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The Role of the Preoptic Area in Response to Cocaine

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The Role of the Preoptic Area in Response to Cocaine

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Dedication

My wife Megan, who, for the last five years has patiently listened me practice (multiple times) every presentation I gave, tolerated my demanding schedule and brought me food on the weekends while I was running behavior.

My parents

The rats

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The Role of the Preoptic Area in Response to Cocaine

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The preoptic area of the hypothalamus is ideally suited to modulate the behavioral and neural response to drugs of abuse such as cocaine. The preoptic area is broken up into two major subregions the medial preoptic area (mPOA) and the lateral preoptic area (LPO), both of which send dense projections to mesolimbic dopamine system. Specifically, both project to the ventral tegmental area (VTA), a brain region implicated in drug-associated reward. Previous work has demonstrated the mPOA is involved in the behavioral and neural response to cocaine in female rats, however the mechanism through which the mPOA modulates response to cocaine is unclear. Whether or not the mPOA also plays a role in response to cocaine in male rats is still not clear. Furthermore the role of the adjacent LPO in response to cocaine is unexplored, despite its anatomical relationship to the VTA and involvement in intracranial self-stimulation. Here I demonstrate that estradiol acts in the mPOA of female rats to modulate response to cocaine. Specifically, microinjections of estradiol directly into the mPOA one day prior to cocaine administration increase cocaine-induced dopamine levels in the nucleus accumbens. The mPOA is also involved in the behavioral regulation of response to cocaine in male rats, as mPOA lesions enhanced cocaine-induced locomotion and reward.

Finally, activation of the LPO, with pharmacology or chemogenetics, potentiates reinstatement of cocaine seeking, an animal model of drug relapse. Together these results demonstrate that the preoptic area as a whole is involved in the regulation of the neural and behavioral response to cocaine and shed light on underlying regulatory mechanisms.

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Chapter 1: General introduction

1.1 Cocaine use and abuse in the United States

The US department of justice estimated that illicit drug use in the United States has an annual societal cost of 193 billion dollars (Center, 2011). Of illicit drugs, cocaine was most frequently rated by state and local law agencies as the greatest threat (Center, 2011). The Drug Abuse Warning Network (DAWN), which tracks drug related emergency room visits, estimated that between 2004-2011 cocaine related emergency room visits accounted for between 40-57% of all non-alcohol illicit drug related visits (2011 low year, 2006 high year); this corresponds to an average of 163.8 cocaine related visits per 100,000 emergency room visits (Netwrok, 2013). In the last 14 years, 85,921 people in the United States died due to cocaine related causes. Additionally, there was a 22% increase in the number of cocaine related deaths in 14 years between 1999 and 2013; increasing from 4,494 in 1999 to 5,495 in 2013, whereas the US population increased by 13% during the same time period (Centers for Disease Control and Prevention). The National Survey on Drug Abuse and Health estimates the lifetime prevalence of cocaine use to be 14.5%(+/- 0.26%) with 1.8% (+/- 0.08%) using cocaine last year; corresponding to 4.4-4.8 million individuals using cocaine within the last year(United States Department of et al., 2014). Taken together these statistics demonstrate that cocaine presents a public health concern for the United States.

Cocaine is an addictive stimulant drug that has been administered by humans for thousands of years. As a stimulant drug, cocaine enhances mental alertness, energy,

sensitivity to external stimuli such as light and sound, decreases the desire for physical needs such as food and sleep, and may facilitates performance on physical tasks(Abuse, 2010). Many individuals in rural South American countries such a Bolivia, Peru, and Colombia, use cocaine products on a daily basis. Specifically, farmers and miners in these countries chew leafs form the *Erythroxylon coca* plant to increase energy and attention in order to work longer hours and increase economic output (Project, 1995). On the other hand, cocaine use in western countries, such as the United States, follows a bimodal pattern of use. Some individuals use cocaine in social situations for its euphoric and stimulant effects. In these cases cocaine hydrochloride is often administered intranasally, although it is also sometimes administered intravenously, and may be combined with other drugs such as alcohol for a synergistic effect (Project, 1995). Intranasally derived highs tend to last for 15-30 minutes followed by a withdrawal state, which may facilitate additional drug use and the development of addiction (Gawin and Ellinwood, 1988; Wise and Koob, 2014). The second group of cocaine users in The United States consists primarily of socially marginalized and minority individuals such as the homeless or unemployed, and sex workers(Project, 1995). These individuals primarily smoke cocaine freebase, also known as crack. While the behavioral effects of smoking cocaine are similar to intranasal administration, the smoking of cocaine reduces the time it takes for cocaine to enter the blood and reach the brain leading to a more pronounced yet shorter, 5-10 minutes, high(Abuse, 2010).

Cocaine use is also associated with a number of adverse physiological and neurological responses. As a stimulant, cocaine constricts blood vessels and this in turn can have negative cardiovascular effects such as heart attacks or cardiac arrhythmia. Heavy cocaine users are also at risk of neurological perturbation such as depression, mood swings, loss of cognitive function as well as seizures and strokes (Abuse, 2010). Finally, individuals, who use cocaine, are at risk of losing control over cocaine use, which may ultimately end in addiction. This behavioral pattern can be viewed as a downward spiral where individuals initially use drugs for their hedonic value but become tolerant to the effects over time and enter into a shifting state of compulsive drug taking and abstinence/withdrawal (Koob and Le Moal, 2001). Eventually this tolerance may lead to an opponent process state where the negative effects of cocaine outweigh the positive effects and an individual is dependent on cocaine to feel normal (Koob et al., 1997). However, dependence and addiction are not necessarily synonymous and other mechanics have also been proposed to explain the progression from moderate use to addiction. Specifically, the incentive-salience model suggests subjects gradually switch from liking a drug to wanting a drug with repeated exposures (Robinson and Berridge, 1993) whereas others have argued that neither dependence nor tolerance are necessary for compulsive drug taking (Wise and Koob, 2014) to occur as recovering addicts easily relapse (Stewart and Wise, 1992). Thus cocaine's addictive potential is well characterized though how exactly it acts in the brain to produce an addicted phenotype is still unclear.

1.2 ANIMAL MODELS OF COCAINE USE

1.2.1 Cocaine-induced locomotion

Animal models have been developed to better study how cocaine acts in the brain to affect behavior. As a psychomotor stimulant, one of cocaine's basic physiological effects is an increase in locomotion (Le Moal and Simon, 1991). Unlike other behavioral paradigms, cocaine-induced locomotion does not require surgery or multiple sessions of conditioning/operant learning. Thus, cocaine-induced locomotion can be used as a rapid screening tool to assess the involvement of various brain regions or neural circuits in cocaine response as well as the efficacy of pharmacological manipulations (Yeh and Haertzen, 1991; Sabeti et al., 2002; Kosten et al., 2014).

1.2.2 Conditioned place preference and self-administration measures of cocaine reinforcement

The reinforcing properties of cocaine are often measured using either conditioned place preference (CPP) or self-administration (Sanchis-Segura and Spanagel, 2006). Self-administration is an operant procedure and holds construct and face validity for human cocaine consumption (Sanchis-Segura and Spanagel, 2006). However, self-administration requires catheterization of the jugular vein, which leaves animal susceptible to loss of catheter patency and infection. Self-administration studies allow researchers to determine the brain regions and neurochemistry that underlying cocaine administration as well as how pharmacological manipulations affect cocaine taking (Ahmed and Koob, 1999; Piazza et al., 2000; Kantak et al., 2001; Robinson et al., 2001; Larson et al., 2010). This procedure can also be used to measure the motivation to administer cocaine using a progressive ratio paradigm (Richardson and Roberts, 1996) or escalation of cocaine

taking (Ahmed and Koob, 1998, 1999). Unlike self-administration, CPP relies on experimenter-administered cocaine; therefore CPP does not require surgery and is a higher throughput procedure. CPP uses a Pavlovian approach where cocaine is paired with distinct unconditioned stimuli; often environmental features such a floor surface, lighting, or odor (Tzschentke, 1998). After several pairing animals spend more time in the cocaine paired environment when given a choice between two or three different environments (Durazzo et al., 1994). Like, self-administration, cocaine CPP has been used to explore the neural regulation of cocaine reward and reinforcement (Tzschentke, 1998, 2007).

1.2.3 Reinstatement of cocaine seeking

A major deleterious component of addiction is the tendency to relapse after drug taking has ceased (Koob and Le Moal, 2001). Reinstatement of drug seeking using animals trained to self-administer cocaine may model relapse in humans. In this paradigm, animals first learn to self-administer cocaine and then undergo a period of extinction (Davis and Smith, 1976; Goldberg et al., 1981), where operant responding no longer yields cocaine, or withdrawal, a period of extended abstinence with a single extinction exposure prior to reinstatement of seeking (Tran-Nguyen et al., 1998). In both cases, cocaine (De Vries et al., 1998; Self and Nestler, 1998), cocaine-related cues (de Wit and Stewart, 1981; Meil and See, 1996), or stress (Stewart, 2000) produce an increase in operant responding behavior, typically nose pokes or lever presses, in the absence of cocaine (Shalev et al., 2002; Bossert et al., 2013). It is also worth mentioning that drug exposure will also reinstate place preference following a period of extinction though there

are far fewer studies employing this methodology to model relapse (Mueller and Stewart, 2000). Reinstatement of drug seeking studies have helped elucidate brain circuits and neurochemistry that might be involved in relapse in humans (Bossert et al., 2005) as drugs(Jaffe et al., 1989), drug cues (Carter and Tiffany, 1999), and stress (Sinha et al., 1999) precipitate cravings and relapse in humans. Furthermore, pharmacological interventions that prevent relapse in humans also block drug, but not stress, induced reinstatement of seeking in animals (Le et al., 1999; Leri et al., 2004; Sorge et al., 2005). However, some caution should be taken when comparing animal models to humans, since human addicts do not often undergo extinction whereby drug based responses (smoking, injecting, drink, etc.) fail to illicit drug delivery and drug cue devaluation does not improve drug relapse rates (Drummond et al., 1995; Marlatt, 1996). The animal models described here have allowed researchers to understand where and how cocaine acts in the brain to modulate a variety of behaviors, ranging from physiological activation to renewed drug taking behavior following extinction, as well as screen for potentially beneficial treatments in a manner we cannot accomplish with human subjects.

