencies in the effects of the estrous cycle on drinking, hormonal effects on ethanol consumption during the estrous cycle may be subtle, and the short duration of the phases may make it difficult to experimentally detect changes in drinking behaviors within a group of rodents who are not synchronized depending on the protocol. Furthermore, circulating hormones during the estrous cycle may have opposing

effects on drinking behaviors. For instance, supplementation of OVX rats with progesterone decreased ethanol intake whereas supplementation with E2 enhanced ethanol intake (Ford, Eldridge, and Samson 2002a).

Many studies in rodents have demonstrated enhancing effects of E2 on drinking behaviors (Ford, Eldridge, and Samson 2004; Rajasingh et al. 2007; Reid, Hubbell, and Reid 2003; Ford, Eldridge, and Samson 2002a; Marinelli, Quirion, and Gianoulakis 2003; Quirarte et al. 2007; Satta, Hilderbrand, and Lasek 2018). However, some studies have reported that E2 decreases drinking in rodents (Almeida et al. 1998; Sandberg, David, and Stewart 1982). These discrepancies may be due to strain or treatment differences. Additionally, E2 injections in female mice increases preference for ethanol in a CPP experiment through activation of ERα and ERβ (Hilderbrand and Lasek 2018). The role of E2 on drinking behaviors in humans is less clear. Blood E2 levels are positively correlated with alcohol consumption in women (Muti et al. 1998), however ethanol consumption might increase circulating E2 levels. Female rats on a chronic liquid diet containing ethanol have increased serum E2 levels (Emanuele et al. 2001), suggesting that ethanol can affect E2 production and secretion (Rachdaoui and Sarkar 2013).

I have also shown that high E2 states increases the sensitivity of VTA DAergic neurons to DA. Because high E2 states increase the sensitivity of VTA DAergic neurons to ethanol (Chapter 2) and increase the rewarding properties of natural rewards and other drugs (Hilderbrand and Lasek 2018; Satta et al. 2018; Oldenburger, Everitt, and de Jonge 1992), the increase in the sensitivity of VTA neurons to inhibition by DA may be compensating for an overall enhanced sensitivity to rewarding and other excitatory stimuli.

D2R activation plays an important role in decreasing the activity of VTA DAergic neurons, DA production, and release (Beckstead et al. 2004; White and Wang 1984; Benoit-

Marand, Borrelli, and Gonon 2001; De Mei et al. 2009), and the expression and activity of D2R is implicated in drug abuse (de Jong et al. 2015; Tacelosky et al. 2015; Ji et al. 2017). The E2-induced enhancement in the sensitivity to DA inhibition could also affect responses to psychostimulants such as cocaine and amphetamine, and E2 effects on DAergic neurotransmission may partially mediate sex differences in psychostimulant abuse. Women begin using cocaine at a younger age than men, more rapidly escalate from drug use to dependence, and experience more severe craving (Griffin et al. 1989; Kosten et al. 1993; Robbins et al. 1999). In rats, females will acquire cocaine self-administration and CPP more rapidly and readily than males (Lynch and Carroll 1999; Carroll et al. 2002; Russo et al. 2003; Hu et al. 2004; Zakharova, Wade, and Izenwasser 2009). Supplementation of E2 in OVX rats increases cocaine and amphetamine stimulated DA release in the nucleus accumbens, cocaine self-administration, and CPP (Lynch et al. 2001; Hu et al. 2004; Segarra et al. 2010; Segarra et al. 2014; Tobiansky et al. 2016; Thompson and Moss 1994). However, an E2-mediated increase in the sensitivity of D_2-like DA receptors to DA inhibition may not be consistent with E2 enhancement of cocaine taking behaviors. While the increase in D2R agonist potency may not be relevant to cocaine self-administration, the increase in DIR would reduce the sensitivity to DA, and decrease the action of D2R on cell bodies and terminals. A decrease in the activity of D2R may promote cocaine taking behaviors. Mutant mice that do not express D2R have increased cocaine self-administration (Cunningham et al. 2000).

A difference between E2 regulation of ethanol and DA sensitivity was demonstrated by the acute in vitro treatment with ICI 182,780. An acute treatment with the ERα/ERβ antagonist reduced the ethanol excitation of VTA DAergic neurons from mice in the diestrus phase, but it had no effect on the inhibition of VTA DA neurons by DA in neurons from mice. These results

suggest that ERs in the VTA acutely affect ethanol excitation of VTA DAergic neurons, but not inhibition by DA. The reversal of enhanced excitation by ethanol of VTA DAergic neurons from mice in diestrus with an acute treatment of an ER antagonist suggests that signaling by membrane-bound ERs in the VTA may be responsible for the enhanced ethanol excitation and 'classical' genomic signaling may be responsible for the enhanced DA inhibition.