1.3 COCAINE PHARMACOLOGY AND MECHANISMS OF ACTION

Cocaine exerts its physiological and behavioral effects by entering the blood where it can then, due to its lipophilic nature (Nayak et al., 1976), freely cross the blood brain barrier and alter behavior by interacting with several neurotransmitter systems. When directly administered into the blood cocaine reaches peak concentration in the brain after 15 minutes and is fully cleared from the system within 6 hours in rats (Nayak et al., 1976). In humans, the half-life of intravenous cocaine ranges between 20 and 60 minutes (Javaid

et al., 1983) with an average of about 45 minutes (Chow et al., 1985) and has a peak brain concentration approximately 4-10 minutes after intravenous administration (Fowler et al., 1989). Once in the brain cocaine acts as a dopamine, norepinephrine, and serotonin agonist by blocking the reuptake of these three neurotransmitters. While cocaine enhances activity in these three neurotransmitter systems, most work on neural regulation of cocaine behavior and addiction has focused on the modulation of dopaminergic neurotransmission (Kuhar et al., 1991; Wise, 2004). This is true partially because dopamine transporter binding affinity correlates with cocaine self-administration in rodents (Ritz et al., 1987) and subjective cocaine highs in humans (Volkow et al., 1997). However, studies using a strain of mice that have been genetically modified to lack dopamine reuptake transporters (DAT) have demonstrated that while DAT are necessary for the expression of cocaine-induced locomotion (Giros et al., 1996) they are not necessary for the expression of cocaine-induced conditioned place preference (Sora et al., 1998) or cocaine self-administration (Rocha et al., 1998); two commonly used measures of cocaine reward in animal models. It is worth noting that animals lacking DAT have chronically elevated dopamine levels, exhibit hyper locomotion, and survive to adulthood at a much lower rate; ~70% DAT knockout survive to adulthood vs. ~98% of wild type reach adulthood (Giros et al., 1996). Genetic knockdown of either norepinephrine reuptake transporters (NERT)(Xu et al., 2000) or serotonin reuptake transporters (SERT)(Sora et al., 1998) failed to attenuate cocaine-induced CPP. On the other hand a combined knockdown of SERT and DAT attenuated cocaine-induced CPP (Sora et al., 2001) whereas a combined knockdown of NERT and SERT (Hall et al., 2002) had little

effect on cocaine-induced CPP suggesting cocaine reward is primarily modulated by dopamine but in the absence of dopamine the serotonin system is able to act in a compensatory manner. Finally, cocaine still increases dopamine levels in the NAc in DAT knockout mice suggesting that cocaine induced-dopamine may still be important for behavioral response to cocaine (Carboni et al., 2001). Thus while genetic studies have demonstrated DAT is not required for cocaine behaviors (Uhl et al., 2002), their interpretation is less clear given the chronic elevation of dopamine levels in these animals and the wealth of pharmacological studies implicating a role for dopamine in the regulation of cocaine behaviors.

1.3.1 The role of the mesolimbic dopamine system in response to drugs of abuse and reward

The mesolimbic circuit is comprised of dopamine neurons in the ventral tegmental area (VTA) that send projections to the nucleus accumbens (NAc) as well as the hippocampus, amygdala, and prefrontal cortex (Marinelli et al., 2006; Pierce and Kumaresan, 2006; Volkow and Morales, 2015). The VTA to NAc projection is thought to be especially important in drug response as all drugs of abuse increase dopamine levels in the NAc: cocaine (Hurd et al., 1989; Caine and Koob, 1994a), amphetamine/methamphetamine (Carboni et al., 1989; Kashihara et al., 1991; Lominac et al., 2014), opioids (Spanagel et al., 1992; Hemby et al., 1995), alcohol (Yoshimoto et al., 1992; Ramachandra et al., 2011), nicotine (Damsma et al., 1989; Nisell et al., 1994), marijuana (Chen et al., 1991; Cheer et al., 2004), and ecstasy (Feduccia and Duvauchelle, 2008). During self-administration of cocaine dopamine levels in the NAc increase in response to cocaine and

return baseline shortly after self-administration ends (Hurd et al., 1989). Thus self-administration may reflect an attempt to maintain extracellular NAc dopamine levels above baseline (Wise et al., 1995). Rodents will learn to self-administer cocaine directly into the NAc (McKinzie et al., 1999), however, this effect appears to be limited to the shell but not the core of the NAc (Rodd-Henricks et al., 2002; Ikemoto, 2003). Additionally, microinjections of cocaine into the NAc produce CPP (Liao et al., 2000). Cocaine microinjections into the striatum produces locomotion, with NAc microinjections producing the greatest increase in locomotion (Delfs et al., 1990). Cocaine, or other drugs that increase dopamine in the NAc, enhance reinstatement of cocaine seeking behavior, a measure of drug relapse in rodents (Shalev et al., 2002). Cocaine also modulates the physiology of dopamine neurons in the VTA that project to the NAc. Specifically, cocaine decreases the firing rate of VTA dopamine neurons (Einhorn et al., 1988), presumably via the activation of autoreceptors on terminals in the NAc (Beaulieu and Gainetdinov, 2011), whereas both single and repeated cocaine administration produces long-term potentiation in VTA dopamine neurons (Ungless et al., 2001; Liu et al., 2005). Furthermore, withdrawal from cocaine self-administration increases the activity of dopamine neurons in the VTA. Additionally, rats that were more sensitive to novel environments display prolonged increases in VTA dopamine neuron firing rate after cocaine withdrawal compared to conspecifics that display less locomotion in response to a novel environment (McCutcheon et al., 2009).

1.3.2 Dopamine signaling in the mesolimbic circuit

Dopamine signaling is accomplished via binding to a family of G protein-coupled dopamine receptors found throughout the brain. Dopamine receptors are classified based on their modulation of cyclic adenosine monophosphate (cAMP) activity via adenylyl cyclase (Vallone et al., 2000). Type-one dopamine receptors (D1R) consist of the D1 and D5 receptors subtypes and are typically found on the postsynaptic membrane (Tiberi et al., 1991). Dopamine binding at D1R activates $G\alpha_{s/olf}$ G proteins, which in turn facilitate the production of cAMP via adenylyl cyclase signaling (Beaulieu and Gainetdinov, 2011). Type-two dopamine receptors (D2R) consist of the D2, D3, and D4 receptor subtypes and are found postsynaptic membrane (Andersen et al., 1990), but also function as autoreceptors on the presynaptic membrane of dopamine neurons (Sokoloff et al., 2006; Beaulieu and Gainetdinov, 2011). D2R activity inhibits the production of cAMP via $G\alpha_{i/o}$ G proteins which blocks adenylyl cyclase production (Beaulieu and Gainetdinov, 2011). Genetic knockdown of D1R produces impairment of psychostimulant-induced locomotion (Crawford et al., 1997) and cocaine self-administration (Caine et al., 2007) with no effect on CPP (Miner et al., 1995). On the other hand knockdown of D2R causes mice to administer more cocaine at higher doses but not lower doses compared to wild-type animals (Caine et al., 2002) and enhances cocaine-induced locomotion (Chausmer et al., 2002). Thus D1R and D2R appear to play opposing roles in the regulation of response to cocaine. Nevertheless manipulation of these receptors has implicated both D1R and D2R in response to cocaine and animals will

learn to self-administer both D1R (Self and Stein, 1992; Self et al., 1996b) and D2R (Wise et al., 1990) agonists in a manner similar to cocaine self-administration.

1.3.3 Pharmacologically manipulation of the mesolimbic dopamine system affects cocaine responsive behaviors

Destruction of dopamine terminals in the NAc with 6-hydroxydopamine (6-OHDA) impairs learned cocaine self-administration (Pettit et al., 1984), while recovery of self-administration following lesions is positively correlated with post-surgery dopamine levels (Roberts et al., 1980). Dopamine depleting lesions of NAc terminals also blocks cocaine induced locomotion (Kelly and Iversen, 1976) and CPP (Sellings et al., 2006). Systemic administration of dopamine antagonists such as haloperidol (D2R) and SCH23390 (D1R) block cocaine-induced CPP and inhibit cocaine-induced locomotion (Adams et al., 2001; Nazarian et al., 2004). Furthermore, systemic administration of dopamine antagonists modulates self-administration behavior. Specifically, receptor antagonism by general, D1R, or D2R antagonists increase the number of cocaine infusions taken in a dose dependent manner, shift the dose response curve for cocaine to the right, an indication of reduced drug efficacy (Piazza et al., 2000), and increase the ED50 of cocaine (Koob et al., 1987; Bergman et al., 1990; Spealman, 1990; Corrigall and Coen, 1991; Caine and Koob, 1994b). Taken together these results demonstrate dopamine antagonists attenuate the reinforcing properties of cocaine. Within the NAc, microinjections of dopamine antagonists inhibit the reinforcing aspects intravenous (Maldonado et al., 1993; McGregor and Roberts, 1993) and intra-NAc (Rodd-Henricks et al., 2002) cocaine self-administration. Dopamine agonists reinstate cocaine-seeking

behavior and facilitate locomotor response when microinjected into the NAc (Self et al., 1996a; Cornish and Kalivas, 2000; Bachtell et al., 2005). Additionally, administration of dopamine antagonists into the NAc shell, but not core, block cocaine primed reinstatement of seeking (Anderson et al., 2003; Bachtell et al., 2005). Thus manipulation of dopamine activity in the NAc affects a wide range of cocaine responsive behaviors ranging from simple physiological response to goal directed behaviors such as cocaine seeking and taking.

Cocaine-induced dopamine in the NAc modulates the activity of GABAergic medium spiny neurons (Koob and Volkow, 2010), which then project to brain regions that regulate motor output. These neurons can be divided into two populations: one that express predominantly D1R and project to substantia nigra and other basal ganglia targets as well as the ventral palladium (Smith et al., 2013), and a second set of neurons that express D2R that innervate the ventral palladium (Smith et al., 2013). While the precise role of D1R and D2R medium spiny neurons in cocaine response and addiction has yet to be elucidated, D1R containing neurons seem to be involved the rewarding aspects of cocaine whereas D2R neurons primarily regulate cocaine seeking behaviors (Self, 2010). Regardless of dopamine receptor subtype, NAc medium spiny neurons project to brain regions involved in locomotor regulation such as the ventral palladium, which serves as a transitional site between the limbic system and basal ganglia and putatively translates motivational signals to motor actions (Mogenson et al., 1980), or the substantia nigra (Zhou et al., 2003) a which is involved in the regulation of motor function (Pollack, 2001;

Mink, 2008). In line with anatomical observations that dopamine sensitive NAc neurons project to the basal ganglia, it has been proposed that dopamine activity in the NAc serves to facilitate approach towards salient stimuli (Ikemoto and Panksepp, 1999). Thus dopamine activity in the NAc regulates the reinforcing properties of cocaine and activity of brain regions involved in motor output allowing dopamine signaling in the NAc to regulate appetitive and consummatory aspects of cocaine behavior.

1.4 THE ROLE OF GONADAL HORMONES IN COCAINE RESPONSE

1.4.1 Clinical results

Clinical and preclinical studies demonstrate there are sex differences in the response to drugs of abuse such as cocaine. Gonadal hormones are at least partially responsible for the enhanced sensitivity females exhibit to cocaine. In humans, self-reported subjective effects of both cocaine and amphetamine are higher during the follicular phase (when estrogen is rising and progesterone is low) than they are during the luteal phase (when estrogen levels are declining and progesterone is high) (Justice and De Wit, 2000; Evans and Foltin, 2010). Figure 1.1A provides a visual description of gonadal hormone fluctuation across the menstrual cycle. Artificial elevation of estrogen to 750 pg/ml during the early follicular phase increases the self-reported desire to obtain amphetamine as well as positive affect in response to amphetamine administration (Justice and De Wit, 2000). Conversely, subjective ratings of cocaine and amphetamine are inversely related to salivary progesterone (White, 2002). Additionally, progesterone administration during the follicular, but not the luteal, phase decreases some of the positive subjective effects of cocaine such as ratings of drug quality and euphoria; however, progesterone does not

modulate drug response in men (Sofuoglu et al., 2002; Evans and Foltin, 2010). Thus evidence from humans suggests that estrogen potentiates and progesterone attenuates the subjective response to cocaine in females but not males.

1.4.2 Preclinical results

Sex differences in response to cocaine are also attributable to gonadal hormones in animal models of drug use (Becker and Koob, 2016). Unlike women, female rodents experience a 4-5 day estrous cycle. This cycle is generally divided into four parts: diestrus 1 (sometimes called metestus); diestrus 2 (sometimes called diestrus); proestrus, and estrus. During diestrus gonadal hormone levels are low, though there is a slow rise in estradiol beginning on diestrus 2 and peaking mid proestrus. Several hours after this peak in estradiol there is a progesterone peak. Four to six hours after the progesterone peak and ten to twelve hours post estradiol peak female rodents will ovulate and enter a state of sexual receptivity known as behavioral estrus (Blaustein and Erskine, 2002; Levine, 2015). It is during estrus phase of the estrous cycle that females exhibit the greatest sensitivity to cocaine. Figure 1.1B provides a visual description of gonadal hormone fluctuation across the rat estrous cycle.

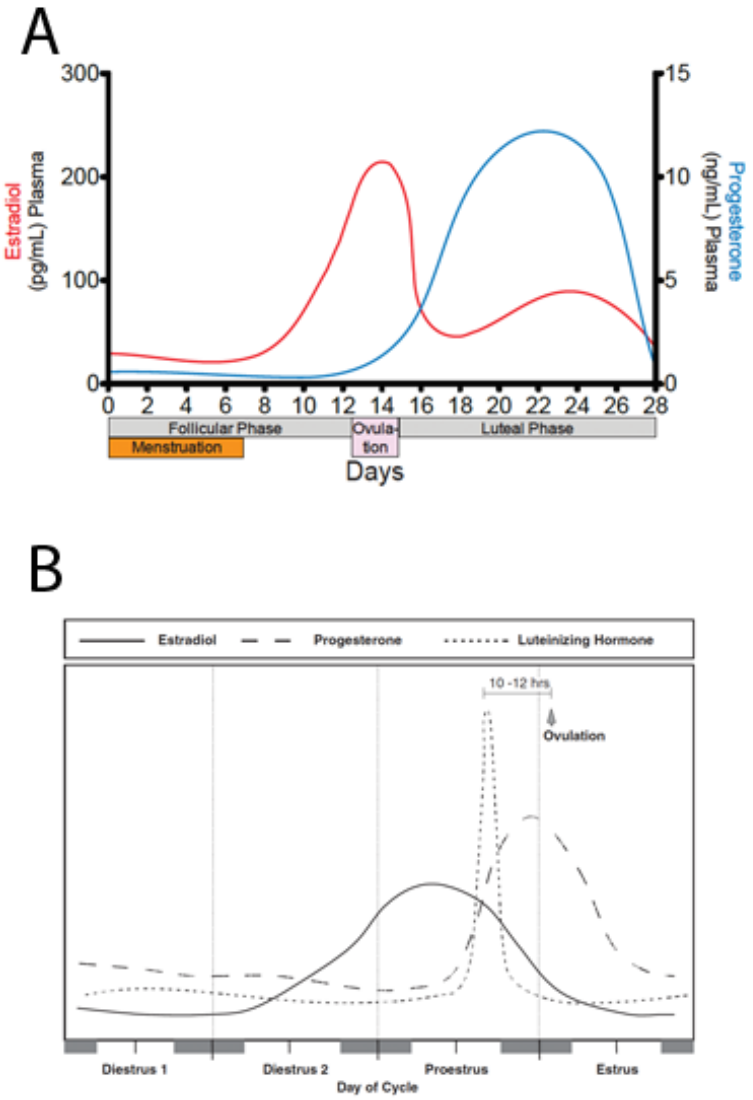

Figure 1.1 Ovulatory Cycles

Diagrams illustrating the fluctuation in gonadal hormones across the menstrual cycle in humans (A) and the estrous cycle in rodents (B). In both cases estrogen raises before progesterone. Menstrual cycle diagram adapted from (Butcher et al., 1974), estrus cycle diagram adapted from (Goldman et al., 2007)

Female rats also show differential responding to drugs of abuse as a function of ovarian hormones. During estrus, females administer more cocaine and reach a higher break point than during proestrus and diestrus (Roberts et al., 1989). Ovariectomized (OVX) females with estradiol, a potent estrogen (Anstead et al., 1997), or estradiol and progesterone replacement exhibit greater cocaine-induced CPP compared to cholesterol-replaced subjects, while replacement with progesterone alone prevents cocaine-induced CPP (Russo et al., 2003b). Estrogen replaced rats that have undergone OVX administer more cocaine at lower doses than OVX controls (Hu et al., 2004). Conversely, progesterone administration decreases cocaine self-administration (Jackson et al., 2006). However, estrogen administration to intact females, males or castrated males does not facilitate cocaine self-administration (Jackson et al., 2006). Estrogen treatment also increases escalation of cocaine administration during long access self-administration in OVX subjects while progesterone treatment decreases cocaine responding during long access as well as in a 2-hour posttest in both sham and OVX-estrogen replaced subjects (Larson et al., 2007). Under a discrete trial procedure, a model of drug binge behavior (Roberts et al., 2002), estrogen replacement increases cocaine intake during the intervals when cocaine was available (Lynch and Taylor, 2005). When given an option between two doses of cocaine during in a long access self-administration paradigm female rats in estrous display a much greater preference for the higher cocaine dose (Lynch et al., 2000). Antagonism of estrogen receptors with tamoxifen attenuates the acquisition of cocaine self-administration to the same degree as OVX (Lynch et al., 2001). Furthermore,

tamoxifen administration decreases cocaine intake in females but not males (Dalton et al., 1986).

Ovarian hormones also modulate cocaine seeking, as cocaine primed reinstatement is highest during estrus and lowest during proestrus (Feltenstein and See, 2007). Additionally, removal of the ovaries decreases cocaine-primed reinstatement of seeking following extinction, whereas acute or chronic estrogen replacement restores the behavior (Larson et al., 2005; Larson et al., 2007). With regards to basic physiological responding to cocaine, female rats in estrus and proestrus display a more pronounced locomotor response compared to rats in diestrus. This study also found that OVX subjects with estrogen or estrogen and progesterone treatment showed greater cocaine-induced locomotion than subjects without hormone replacement (Sell et al., 2000). However, estrogen does not modulate behavioral sensitization that occurs with repeated cocaine administration (Sell et al., 2002). There is also evidence that cocaine and estrogen interact to make the brain more sensitive to rewarding stimulation since the combination of systemic estrogen and cocaine lowers the threshold required for intracranial self-stimulation (Galankin et al., 2010).

There is mixed evidence from non-human primate studies on the relationship between ovarian hormones response to cocaine. One group found there was no difference in cocaine self-administration across the menstrual cycle of five rhesus macaques (Cooper et al., 2013), whereas a different group found greater cocaine responding, measured with

a progressive ratio break point, during the follicular phase but only at the lowest dose of cocaine (0.0032 mg/kg/injection) in crab-eating macaques (Mello et al., 2007). This effect may be dose dependent as the lowest dose used by Cooper *et al*. was 0.0125 mg/kg/infusion. Similar to human studies reported above (Evans and Foltin, 2010) progesterone treatment decreases cocaine self-adminstration and shifts the dose response curve down and to the right in female rats (Evans and Foltin, 2010). Thus there is evidence in both rodents and nonhuman primates that estrogen potentiates and progesterone attenuates response to cocaine.