ICI 182,780 actions on ERα include interfering with the dimerization of ERα, blocking nuclear translocation, increasing ERα degradation, and blocking rapid signaling of ERα from the membrane (Howell et al. 2000; Dauvois, White, and Parker 1993; Wade et al. 2001). ICI 182,780 can block the transcriptional effects of ERβ (Tremblay et al. 1997), and may also act as an agonist for GPER (Thomas et al. 2005). I believe that the ability of ICI 182,780 to block ethanol-induced excitation of VTA DA neurons during diestrus is likely due to its inhibition of ERα and/or ERβ because activation of GPER1 by ICI 182,780 would have an estrogenic action rather than reversal of the estrogen effect on ethanol potency that we observed. Additionally, GPER was not observed in the VTA using immunohistochemistry in male and female rats (Hazell et al. 2009). Few other studies have investigated the effects of ICI 182,780 on the reward circuitry and behavior. Intracerebroventricular injection of ICI 182,780 in female rats decreases cocaine sensitization and CPP (Segarra et al. 2014). The E2-induced paced mating behavior in female rats is reduced with ICI 182,780 in the striatum (Xiao, Jackson, and Becker 2003). Additionally, the E2-induced increase in striatal DA release in response to amphetamine and KCI is also reduced with ICI 182,780 in female rats (Xiao, Jackson, and Becker 2003; Song et al. 2019). Interestingly, this E2-induced enhancement of DA release in response to amphetamine is also blocked by a mGluR5 antagonist (Song et al. 2019).

To summarize, the sensitivity of VTA DA neurons to DA and ethanol is enhanced by E2 in female mice. The differences between DA and ethanol sensitivity in response to acute treatment with ERα/ERβ antagonist suggests that the E2 effects on ethanol sensitivity may be due to rapid signaling mechanisms, and the E2 effects on DA sensitivity may be due to genomic mechanisms. I next investigated which ER and which downstream signaling pathways might mediate E2 modulation of ethanol and DA sensitivity of VTA DA neurons in female mice.

6.3 Estradiol enhances ethanol excitation of VTA DA neurons through activation of estrogen receptor α and metabotropic glutamate receptor 1

Using OVX mice supplemented with agonists specific for ERα and ERβ and ER antagonists in freely-cycling female mice, I showed that E2 enhances the ethanol-induced excitation of VTA DA neurons through activation of ERα. The concentrations of PPT and DPN were chosen to balance receptor selectivity with occupancy (Sepehr et al. 2012). Since only one dose of DPN was used in these experiments, it is still possible that ERβ may still play a role in modulating the sensitivity of VTA DA neurons to ethanol. In order to further investigate the role of ERα and ERβ in modulating the sensitivity of VTA DA neurons to ethanol, future experiments could knockdown/knockout or upregulate expression of *Esr1* and *Esr2* in the VTA. In addition, it would be useful to determine if treatment with an ERα-selective antagonist blocks enhancement of ethanol-stimulated increase in firing of VTA DA neurons in OVX mice supplemented with E2 to more conclusively establish that E2 is acting through ERα to enhance ethanol excitation.

Because treating slices acutely with ICI 182,780 and an ERα-selective antagonist (MPP) reversed the enhanced ethanol sensitivity of VTA DA neurons from mice in diestrus, this suggests that signaling from membrane-bound ERα may mediate the enhanced excitation by

ethanol, rather than 'classical' genomic effects of ERα. ER localized at the plasma membrane in neurons can rapidly signal through PKA, PLC, and MAPK pathways (Nabekura et al. 1986; Minami et al. 1990; Mobbs et al. 1991; Aronica, Kraus, and Katzenellenbogen 1994; Watters et al. 1997). Membrane bound ERα and GPER promote the sexual behavior lordosis in rats (Long et al. 2017; Dewing et al. 2007). In the medial preoptic nucleus, membrane bound ERα promotes sexual receptivity of female rats through activation of mGluR1 (Dewing et al. 2007). The role of rapid ERα signaling should be investigated in future studies by acutely applying E2 or specific ER agonists onto slices containing the VTA. I was unable to keep E2 in solution in the aCSF used in the slice preparation, so future work will be needed to troubleshoot this issue.