1.4.3 Neural circuits involved in gonadal hormone dependent response to cocaine

In naturally cycling female rodents, basal extracellular dopamine concentration in the striatum is highest during estrus (Xiao and Becker, 1994) whereas the binding affinity of D2 agonists in the striatum increases during proestrus and diestrus (Di Paolo et al., 1988). Estradiol administration to OVX rats increases basal, amphetamine- (Becker, 1990a), cocaine- (Peris et al., 1991), nicotine-(Dluzen and Anderson, 1997), and KCl (Becker, 1990b) invoked dopamine release in the striatum; a brain region which receives dopaminergic input and contains the NAc (Nicola et al., 2000). Direct administration of estradiol into the NAc of OVX rats modulates potassium-induced dopamine release in a triphasic manner. Specifically, dopamine levels increase two minutes after estradiol treatment and then decrease fifteen minutes post treatment rising again one to two hours after estradiol administration (Thompson and Moss, 1994). DAT expression in the NAc is down regulated by short term OVX but increases 3 months following OVX; estrogen replacement does not modulate DAT levels at either time point (Bosse et al., 1997).

Furthermore, D1 and D2 receptor expression in the striatum is decreased 2 weeks and 3 months following OVX, though there were no differences in receptor affinity (Bosse and Di Paolo, 1995). Progesterone administration to OVX rats increases and then decreases extracellular dopamine in the striatum over the course of an hour (Di Paolo et al., 1986), while low doses of progesterone decreases potassium (Cabrera et al., 1993) and amphetamine-induced dopamine release in the striatum (Dluzen and Ramirez, 1989). Acute estradiol treatment 30 minutes prior to cocaine administration increases cocaine-stimulated dopamine in the dorsal lateral striatum but not NAc of OVX females (Cummings et al., 2014). Cocaine induced inhibition of dopamine reuptake as well as number of dopamine neurons in the VTA and substantia nigra is decreased by OVX (Walker et al., 2012). Taken together estrogen enhances dopamine activity in the NAc of female rodents.

Estradiol treatment also attenuates GABA release from medium spiny neurons, the primary target of VTA dopamine neurons in the NAc (Hu et al., 2006), suggesting that estrogen may attenuate the activity of medium spiny neurons. Inhibition of these medium spiny neurons has been suggested to be the final common pathway in drug reward as depression of these neurons by opiates (Olds, 1982; David and Cazala, 2000; Margolis et al., 2003) or glutamate antagonists (Carlezon and Wise, 1996) support self-stimulation (Wise, 2002). However, it is worth noting that this conclusion is not fully supported by the dopamine literature. While neither D1 nor D2 agonists alone are sufficient to produce self-administration when microinjected into the NAc a cocktail of the two does

suggesting both the excitatory and inhibitory effects of D1R and D2R are required for self-administration (Ikemoto et al., 1997). Conversely, D2R knockout animals are more sensitive to cocaine (Caine et al., 2002); the opposite of what would be expected if depression of medium spiny neurons in the NAc modulated response to drugs of abuse. Recent optogenetic studies demonstrate that activation of D1R medium spiny neurons enhances cocaine CPP while activation of D2R medium spiny neurons inhibits cocaine CPP (Lobo et al., 2010). Taken together, these studies demonstrate that cocaine reward likely results from inhibition of D2R medium spiny neurons and excitation of D1R medium spiny neurons. Thus estrogen may enhance responsiveness to cocaine by inhibiting medium spiny neurons, though the dopamine receptor profile of these neurons is currently unknown.

1.4.4 Summary

In summary, females are more sensitive to the reinforcing and addicting effects of drugs of abuse such as cocaine. In both humans and non-human animals sensitivity to cocaine varies as a function of the menstrual/estrous cycle. This combined body of literature suggests that estrogen faciliates and progesterone attenuates the behavioral and neurological response to cocaine. Finally, in animal models, estrogen treatment restores both behavioral and neuronal sensitivity to dopamine in animals that have undergone ovariectomies.

1.5 THE PREOPTIC AREA OF THE HYPOTHALAMUS: A POTENTIAL MODULATOR OF RESPONSE TO COCAINE

1.5.1 Circuitry of the preoptic area

One area of the brain that may modulate sex differences in drug response is the preoptic area, specifically the medial preoptic area (mPOA). The preoptic area is a heterogeneous structure in the rostral hypothalamus that has been divided into a medial and lateral region; lateral preoptic area (LPO). The major visible anatomical difference between these two regions is that the mPOA contains the sexually dimorphic nucleus or median preoptic nucleus, which is larger in males than females (Dohler et al., 1984). Both regions contain neurons expressing γ-Aminobutyric acid (GABA) (Zhao et al., 2013; Kallo et al., 2015), glutamate (Geisler et al., 2007; Kallo et al., 2015), and neurotensin (Zahm et al., 2001; Geisler and Zahm, 2006). Stimulation of both structures promotes locomotion (Sinnamon, 1993) and both regions send GABAergic and glutamatergic projections to the VTA (Kallo et al., 2015). While there are few if any structural divisions between the mPOA and LPO, the mPOA contains predominantly GABAergic neurons (Zhao et al., 2013) and contains a large number of neurons expressing estrogen and progesterone receptors (Simerly et al., 1990; Lauber et al., 1991) whereas the LPO has few neurons that express hormone receptors. Furthermore, the mPOA contains a population of galanin producing neurons (Wu et al., 2014) that are regulated in females by estrogens (Mitchell et al., 2004). The LPO also contains galanin neurons though they are far fewer in number and expressed along the transition zone between the mPOA and LPO (Cheung et al., 2001). While both the mPOA and LPO send projections to the VTA as well as the mesencephalic locomotor region (Swanson et al., 1984; Swanson et al., 1987), a

subdivision of the basal ganglia (Shik et al., 1966), they receive input from different brain regions. The mPOA is innervated directly by the bed nucleus of the stria terminalis and medial amygdala (Hull and Dominguez, 2012), two regions that receive chemosensory input (Baum and Everitt, 1992) and are involved response to cocaine (Hurd et al., 1997; Erb and Stewart, 1999), as well as indirectly by all sensory modalities (Simerly and Swanson, 1986). The LPO receives inhibitory projections from the septal nuclei (Swanson and Cowan, 1979) and NAc (Mogenson et al., 1983); both regions implicated in reward (Olds and Milner, 1954). Thus while both of these regions innervate the mesolimbic dopamine system via projections to the VTA, the mPOA is sexually dimorphic and has a high concentration of gonadal hormone receptors raising the possibility that estrogen and progesterone may modulate cocaine responsiveness by acting in the mPOA.

1.5.2 The medial preoptic area regulates hormonally sensitive naturally rewarding behaviors in female rodents

The mPOA modulates reproductive behaviors (Graham and Pfaus, 2013) and maternal care (Numan et al., 1977; Numan and Smith, 1984; Numan, 1988; Stack et al., 2002; Stolzenberg and Numan, 2011); both of which are regulated by hormonal activity within the mPOA. Electrical stimulation of the mPOA enhances maternal behavior (Morgan et al., 1997) whereas lesions or pharmacological inactivation of the mPOA disrupts aspects of maternal care such as pup retrieval and nursing (Gray and Brooks, 1984; Numan et al., 1988; Morgan et al., 1997). Crystalline estradiol implants in the mPOA restore maternal behavior in pregnant rats following a hysterectomy and OVX (Numan et al., 1977;

Stolzenberg and Numan, 2011). Estrogen in the mPOA may modulate maternal care via dopamine signaling as D1R agonists can facilitate maternal care in pregnant rats that have undergone a hysterectomy and OVX in manner similar to estradiol replacement described above (Numan et al., 1988).

Estrogen in the preoptic area facilitates lordosis; a measure of sexual receptivity in female rodents (Pfaus et al., 2015). Specifically estrogen implants in the mPOA produce lordosis in response to a sub-threshold systemic dose of estradiol (Barfield and Chen, 1977). Lesions of the mPOA also facilitate lordosis (Nance et al., 1977), while electrical stimulation of the mPOA suppresses lordosis (Pfaff and Sakuma, 1979). However, this enhancement is only observed when females are not able to pace the rate of copulation (Whitney, 1986). Finally, reduction of estrogen receptor alpha in the mPOA with shRNA inhibits lordosis in OVX and hormonally treated females (Spiteri et al., 2012). The same study also found that females with reduced estrogen receptor alpha spent more time around castrated males than did control female.

Maternal care and female sexual behavior are likely modulated through direct or indirect interactions between the mPOA and the mesolimbic dopamine system. The mPOA is able to modulate activity of the mesolimbic dopamine system as it projects to the VTA; however, the mPOA does not directly innervate the NAc (Simerly and Swanson, 1988; Tobiansky et al., 2013a; Ikemoto and Bonci, 2014). Specifically, 2.68(+/- 0.2) percent of neurons projecting to the VTA are mPOA efferents (Yetnikoff et al., 2015). Transient

inhibition of the mPOA attenuates conditioned behaviors associated with pups (Pereira and Morrell, 2010). Maternal care increases dopamine activity in the NAc (Champagne et al., 2004), whereas inhibition of the VTA impairs pup grooming (Seip and Morrell, 2009). Furthermore, unilateral lesions of the mPOA attenuated pup-induced cellular activity in the ipsilateral NAc (Stack et al., 2002), and the combination of unilateral mPOA denervation and contralateral VTA lesion impairs maternal behaviors (Numan and Smith, 1984). Estrogen sensitive neurons project to the VTA where they modulate lordosis (Sakuma, 2015). Furthermore oxytocin is needed in both the VTA and mPOA in order to induce the onset of postpartum maternal care (Pedersen et al., 1994). Thus, there is ample evidence that mPOA and mesolimbic system interact to regulate hormone-responsive naturally rewarding behaviors.

1.5.3 The medial preoptic area regulates homeostatic and physiological behaviors

The mPOA is also involved in the modulation of male sexual behavior (Hull and Dominguez, 2012; Will et al., 2014), thermoregulation (Roberts and Martin, 1977), osmoregulation (McGowan et al., 1988), slow wave sleep (Kaushik et al., 2011), offensive(Fuchs and Siegel, 1984) and defensive behaviors (Fuchs and Siegel, 1984), as well as locomotion (Reynolds et al., 2006). Excitation of the preoptic area with glutamate or blocking GABA with picrotoxin increases locomotion in anesthetized rats. That this effect is observed in anesthetized rats suggests the preoptic area may be involved in the mechanical regulation of locomotion independent of motivational stimuli (Sinnamon, 1993). However, these results are equivocal as pharmacological inactivation of the mPOA with the GABA-A receptor agonist increases both locomotion and NAc dopamine

levels (Osborne et al., 1993). Additionally, cholinergic activation of the mPOA with Carbachol decreases locomotor activity in a dose dependent manner (Brudzynski and Mogenson, 1986). Estrogen implant into the preoptic area increases locomotor activity when measured by wheel running, behavior that supports operant responding (Collier and Hirsch, 1971), but does not impact open field locomotion (Fahrbach et al., 1985).