Because ERα activates mGluRs in a glutamate-independent manner and can regulate many neuronal functions, signaling pathways, and synaptic function (Boulware et al. 2005; Dewing et al. 2007; Huang and Woolley 2012; Boulware, Kordasiewicz, and Mermelstein 2007), I investigated whether transactivation of mGluR1 plays a role in the E2-induced enhancement of ethanol excitation in VTA DA neurons. The inhibitory effects that the mGluR1 antagonist (JNJ16259685) had on ethanol sensitivity of VTA neurons in high E2 states indicates that E2-mediated enhancement of ethanol sensitivity of the VTA DA neurons in females is dependent upon activation of mGluR1. ERα/mGluR1 signaling promotes sexual behaviors in female rodents and ERβ/mGluR1 signaling promotes sexual behaviors in male Japanese quail (Dewing et al. 2007) (Seredynski et al. 2015). Most of the published literature investigating the role of ER/mGluR signaling in drug abuse has focused on mGluR5. In rodents, ER/mGluR5 activation promotes cocaine intake, cocaine-induced locomotor sensitization, and amphetamine-induced DA release (Martinez et al. 2014; Martinez et al. 2016; Song et al. 2019).

Future studies should more thoroughly investigate the interaction between ERα and mGluR1 in the VTA. ERα co-immunoprecipitates with mGluRs in experiments with cultured cells and are these receptors are colocalized in the hypothalamus (Dewing et al. 2007), so a future study should investigate if there is a physical interaction between ERα and mGluR1 in the VTA. The mGluR1 antagonist experiments should also be repeated in OVX mice supplemented with an ERα agonist. Additionally, it would be interesting to investigate whether this effect is specific for mGluR1, or if antagonists for mGluR5 would have a similar effect. VTA DA neurons in mice express both mGluR1 and mGluR5 (Merrill et al. 2015). It is unknown what downstream pathways following mGluR1 activation modulate ethanol sensitivity of VTA DA neurons, so future studies should also investigate this.

Based on my results, I hypothesize that endocannabinoid signaling following ERα activation of mGluR1 may regulate ethanol sensitivity of VTA DA neurons. It has been shown in hippocampal neurons from female rats that downstream of ERα activation of mGluR1, endocannabinoids retrogradely act on GABAergic synapses to decrease GABA release, thereby disinhibiting CA1 pyramidal cells (Huang and Woolley 2012). Although GABA antagonists had no effect on ethanol sensitivity of neurons from OVX female mice treated with E2 or VEH, it is possible that an effect was not seen because there is a floor effect, i.e. that there was already reduced GABA release onto VTA DA neurons that could not be driven further by GABA antagonists. An alternative explanation might be through regulation of glutamatergic synapses to increase glutamate release. mGluR7 and endocannabinoids can regulate excitatory synapses onto VTA neurons by reducing presynaptic release of glutamate onto VTA DA neurons (Melis et al. 2004; de Rover, Meye, and Ramakers 2008), however this would have the opposite effect on the ethanol excitation of VTA DA neurons. It is possible that ERα/mGluR1 activation may modulate

ethanol excitation of VTA DA neurons through decreasing GABA release or increasing glutamate release. A more thorough dissection of synaptic circuits using patch-clamp recordings could begin to elucidate whether these mechanisms play a role in the E2-mediated enhancement of ethanol sensitivity in the VTA.

mGluR1 could also directly regulate the excitability of VTA DA neurons by acting on postsynaptic ion channels such as HCN or GIRK. mGluR1 is a $G_{q/11}$-linked GPCR which induces the release of Ca^{2+} through PIP_3 signaling. Calcium can regulate HCN and firing of neurons (Schwindt, Spain, and Crill 1992). mGluR1 inhibits I_h and enhances the excitability of retinal ganglion cells and cingulate pyramidal neurons (Li et al. 2017; Gao et al. 2016). However, blocking I_h was found to decrease ethanol excitation in VTA DA neurons in male mice (Okamoto, Harnett, and Morikawa 2006), but did not occur in rat VTA neurons (Appel et al. 2003). GIRK channels are activated by ethanol and are important regulators of the excitability of VTA DAergic neuron (Lewohl et al. 1999; Kobayashi et al. 1999; Aryal et al. 2009). A global knockout of GIRK3 decreases the ethanol excitation of VTA DA neurons and ethanol-stimulated DA release in the nucleus accumbens (Christensen et al. 2011). E2 increases the function and mRNA expression of GIRK channels in primary rat lactotrophs (Christensen et al. 2011). Future projects should investigate downstream signaling of mGluR1 activation by ERα and potential mechanisms for the regulation of ethanol sensitivity in the VTA.

6.4 Estradiol increases the sensitivity of VTA DA neurons to DA through co-activation of estrogen receptor α and estrogen receptor β

I have shown that activation of both ERα and ERβ is required to enhance VTA DA inhibition in OVX female mice. These results suggest that the ability of E2 to increase the inhibitory effect of DA on VTA neurons is through activation of both of these receptors.

Interestingly, OVX female mice treated with a combination of DPN and PPT, but not treated with DPN or PPT alone, exhibited enhanced ethanol CPP (Hilderbrand and Lasek 2018). Expression of ethanol CPP is dependent upon mechanisms within the VTA including ionotropic glutamate, opioid, and GABA receptors (Bechtholt and Cunningham 2005; Pina and Cunningham 2016). Gonadally-intact females exhibit stronger ethanol CPP compared to males and OVX females (Torres et al. 2014), indicating that gonadal hormones such as E2 play an important role in the sex differences in the rewarding effects of ethanol. These findings may begin to elucidate the physiological changes in the reward circuitry which may be involved in the modulation of ethanol CPP in female mice, however future work will need to further investigate if there is a role for the E2 modulation of DA sensitivity in the acquisition of ethanol CPP.

6.5 Estradiol promotes the desensitization of DA D2 receptor in VTA DA neurons

OVX mice supplemented with E2 exhibit D2R desensitization upon prolonged agonist stimulation, whereas VEH-treated OVX mice do not. E2 also mediates an increase in the desensitization of D2R in the anterior pituitary in OVX rats (Pasqualini, Bojda, and Kerdelhue 1986). OVX decreases D2R and D1R density in the striatum of rats, and E2 supplementation restores D2R, but not D1R, densities (Bosse and DiPaolo 1996). The E2-mediated increase in the density of D2R is through activation of ERβ, and there are no changes in gene expression, suggesting that E2 may regulate the trafficking of D2R to the plasma membrane (Le Saux, Morissette, and Di Paolo 2006). In contrast to the enhancing effects of E2 on D2R density in the striatum, E2 decreases D2R density in the caudate nucleus and nucleus accumbens in OVX rats (Chavez et al. 2010). Additionally, E2 induces the internalization of the mu-opioid receptor in the medial preoptic nucleus in females through the neuropeptide Y-Y1 receptor (Mills, Sohn, and Micevych 2004). The mechanisms through which E2 regulates the internalization and trafficking

of D2R in the VTA, and other GPCRS such as MORs, require further investigation. It would be useful to know if E2 alters the surface expression of D2Rs in the female mouse VTA.

Phosphorylation sites on D2R regulate the desensitization and trafficking of D2R (Bofill-Cardona et al. 2000; Namkung and Sibley 2004). Desensitization of D2R following agonist activation involves GRKs or second messenger pathways such as PKC (Namkung et al. 2009; Namkung and Sibley 2004; Nimitvilai, McElvain, and Brodie 2013). GRK phosphorylates D2R and increases the affinity for arrestin-binding and subsequent clathrin-mediated receptor internalization (Namkung et al. 2009). PKC also phosphorylates D2R and can also lead to the internalization of D2R through arrestin and dynamin (Namkung and Sibley 2004).

E2-mediated enhancement of D2R desensitization and decreased D2R function may have effects on drug taking behaviors such as cocaine CPP. Cocaine induces a functional loss of D2R in slices containing the VTA, and this loss is critical for the cocaine-induced changes in glutamatergic potentiation in the VTA (Madhavan et al. 2013). Cocaine effects on excitatory synapses in the VTA play a critical role in the acquisition of cocaine CPP (Dong et al. 2004), so E2-mediated enhancement of D2R desensitization may reinforce cocaine effects in the VTA and increase cocaine CPP.