The modulation of locomotion by the mPOA is of particular interest given the role of dopamine activity in the NAc on locomotion. Dopamine depleting lesion of the NAc with 6OHDA attenuates dopamine and cocaine induced locomotion (Kelly and Iversen, 1976). Conversely, dopamine (Pijnenburg and van Rossum, 1973), cocaine (Delfs et al., 1990), and amphetamine (Essman et al., 1993), when injected into the NAc increase locomotion. VTA dopaminergic neurons that project to the NAc regulate locomotion (Swanson and Kalivas, 2000). Thus the ability of the mPOA to regulate locomotion provides further evidence that this structure is able to modulate dopamine activity in the NAc.

1.5.4 The medial preoptic area regulates cocaine response in females

Lesions of the mPOA enhance the behavioral and neurochemical response to cocaine. Previous work by a graduate student in the Dominguez lab, Dr. Tobiansky, determined that radiofrequency lesions of the mPOA enhance cocaine-induced CPP as well as c-Fos expression in the NAc in female rats (Tobiansky et al., 2013a). Furthermore, Dr. Tobiansky found that neurons that project from the mPOA to the VTA are at least partially GABAergic, a find that has since been replicated by others (Kallo et al., 2015). Dr. Tobiansky also demonstrated that unilateral neurochemical lesions of the mPOA

elevated ipsilateral cocaine-induced dopamine release. Finally, Dr. Tobiansky showed that estrogen receptor alpha (ERa) and G protein coupled estrogen receptor 1 (GPER) were both found on mPOA-VTA efferents whereas there was minimal progesterone receptor (PR) expression on these same neurons. Thus, estrogens are far more likely to modulate response to cocaine via the mPOA than is progesterone in female rats.

1.5.5 The medial preoptic area in males: a potential role in cocaine reward

Up to this point I have discussed reasons why gonadal hormones such as estrogens may enhance response to cocaine response in females. However, it is also possible the mPOA also regulates response to cocaine in males. As with females the mPOA is also involved in the modulation of naturally rewarding behaviors in males. There is an extensive literature on the effects of the mPOA manipulation on male sexual behavior, response, and motivation in a variety of species (Hull and Dominguez, 2006, 2007; Will et al., 2014). Additionally, manipulation of the mPOA with gonadal hormones such as testosterone (Hull et al., 2006) or estradiol (Putnam et al., 2003) reinstates copulatory behavior in castrated males suggesting that gonadal hormones modulate reinforcing behavior in both males and females. While it is possible that gonadal hormones also act in the mPOA of males to modulate response to cocaine, this hypothesis is unlikely for several reasons. First castrated males do not differ from gonadally intact males with regards to cocaine-induced CPP whereas OVX females exhibit attenuated cocaine-induced CPP (Russo et al., 2003b). Furthermore, testosterone replacement does not facilitate cocaine-induced CPP in male rodents (Minerly et al., 2008). Castrated and intact males do not differ with regards to cocaine self-administration either (Hu et al.,

2004) and estradiol replacement does not enhance cocaine self-administration in castrated males as it does in OVX females (Jackson et al., 2006). Taken together these studies demonstrate that gonadal hormones do not modulate response to cocaine in males. However, it is possible, given the innervation of the VTA, that the mPOA is able to modulate response to cocaine in a hormonally independent manner. As evidence of this possibility pharmacological inhibition of the mPOA increases dopamine release in the NAc (Osborne et al., 1993) and electrical stimulation of the mPOA suppresses putative dopaminergic and GABAergic neurons in the VTA (Maeda and Mogenson, 1980).

1.5.6 The lateral preoptic area: a potential role in cocaine reward

While I have focused mostly on the role mPOA the anatomical division between the LPO and mPOA is not clear. There are few estrogen and progesterone receptors in the LPO compared to the mPOA suggesting that gonadal hormones likely do not regulate response to cocaine via the LPO. However, given the anatomical and functional similarities between the two regions it is still possible that the LPO may regulate response to cocaine: either behavioral or neurological. Specifically, both regulate aspects of sleep (Szymusiak et al., 2007; Kaushik et al., 2011) as well as locomotion (Sinnamon, 1993), a behavior sensitive to mesolimbic dopamine activity (Kelly and Iversen, 1976). Additionally, both send projections to the VTA. In fact the LPO accounts for ~5.24% of the afferents the VTA receives from other brain regions; the only region from which the VTA receives more afferents is the NAc (Yetnikoff et al., 2015). Since activity of VTA dopamine neurons have a profound effect on cocaine taking (Einhorn et al., 1988; Brebner et al., 2000a; Ungless et al., 2001) and seeking behaviors (Stewart, 1984; Stewart and Vezina,

1988), the abundance of projections from the LPO to the VTA suggest that activity in the LPO may also module response to cocaine. Furthermore, projections from the LPO to the VTA exhibit increased activity in response to systemic amphetamine administration (Colussi-Mas et al., 2007). Finally, the LPO supports intracranial self-stimulation further raising the possibility that this hypothalamic structure functionally modulates the mesolimbic dopamine pathway (Elder and Work, 1965).

1.5.7 Summary

The preoptic area is a heterogeneous region of the hypothalamus that contains two main subdivisions the LPO and mPOA. Both of these regions are implicated in the regulation of variety of behaviors although the mPOA has been more thoroughly studied given its sexually dimorphic nature and clear involvement in the regulation of reproductive behavior and maternal care. Given that the mPOA contains a larger number of gonadal hormone receptors it is possible that gonadal hormones act in the mPOA to modulate sensitivity to cocaine in female rodents. Previous work in our lab has demonstrated that lesions of the mPOA enhance the behavioral and neurologic response to cocaine, though the exact mechanism through which the mPOA regulates cocaine response remains unclear. Additionally, given that both of the mPOA and LPO heavily project to the mesolimbic dopamine system, accounting approximately eight percent of the afferents this region receives, it is also possible that they can modulate response to cocaine independent of hormonal action.

Chapter 2: Estrogen in the medial preoptic area modulates cocaine-induced dopamine in the NAc.

Data presented in this chapter was published along with other data in (Tobiansky et al., 2015)

2.1 Abstract

Estradiol enhances the behavioral and neurochemical response to cocaine in females. The medial preoptic area (mPOA), a region in the hypothalamus, contains one of the richest concentrations of estrogen receptors in the central nervous system and plays a key role in the regulation of two naturally rewarding behaviors: reproductive and maternal behaviors. Recent work in our lab has demonstrated that the mPOA regulates cocaine-induced dopamine levels in the nucleus accumbens (NAc) and that projections from the mPOA to the ventral tegmental area (VTA) are sensitive to estradiol. However, whether estradiol enhances the dopaminergic response to cocaine by acting in the mPOA is still unclear. To answer this question, here we examined whether microinjections of estradiol directly into the mPOA modulated cocaine-induced dopaminergic activity in the NAc. Results show that microinjections of estradiol directly into the mPOA increased cocaine-induced release of dopamine in the nucleus accumbens. These findings point to a novel estradiol-dependent pathway that modulates cocaine-induced dopaminergic activity in the mesolimbic system.

2.2 Introduction

Compared to men, women are more susceptible to the reinforcing and addictive properties of drugs of abuse such as cocaine (Becker and Hu, 2008). These sex differences are at least partially attributable to sex steroid hormones. Women self-report

lower cocaine craving during the luteal phase of the menstrual cycle, when progesterone is high, compared to the follicular phase, when estrogen is rising and progesterone is low. Administration of progesterone decreases the perceived hedonic value of cocaine in women during the follicular phase, but does not alter cocaine response in men (Evans and Foltin, 2010). Similar observations have been made in studies using rodents. Female rats develop cocaine self-administration faster and at lower doses than males (Hu et al., 2004). During estrus, females administer more cocaine and reach a higher break point than during proestrus and diestrus (Roberts et al., 1989). Additionally, removal of ovaries impairs cocaine self-administration, whereas estradiol (E2) replacement restores cocaine self-administration (Hu et al., 2004). Conversely, treatment with progesterone attenuates cocaine self-administration in estrogen-replaced animals (Jackson et al., 2006). Thus, in both humans and rodents, estradiol potentiates and progesterone attenuates cocaine responses.

One brain region where hormones may act to influence cocaine response is the mPOA. Sex steroid hormones in the mPOA influence naturally rewarding behaviors such as maternal care (Stolzenberg and Numan, 2011) and reproductive behaviors in females (Graham and Pfaus, 2013). While reward related regions such as the VTA, NAc, or striatum contain sex-steroid hormone receptors (Maus et al., 1989; Schultz et al., 2009; Frye et al., 2013), the mPOA contains one of the highest concentrations of cells expressing sex-steroid hormone receptors including but not limited to estrogen receptor alpha (ERα), progesterone receptor (PR) and membrane bound g-protein estrogen

receptor (GPER) (Lauber et al., 1991; Brailoiu et al., 2007). These receptors are behaviorally relevant, as crystalline estradiol implants in the mPOA restore maternal behavior in pregnant rats following a hysterectomy and OVX (Numan et al., 1977; Stolzenberg and Numan, 2011). Additionally, viral-mediated knockdown of ERα in the mPOA of OVX female rats inhibits lordosis and increases the time spent around castrated males (Spiteri et al., 2012).