E2 regulation of D2R desensitization may be mediated by the receptor tyrosine kinase ALK. *Alk* expression was increased in E2-treated OVX mice. *Alk* is also increased in the striatum of male and female mice (Lasek et al. 2011). Because inhibiting ALK blocks D2R desensitization in the VTA of male mice (Dutton et al. 2017), an increase in ALK may promote the desensitization of D2R. ALK can activate PKC signaling through phospholipase C-γ (Bai et al. 1998), so ALK may enhance D2R desensitization through increased PKC signaling. In order to link the increase in ALK to DIR in E2-treated OVX mice, future experiments should

investigate whether inhibiting ALK can block the E2-mediated DIR in females and increasing/activating ALK can reproduce the enhancement of DIR in females in a low E2 state.

6.6 No hormone-dependent differences in basal firing rates in VTA DAergic neurons

I did not see significant differences in basal firing rates of VTA neurons between estrus and diestrus from freely cycling female mice, except for in Chapter 3 where the basal firing rates of neurons from estrus were higher than neurons from mice in diestrus. In experiments described in Chapter 2, I did not observe a significant difference in basal firing rates between neurons from mice in diestrus and estrus ($P = 0.09$). Additionally, when I pool all of the results from recorded neurons (Chapters 2-5), I do not find significant differences in basal firing rates between neurons from mice in the estrus and diestrus phases from Chapters 2 through 5 ($n = 49$-52 per group, $t_{99}=1.69$, $P = 0.0984$). I also did not observe significant baseline rate differences between DPN-, PPT-, E2-, and VEH-treated OVX female mice. These discrepancies may be due to unknown environmental variables affecting the basal firing properties of the neurons from Chapter 3. The basal firing properties of the neurons depend on factors such as the viability of the slice preparation.

Other studies have reported differences in basal firing activity according to the estrus phase of intact rodents with single-unit extracellular *in vivo* measurements. Basal firing rate and bursting were found to be greater in estrus compared with diestrus (Zhang et al. 2008; Calipari et al. 2017). One potential explanation for this discrepancy is that we recorded from *in vitro* brain slices. The ionic, chemical, and synaptic environment of the neurons changes during slice preparation. The axotomy that occurs during slice preparation can alter the membrane properties, excitability of neurons through changes in potassium currents, (Ungless, Gasull, and Walters 2002), and synaptic inputs (Gariano and Groves 1988). The excitatory inputs responsible for

burst firing in the VTA are disrupted (Gariano and Groves 1988). Other hormones besides estrogen may contribute to changes in baseline firing. Progesterone increases the basal firing rate of VTA DAergic neurons in OVX rats (Zhang et al. 2008). Furthermore, these differences may not be due to direct actions of ER activation in the VTA but may be due to changes in inputs to the VTA induced by the effects of ER activation in other brain regions.

6.7 Summary

The results from this dissertation support a role for E2 in the regulation of DIR and sensitivity to DA and ethanol in the VTA. E2 enhances ethanol sensitivity of VTA DA neurons. High E2 states also results in a change in the balance of D2R autoinhibition, with enhanced acute DA inhibition but also enhancement of the desensitization of D2R, as measured by DIR. The ERs that mediate the enhanced sensitivity to ethanol and DA have been identified, and potential downstream pathways mediating these effects have been proposed, although the mechanism for enhanced DA sensitivity is less clear. The work from this dissertation paves the way for a more in-depth investigation into the mechanistic actions downstream of ER and how E2 enhances behavioral responses to ethanol and other drugs of abuse.

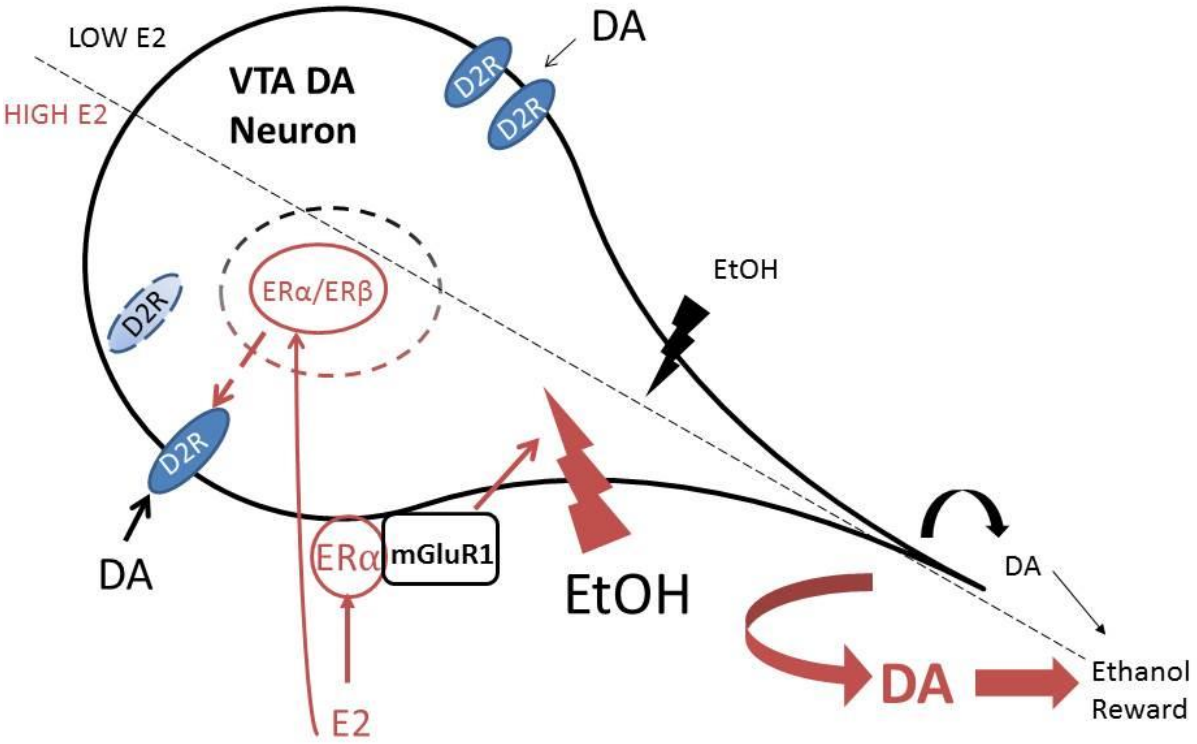

Figure 18. Summary of findings on E2 regulation of VTA DA neurons in female mice

In low E2 states (black), ethanol (EtOH) excites (lightning bolt) VTA DAergic neurons, which leads to the release of DA in target regions and is thought to mediate the rewarding effects of EtOH. DA inhibits the firing of DA neurons through the autoinhibitory D2R. In high E2 states (red), EtOH excitation is enhanced, which may lead to an increase in DA release in target regions and enhancement of ethanol reward. Although inhibition by DA is also enhanced under high E2 states, D2R desensitization may also be enhanced by E2. Together, this may cause a change in the balance of DA neurotransmission in the VTA.

6.8 Future Directions

Although many questions were answered in this dissertation, much remains unknown about E2 actions in the VTA. Future work should primarily focus on (1) how all the phases of the estrous cycle affect VTA DA DIR and sensitivity to DA and ethanol, (2) the mechanisms by which ERα/mGluR1 signaling regulate ethanol sensitivity in the VTA, (3) the mechanisms by which ERα/ERβ and GPER regulate DA sensitivity in the VTA, and (4) the mechanisms by which E2 regulates VTA DA DIR.

Mice were staged during estrus and diestrus due to the differences in circulating E2 levels and the longer duration of the phases compared to metestrus and proestrus, however E2 levels are highest during proestrus and actions of the other gonadal hormones fluctuating throughout the estrous cycle may interact with the E2 effects (Nelson et al. 1982). Future studies should expand on this work by thoroughly investigating DIR and the sensitivity to DA and ethanol throughout all phases of the estrous cycle. In mice, circulating E2 is lowest during the estrus phase at less than 0.3 pg/ml, and remain low during metestrus. Serum E2 levels increase during diestrus to around ~6 pg/ml, and then peak during proestrus at ~8 pg/ml (Nilsson et al. 2015). Measuring the electrophysiological properties in all the phases will provide more physiological relevant insight into how E2 interacts with the other naturally occurring hormonal changes in a gonadally-intact female mouse.

This dissertation has provided evidence for the regulation of ethanol sensitivity by ERα signaling through mGluR1 in female VTA neurons, but it remains unknown if ERα is directly interacting with mGluR. Immunohistochemistry can be used to determine if ERα and mGluR1 colocalize in VTA DA neurons, and direct physical interactions can be further demonstrated using co-immunoprecipitation experiments. In order to investigate if mGluR5 also plays a role in

the regulation of ethanol sensitivity, these experiments can be repeated using a selective antagonist for mGluR5 such as 2-Methyl-6-(phenylethynyl)pyridine (MPEP). Finally, in order to determine if endocannabinoids regulate ethanol sensitivity downstream of ERα/mGluR1 activation, perhaps through a retrograde signaling mechanism, future studies could use agonists and antagonists for CB1Rs and CB2Rs.