These sex steroid hormone-sensitive behaviors may be modulated through direct or indirect interactions between the mPOA and the mesolimbic dopamine system; specifically the VTA (Simerly and Swanson, 1988). This is not surprising given the mesolimbic dopamine system's role in regulating reinforcing behaviors (Alcaro et al., 2007). Transient inhibition of the mPOA attenuates conditioned behaviors associated with pups (Pereira and Morrell, 2010). Maternal care also increases dopamine activity in the NAc (Champagne et al., 2004), whereas inhibition of the VTA impairs pup grooming (Seip and Morrell, 2009). Furthermore, unilateral lesions of the mPOA attenuated pup-induced cellular activity in the ipsilateral NAc (Stack et al., 2002), and the combination of unilateral mPOA denervation and contralateral VTA lesion impairs maternal behaviors (Numan and Smith, 1984). Thus, the mPOA and mesolimbic system interact to regulate maternal care.

Previous work in our lab has demonstrated that the mPOA modulates behavioral and neurological responses to cocaine in female rats. Namely, lesions of the mPOA enhanced

cocaine-induced conditioned place preference (CPP) as well as Fos-immunoreactivity in the NAc following administration of acute systemic cocaine injections (Tobiansky et al., 2013a). In line with the behavioral results, we also discovered that unilateral lesions of the mPOA enhance cocaine-induced dopamine in the NAc ipsilaterally to mPOA lesion, and that the projections from the mPOA to the VTA are at least partially GABAergic and sensitive to estrogen signaling (Tobiansky et al., 2015). These results were based on immunohistochemical colocalization study that demonstrated there were a substantial number of ERα and GPER receptors but a paucity of PR receptors on neurons that project to the VTA. Thus sex-steroid hormones acting in the mPOA to modulate activity response to cocaine via projections to the mesolimbic system it is likely via estrogenic signaling. The goal of the present study is to address this question, specifically if estradiol administered directly into the mPOA is able to modulate the cocaine-induced dopamine in the NAc.

2.3 GENERAL METHODS

2.3.1 Subjects

Thirty-two adult female Sprague-Dawley rats (PN 65-74, 200-224 g; Harlan Laboratories, Indianapolis, IN) were double housed in a temperature-controlled room (22°C, 30-70% humidity) on a reverse light/dark cycle (12 hours light/12 hours dark; lights off at 10 a.m.) with food and water freely available. All experiments were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin and were in accordance with the National Institutes of Health Guidelines for the Use of Animals in Research.

2.3.2 Stereotaxic surgery and ovariectomies

All rats were anesthetized with isofluorane (2-5%) and mounted on a stereotaxic apparatus with non-piercing earbars (Stoelting). For microinjections into the mPOA, a guide cannula (23-gauge thin wall hypodermic tubing, 15mm length; Vita Needle, Needham, MA) was lowered to 2mm above the mPOA (AP, -0.3mm; ML, +3.1mm; DV, -6.8mm; angle 18 degrees (Paxinos and Watson, 2007)). In order to prevent spatial conflict with the microdialysis probe, the mPOA guide cannula was inserted at an angle. A microdialysis guide cannula was then lowered to 4 mm above the NAc shell-core border (AP, +1.7mm; ML, +1.0mm; DV, -4.0mm (Paxinos and Watson, 2007)) ipsilaterally to the mPOA guide cannula. Three small screws were inserted in the skull and then cannulae were fixed in place with dental resin (Hygenic Corporation, Cuyahoga Falls, OH). A 15mm 27-gauge dummy cannula was then inserted into the guide cannula in order to maintain patency. Immediately following cranial surgery all rats were OVX. A single medial incision was made through the abdominal wall, the uterine horns were then ligated with absorbable suture and the ovaries manually ablated. The abdominal wall was tied with absorbable suture and the skin incision was closed with sterile surgical staples. Aseptic techniques were used throughout surgery.

2.3.3 Microdialysis and cocaine administration

Concentric microdialysis probes were constructed using a semipermeable dialysis membrane (13,000 MW cutoff; Spectrum Labs, Rancho Dominguez, CA) with an outer diameter of 216 μm and an inner diameter of 200 μm. The active dialyzing length was 2 mm. The extraction fraction of each probe was measured *in vitro* before use to ensure

maximal recovery of analytes. Only probes with recovery rates of > 10% were used. On the morning of microdialysis, probes were inserted through the guide cannulae. Filtered and degassed Dulbecco's PBS (in mM: 138 NaCl, 2.7 KCl, 0.5 $MgCl_2$, 1.5 KH_2PO4, and 1.2 $CaCl_2$; pH 7.4; Sigma Aldrich, St. Louis, MO) was perfused through the probe at a rate of 1 μL/min using a 1mL gas-tight syringe on an infusion pump (PHD 22, Harvard Apparatus, Holliston, MA). Animals were allowed to acclimate to the probe insertion in the testing chamber for 3 hours prior to sample collection. Food and water were available during this time period and removed once sample collection began. Once the acclimation period was over, four 15-minute baseline samples were taken. Subjects were first injected intraperitonally with 1 mL/kg of 0.9% NaCl, to control for injection stress, followed 60 minutes later by 10mg/kg/ml cocaine hydrochloride (Sigma-Aldrich, St. Louis, MO) and another 90 minutes in the test chamber; 15-minute dialysate samples were collected throughout this microdialysis process. All samples were immediately frozen on dry ice and then stored at -80°C until analysis of dopamine concentration with high performance liquid chromatography with electrochemical detection (HPLC-EC).

2.3.4 Microinjections

Twenty-four hours prior to microdialysis, 2.5 μg of water-soluble estradiol (Sigma-Aldrich St. Louis, Mo), dissolved in artificial cerebrospinal fluid (aCSF (Harvard Apparatus, Holliston, MA.)), or equal volume vehicle was microinjected into the mPOA. The dummy cannula was removed and a 27-gauge microinjector that extended 2.0 mm past the guide was inserted into the mPOA. After forty-five seconds of equilibration, 0.3 μL of solution was injected over the course of one minute using a microinfusion pump

(Harvard Apparatus, Holliston, MA.). The injection volume was chosen based in part on earlier studies that showed that 1 μL volume injections into the hypothalamus of rats remains within ~1 mm of the injection site (Grossman and Stumpf, 1969). The injector was left in place for ninety seconds after the infusion was finished to allow for diffusion before it was removed and the dummy cannula reinserted.

2.3.5 Tissue collection: microinjection and probe placement

Immediately following microdialysis, subjects were euthanized with a lethal dose of Euthasol (0.3 mL/animal; Virbac Animal Health, Inc.; Fort Worth, TX). Brains were then extracted and post-fixed in 4% PFA for 48 hours at 4°C before being transferred to a 30% sucrose solution and stored at 4°C until sectioning (at least 48 hours). Coronal sections were cut at 100 μm at the level of the NAc and the mPOA using a freezing microtome (Microm HM 450, ThermoFisher Scientific, Waltham, MA) and were stored at -20°C in a cryoprotectant solution. Tissue was then mounted onto slides, washed in 0.1M PB, stained with methyl green, dehydrated, and cover slipped to assess placement. Subjects with placement outside of with either the mPOA or NAc were excluded as well as subjects who had extensive damage to either structure.

2.3.6 High performance liquid chromatography with electrochemical detection

HPLC-EC was used to determine the levels of doapmine present in the NAc following saline or cocaine administration. Samples were individually thawed and then injected into a 6μL loop. Monoamines were separated by an Acclaim PA2 reversed phase column (2.1 X 100mm, 2.2 μm packing) and detected using an Antec VT-03 electrochemical flow cell with ISAAC reference electrode and a working potential of 550mV at 35ºC. The mobile

phase consisted of 12.5% MeOH, 50 mM phosphoric acid, 8 mM NaCl, 0.1 mM EDTA, and 150 mg/L octanesulfonic acid, with a pH of 5.6, and pumped at a rate of 0.225 mL/min. Chromatography was performed using an Antec Decade 2 microelectrochemical detector attached to a PC running Clarity chromatography software (Data Apex, Prague, Czech Republic).

2.3.7 Experimental design

In order to determine whether estradiol in the mPOA modulates cocaine-induced dopamine in the NAc, a group of 32 females were ovariectomized and then received cannulation of the mPOA and NAc core-shell border. Because we were interested in the role of estradiol in the mPOA, animals did not receive chronic systemic estradiol replacement. One group received systemic estradiol injections (n=16) nine days and five days prior to microdialysis to mimic estrogen surges in a naturally cycling female, while a second group received vehicle (n=16) during these days and were without systemic estradiol for 23-25 days prior to microdialysis. One day prior to microdialysis, animals were microinjected with estradiol (n=16) or aCSF (n=16) into the mPOA. Hormone pretreatment and hormone microinjection were performed with a balanced 2x2 design. Microdialysis and probe placement were performed as described above. Figure 2.1 provides an experimental timeline

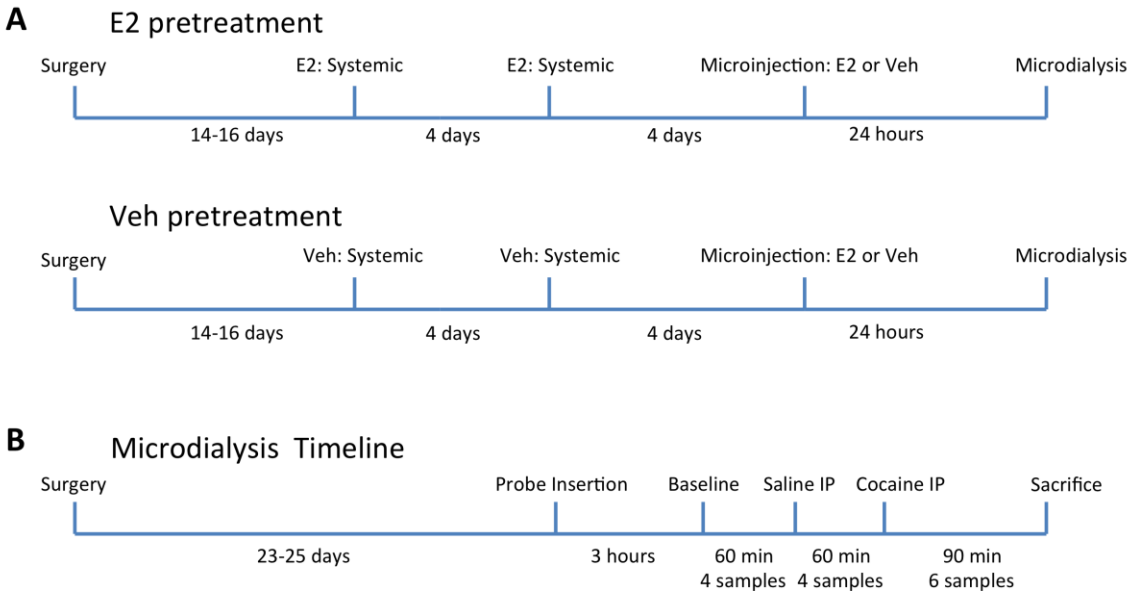

Figure 2.1 Experimental timeline of hormone treatment and microdialysis.

(A) Timeline of systemic hormone treatment. Subjects either received systemic estradiol (top panel) or sesame oil vehicle (middle panel) nine days and five days prior to microdialysis. On the day before microdialysis subjects received a unilateral injection of either water-soluble estradiol or aCSF directly into the mPOA. Subjects were treated in a balanced 2x2 manner such that half the systemic estradiol and half the sesame oil subjects received one of two-microinjection conditions. (B) Microdialysis timeline for all subjects. After probe insertion subjects were allotted 3 hours to acclimate followed by 3.5 hours of sample collection every 15 minutes.

2.3.8 Statistics

All statistical tests were conducted in R version 3.2.2 (2015-08-14) Fire Safety. NAc dopamine was analyzed as percent change from baseline since there were no significant differences between average dopamine levels across the different treatment groups at baseline. Analysis of the microinjection study employed a four-way mixed effects design, comparing the effects of hormone pretreatment, drug, hormone microinjection, and time on percent dopamine change in the NAc. Hormone microinjection and hormone pretreatment were fixed factors while time and drug were allowed to vary as a function of subject. For this analysis only the first four time points from the cocaine group were used in order to have a balanced design with time in the saline group. Third order and second order interactions were evaluated by pooling the non-significant 4-order interaction into the error term. For all analyses, main effects were not interpreted in the presence of an interaction. Since there was a complex relationship between several of the variables in the study, a three-way interaction, and interactions were interpreted through decompositions by examining a factor at each level of another factor. The goal of this process is to aid the reader in interpreting the effect of a three-way interaction and not to definitively test simple main effects. As such multiple corrections were limited to within significant two-way interactions and main effects involving time. Post hoc tests for the main effect of time at various factor levels were conducted using a general linear hypothesis test with Tukey contrasts. A secondary analysis was conducted to determine if the any of the groups differed at the last two cocaine time points. This analysis consisted of growth curve modeling as a function of time and treatment to determine if rate of dopamine

decrease changed as a function of hormone treatment as well as one factor ANOVAs with hormone groups pooled to determine if there were group differences at either of these two time points. Alpha was set at 0.05, though, in the case of interaction decompositions, marginally significant results are discussed to assist in the description of interactions.

2.4 RESULTS

Microinjection and microdialysis-probe placement were histologically verified for all subjects (Figure 2.2). 26 of 31 microinjections were located in the mPOA; however, two of these animals contained visibly infected brain tissue and were excluded. Additionally, one subject had a moderately sized lesion in the mPOA and was excluded. Interestingly this subject had a greater percent increase in dopamine in response to cocaine than most other subjects. However, direct comparison to the previous lesion study conducted in our lab by Tobiansky et al. would be unwarranted, though it is interesting and reassuring that this effect was directionally equivalent to the previous results (Tobiansky et al., 2015). Microdialysis probes were all placed in the NAc with the majority (47.8%) in the shell region of the NAc, 43.5% in the shell-core border, and 8.7% in the core. There were no regional differences in average percent change in dopamine in response to either saline ($F_{(2,20)} = 0.26$, $p = 0.77$) or cocaine ($F_{(2,20)} = 0.06$, $p = 0.94$). Finally, there were no group differences in average baseline dopamine levels across the four treatment groups ($F_{(3,17)} = 0.89$, $p = 0.47$), thus NAc dopamine response to saline and cocaine was analyzed as a percent change in baseline.

A 4-way mixed ANOVA was used to examine the effects of hormone pretreatment, drug, estradiol microinjection, and time on percent change in NAc dopamine (Figure 2.3). There was no significant 4-way interaction ($F_{(3,133)} = 2.03$, $p = 0.11$). However, there was a significant 3-way interaction and several 2-way interactions present in the saturated model. These interactions were subsequently interpreted using models lacking higher order interactions.

A mixed ANOVA, excluding the 4th order term, revealed the only significant 3rd order interaction was between hormone pretreatment, drug, and time ($F_{(3,136)} = 3.83$, $p < 0.05$; Figure 2.3). Decomposition of this interaction revealed that there were no two-way interactions between hormone pretreatment and drug at any of the 4 time points. There was an interaction between time and hormone pretreatment in response to cocaine ($F_{(3,63)} = 5.14$ $p < 0.01$) but not saline ($F_{(3,63)} = 0.72$ $p = 0.54$). Furthermore, there was a significant drug × time interaction ($F_{(3,77)} = 6.40$, $p < 0.001$) for subjects pretreated with estradiol and a marginal effect for subjects pretreated with vehicle ($F_{(3,70)} = 2.30$, $p = 0.08$). Decomposition of the drug × time interaction revealed that estradiol pretreatment modulated the time course of dopamine response to cocaine but not saline. Specifically, post hoc tests revealed that the percent change in dopamine was greater 15-minutes post cocaine compared to 45-minutes ($z = -4.47$, $p < 0.001$) and 60-minutes ($z = -4.91$, $p < 0.001$) post-cocaine for subjects that received estradiol pretreatment leading up to microdialysis. These results are recapitulated by the hormone treatment by time interaction described above. In summary, decomposition of the three way hormone

pretreatment x drug x time interaction suggests that there is a greater initial increase in dopamine in response to cocaine in E2 pretreated, compared to vehicle pretreated, subjects.

There was also a significant drug × estradiol microinjection interaction ($F_{(1,146)} = 4.71$, $p < 0.05$), whereas neither the hormone pretreatment × drug ($F_{(1,146)} = 0.58$, $p = 0.46$) nor the hormone pretreatment × estradiol microinjection ($F_{(1,19)} = 1.64$, $p = 0.21$) interactions were significant. All other significant second-order interactions included the time factor and have already been provided in the results described above. Decomposition of the drug × estradiol microinjection interaction revealed that, in response to cocaine, estradiol treated animals exhibited a greater percent increase in dopamine compared to vehicle treated animals ($p < 0.05$), but there was no effect of estradiol microinjection in response to saline ($p = 0.51$). Compared to saline, cocaine increased percent change in dopamine for both E2 ($p < 0.001$) and vehicle ($p < 0.05$) treated animals. In summary, decomposition of the drug x estradiol microinjection interaction suggests that regardless of estradiol microinjection cocaine increases NAc dopamine, as has been established numerous times in the past, and that this increase in NAc dopamine is greater in subjects that received estradiol microinjections into the mPOA.

Visual examination of the data also suggested that combination of estradiol pretreatment and an estradiol microinjection facilitated cocaine-induced dopamine in the NAc, specifically at C1in Figure 2.3. However, as noted above, there were no 4-way interaction

and as such results should be interpreted as a secondary analysis. Tukey's corrected comparisons of revealed no significant differences between subjects in estradiol pretreatment / estradiol microinjection group and the other 3 groups at time point C1. However, there were notable trend differences between several groups: estradiol / estradiol vs. Veh/Veh ($p = 0.0503$), estradiol / estradiol vs. estradiol pretreatment/ Veh microinjection ($p = 0.0906$).

As stated in the methods section the primary goal of the present study was to examine the effect of various hormonal treatments on NAc dopamine release in response to cocaine and saline. In order to run a fully factorial analysis I only analyzed the first 4 cocaine samples in the primary analysis, however, I did collect two additional cocaine samples that were not analyzed in the primary analysis. These additional samples were collected in order to determine if hormone treatment modulated the rate at which cocaine induced dopamine returned to baseline. Growth curve analysis suggested that while rates of dopamine decay were variable across subjects (accounting for ~31% of total model variability) there was not a significant effect of hormone treatment condition on the dopamine decay rate ($F_{(3,38)} = 2.15$, $p = 0.11$). However, analysis of the effect of hormone treatment on dopamine levels at the last two time points revealed a significant main effect of hormone pretreatment 75 minutes after cocaine administration ($F_{(1,17)} = 8.95$, $p < 0.01$), whereas there was not a significant effect of either hormone microinjection ($F_{(1,17)} = 3.70$, $p = 0.07$) nor hormone pretreatment ($F_{(1,17)} = 1.43$, $p = 0.25$) 90 minutes after cocaine administration.