The work completed thus far has demonstrated that the E2 enhancement of DA sensitivity is through activation of both ERα and ERβ, but there was no change in the expression of *Th*, *Drd2s*, *Drd2l*, or *Drd3.* Although there were no changes in the expression of these genes critically involved in DA neurotransmission, there may be changes at the translational or protein level. Future studies could use immunohistochemistry or western blot in order to measure protein levels. E2 may also regulate the expression of other proteins involved in DA neurotransmission, such as DAT, so it may be beneficial for future studies to conduct gene expression profiling in the VTA from female mice in low and high E2 states. Finally, E2 may be regulating the density of D2R on the plasma membrane without regulating the expression of D2R. In order to investigate this, future studies should use radioligand binding autography to measure the density of D2R. These experiments would help determine how DA sensitivity is enhanced in high E2 states.

Finally, I have established a novel role of E2 in regulating the desensitization of D2R in the VTA DA neurons of female mice, and this may be due to increased levels of ALK since inhibiting ALK blocks D2R desensitization in male mice (Dutton et al. 2017). In order to begin investigating if the increased levels of ALK contribute to the enhanced desensitization of D2R in the VTA, the effects of inhibiting ALK expression and activity on DIR can be determined. Then, studies should determine if overexpression or activation of ALK can reverse the blocked DIR in

low E2 states. Future studies should also investigate D2R desensitization in the VTA throughout the estrous cycle. Changes in the desensitization of D2R may not completely correlate with changes in circulating E2 levels if other gonadal hormones effect D2R desensitization. Progesterone blocks the E2-mediated enhancement of the μ-opioid receptor internalization (Mills, Sohn, and Micevych 2004). Interestingly, DIR also occurs in male mice, without circulating E2, but local synthesis of E2 may be occurring in the VTA. Aromatase activity is detected in the rodent midbrain of from birth to adulthood (MacLusky et al. 1994), and aromatase protein as measured by immunofluorescence is present in the VTA of adult male mice (MacLusky et al. 1994). In order to determine if locally synthesized E2 plays a role in regulating DIR in both sexes, aromatase inhibitors can be administered onto slices containing the VTA to block local synthesis of E2, and DIR can be measured. If there is no evidence of E2 regulating DIR in male mice, this would be a very intriguing finding and would suggest sex-differences in the mechanisms for the desensitization of D2R.

Taken together, these experiments could provide greater insight into how E2 and the estrous cycle regulates the VTA in females, and could be therapeutically useful in treating alcoholism and other psychiatric disorders involving the VTA in women.

6.9 Overall Conclusions

This body of work is the first to comprehensively explore the role of ER in regulating the physiology of VTA DA neurons, particularly how ERs regulate the sensitivity of VTA DA neurons to DA and ethanol. These changes in VTA functioning may have significant behavioral effects to drive binge drinking and ethanol reward in females. Taken together, these data have provided important information about the VTA in females.

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APPENDICES

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (OACIB) (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

5/16/2018

Amy Lasek
Psychiatry
M/C 912

Dear Dr. Lasek:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and **renewed on 5/16/2018.**

Title of Application: Hormonal Regulation of Ethanol Binge Drinking and Reward
ACC NO: 17-081
Original Protocol Approval: 5/26/2017 **(3 year approval with annual continuation required).**
Current Approval Period: 5/16/2018 **to** 5/16/2019

Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 3

Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Involvement of brain estrogen receptors in alcohol consumption and reward (Inst # 00339614)*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
R01 AA026300	*Pending*		*UIC*	*Amy Lasek*
Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*The role of estrogen receptors in the ventral tegmental area*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
F31 AA026168	*Pending*		*UIC*	*Bertha Vandegrift*
Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*The Role of Amygdalar Estrogen Receptors in Ethanol Reward and Binge Drinking (Inst # 00016554)*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
F31 AA024344 (A1 v.)	*Funded*		*UIC*	*Elisa Hilderbrand*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

Timothy J. Koh, PhD
Chair, Animal Care Committee
TJK/*kg*
cc: BRL, ACC File, Elisa Hilderbrand, Bertha Vandegrift

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VITA

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EDUCATION: B.S., Molecular Biochemistry and Biophysics, Illinois Institute of Technology, Chicago, Illinois, 2011

Ph.D. Physiology & Biophysics, University of Illinois at Chicago, Chicago, Illinois, 2019

HONORS: Dean's List, Illinois Institute of Technology, Chicago, Illinois, 2011

Kate Barany Award, University of Illinois at Chicago, Illinois, 2016

Student Presenter Award, Graduate College, University of Illinois at Chicago, 2016

Mark R. Lambrecht Award for Scholarship and Commitment, University of Illinois at Chicaog, Illinois, 2017

Pre-doctoral Education for Clinical & Translational Scientists Supplemental Program Award, Center for Clinical and Translational Science at University of Illinois at Chicago, Chicago, Illinois, 2017

PUBLICATIONS: Nimitvilai, S., You, C., Arora D.S., McElvain M.A., **Vandegrift, B.J.**, Brodie, M.S**.**, Woodward, J.J., Differential effects of toluene and ethanol on dopaminergic neurons of the ventral tegmental area. *Frontiers in Neuroscience*;10: 434, Sep 22, 2016. PMID: 27713687

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You, C., Savarese, A., **Vandegrift, B.J.,** He, D., Pandey, S.C., Lasek, A.W**.,** Brodie, M.S., Ethanol acts on KCNK13 potassium channels in the ventral tegmental area to increase firing rate and modulate binge–like drinking, *Neuropharmacology* 144: 29-36, 2018. PMID: 30332606

SELECTED ABSTRACTS:

Vandegrift, B.J., You, C., Satta,R., Brodie, M.S., Lasek, A.W., High estrogen states increase ethanol and dopamine effects in ventral tegmental area neurons from free-cycling and ovariectomized female mice, *Alcoholism: Clinical and Experimental Research 40 (S1)*:17A, 2016.

Vandegrift, B.J., You, C., Satta, R., Brodie, M.S., Lasek, A.W., Estrogen receptors in the ventral tegmental area affect neuronal responses to ethanol and dopamine. *Alcoholism: Clinical and Experimental Research 41 (S1)*:abstract 32, 2017.

B.J. Vandegrift, M.S. Brodie, A.W. Lasek, Activation of estrogen receptor alpha enhances ethanol excitation of ventral tegmental area neurons in female mice, *Alcoholism: Clinical and Experimental Research 42 (S1)*:abstract 531, 2018.

BJ Vandegrift, MS Brodie & AW Lasek, Activation of estrogen receptor alpha enhances ethanol excitation of ventral tegmental area neurons in female mice, , Program No. 199.30, 2018 Neuroscience Meeting Planner, Washington, D.C., Society for Neuroscience, 2018. Online. Selected as a *Hot Topic* for the conference

TEACHING:

Physiology & Biophysics, University of Illinois at Chicago College, Training of high school student volunteer, 2016

Graduate Education in Medical Sciences, University of Illinois at Chicago College of Medicine, Teaching Assistant for Integrative Biology, 2016

Urban Health Summer Prematriculation Program (SPP), University of Illinois at Chicago Medical School, Chicago, Illinois; Muscle Physiology & Neurophysiology, 2017-2019

Graduate Education in Medical Sciences, University of Illinois at Chicago College of Medicine, Teaching Assistant for Physiology, 2017

Graduate Education in Medical Sciences, University of Illinois at Chicago College of Medicine, Teaching Assistant for Cell Biology, 2017

INVITED COLLOQUIA:

Seminar at the Department of Psychiatry, Neuroscience Seminar Series, University of Illinois at Chicago, Seminar title: "High estrogen states increase ethanol and dopamine effects in ventral tegmental area neurons from freely-cycling and ovariectomized female mice". Chicago, IL, 7/11/2016

Seminar at the Department of Physiology & Biophysics, University of Illinois at Chicago, Seminar title: "Estrogen Receptor Function in the Ventral Tegmental Area". Chicago, IL, 11/4/2016

Seminar at the Department of Psychiatry, Neuroscience Seminar Series, University of Illinois at Chicago, Seminar title: "Estradiol increases the sensitivity of the ventral tegmental area to dopamine and ethanol". Chicago, IL, 9 /11/2017

Seminar at the Department of Psychiatry, Neuroscience Seminar Series, University of Illinois at Chicago, Seminar title: "Estradiol enhances ethanol sensitivity of VTA DA neurons through a mGLUR1 dependent mechanism in female mice". Chicago, IL, 12/10/2018

PROFESSIONAL MEMBERSHIP: Society for Neuroscience 2015, 2018

Research Society for Alcoholism 2017