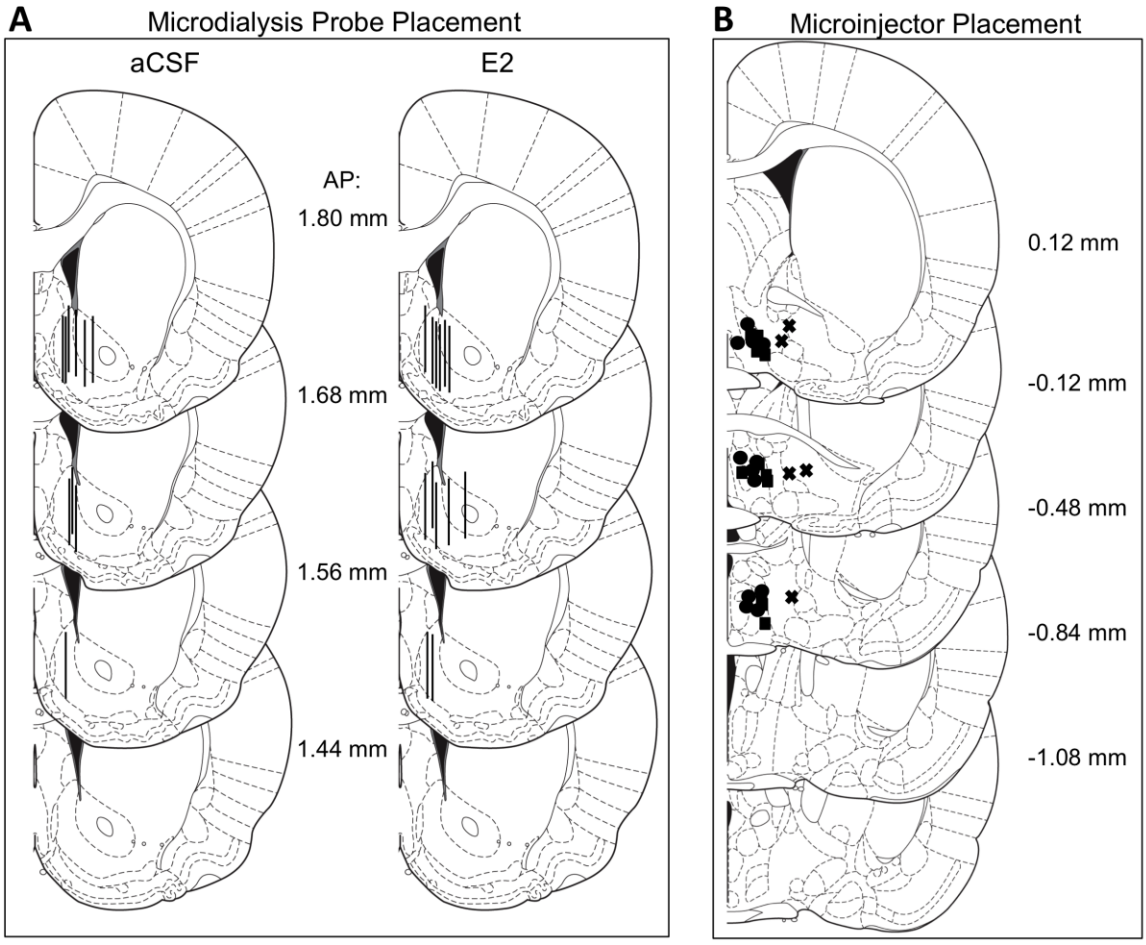

Figure 2.2 Microdialysis probe and cannula placement

(A) Coronal hemisections through the right side of the nucleus accumbens (NAc; 1.44 to 1.80 mm anterior to the bregma). Lines represent placement of microdialysis probes in E2 and aCSF microinjected animals. (B) Coronal hemisections through the medial preoptic area (mPOA; 0.12 mm anterior to 1.08 mm posterior to the bregma). Circles and square represent microinjector placement for estradiol and aCSF treated animals respectively and X's represent misses, adapted from (Paxinos et al, 2007).

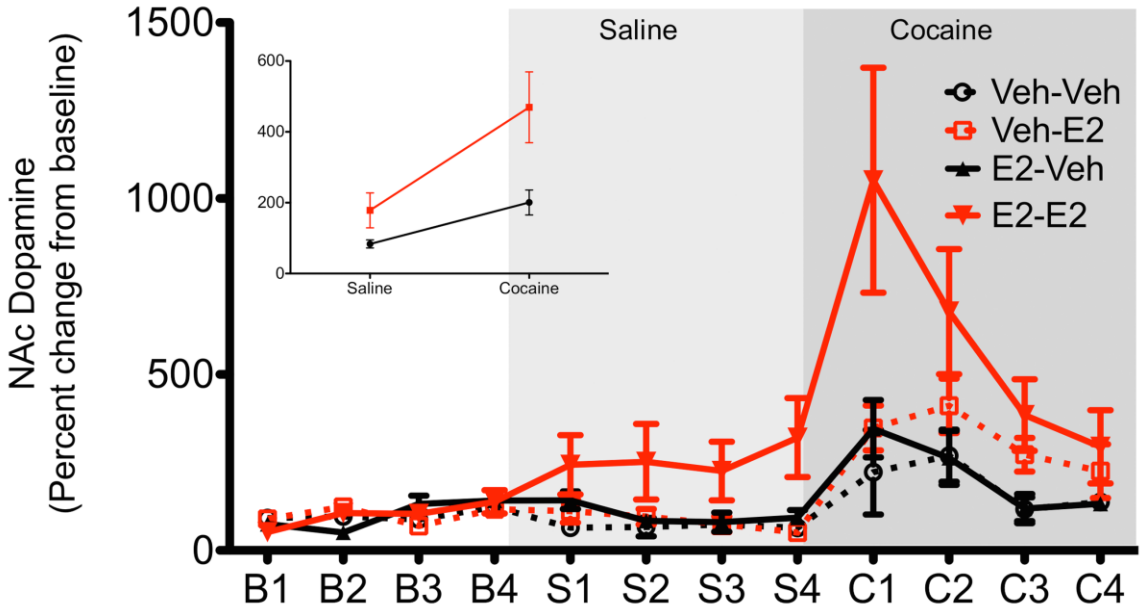

Figure 2.3 Estradiol in the mPOA enhance cocaine-induced dopamine release in the NAc Estradiol administration increases cocaine-induced dopamine release. Dopamine release (percent baseline) differs between subjects who received aCSF mPOA microinjection and were pretreated with vehicle (n = 5; black circle), estradiol mPOA microinjection and pretreatment with vehicle (n=6; red square), aCSF mPOA microinjection and were pretreated with estradiol (n=5; black triangle), or estradiol mPOA microinjection and were pretreated with estradiol (n=7; red triangle). Analysis revealed a significant three-way interaction between time, drug, and hormone pretreatment; decomposition of interaction present in results section. Inset shows significant interaction ($F_{(1,146)} = 4.71$, $p < 0.05$) between hormone microinjection and drug (saline and cocaine) collapsed across time. Values are expressed as mean ±SEM.

2.5 DISCUSSION

The present results expand upon previous work conducted by our lab establishing the mPOA in the regulation of response to cocaine in female rats. The mPOA regulates naturally rewarding behaviors in females, such as maternal care (Stolzenberg and Numan, 2011), and contains a high concentration of estrogen receptors. The influence circulating sex steroid hormones have on cocaine-induced behavioral and neurochemical response is well established (Becker and Hu, 2008; Becker and Koob, 2016). Additioanlly, previous work in our lab established that lesions of the mPOA increases the behavioral response to cocaine as well as cocaine-induced dopamine levels in the NAc (Tobiansky et al., 2013a; Tobiansky et al., 2015). However, whether E2 modulates cocaine response by acting in the mPOA was, until now, unclear. Here, we demonstrated E2 microinjections into the mPOA 24 hours before cocaine administration increased cocaine-induced dopamine release in the NAc. We also replicated previous findings (Peris et al., 1991) that subjects that received systemic estrogen replacement exhibited a greater increase in cocaine-induced dopamine in the NAc than vehicle treated subjects.

One mechanism through which the mPOA might regulate cocaine response is via its projections to the mesolimbic dopamine system, particularly inhibitory projections to the VTA. The mPOA has reciprocal connections with the VTA (Miller and Lonstein, 2009), but does not send efferents directly to the NAc (Simerly and Swanson, 1988) suggesting an indirect regulation of the NAc via the VTA. These projections from the mPOA to the VTA are likely inhibitory, as the primary amino acid produced by preoptic neurons is γ-

aminobutyric acid (GABA) (Herbison et al., 1992; Unda et al., 1995). A recent *in situ* hybridization study in mice found that approximately 90% of the neurons in the mPOA express glutamate decarboxylase (GAD)-65 an enzyme that catalyzes the synthesis of GABA (Zhao et al., 2013); suggesting the majority of mPOA neurons are GABAergic. Additionally, cells containing GAD-67 are more prevalent than glutamate-producing cells in the mPOA (Tsuneoka et al., 2013). Expressions of genes that encode glutamate decarboxylase are also highly upregulated in the mPOA (McHenry et al., 2015). Maternal behavior increases Fos-ir of GABA-producing cells in the mPOA of virgin (Tsuneoka et al., 2013) and lactating female rats (Lonstein and De Vries, 2000), suggesting that naturally rewarding behaviors are also modulated via GABAergic projections from the mPOA. Inhibition of the mPOA with GABA agonists increases locomotion (Arrati et al., 2006), a behavioral correlate of increased dopamine release in the NAc (Costall et al., 1984). These results are in line with previous work demonstrating that lesions of the mPOA increase cocaine-induced dopamine in the NAc. However, it is worth noting that baseline differences in NAc dopamine were not assessed with no-net flux microdialysis in this study (Tobiansky et al., 2015). Lesions studies conducted by our lab suggest the mPOA plays an inhibitory role in the regulation of neural and behavioral responses to cocaine (Tobiansky et al., 2013a; Tobiansky et al., 2015). Thus, if the mPOA is regulating the VTA via inhibitory projections, it is likely that these inhibitory projections are synapsing on dopamine neurons in the VTA and not GABAergic interneurons (Johnson and North, 1992; Margolis et al., 2006). However, it is also possible that the mPOA sends expiatory projections to the VTA that project onto inhibitory GABAergic

interneurons. Recent results from our lab suggest that projections from the mPOA to the VTA that are immunopositive for GABA appose both putative dopamine-positive neurons, based on tyrosine hydroxylase immunoreactivity, and putative dopamine-negative neurons (Tobiansky et al., 2015).

Given the similarity in results between the previous lesion study (Tobiansky et al., 2015) and estradiol microinjection study presented here, namely increased release of cocaine-induced DA, we propose that estrogen in the mPOA facilitates cocaine-induced dopamine in the NAc by inhibiting GABAergic projections from the mPOA to the VTA. GABAergic neurons within the mPOA are sensitive to estrogens (Sar et al., 1983). Estrogen increases GABA concentration, the reuptake of GABA, and the expression of $GABA_A$ receptors within the mPOA (Herbison, 1997). Additionally, studies in the hippocampus demonstrate that estrogen modulates synaptic plasticity of GABAergic neurons (Naftolin et al., 1996). Thus, increased levels of estradiol in the mPOA likely enhance the sensitivity of mPOA GABAergic projection neurons to inhibition from other structures and alter the synaptic connections in these regions. Estrogen treatment has also been shown to modulate the electrophysiology of GABAergic neurons in the mPOA in a biphasic manner (Wagner et al., 2001). Specifically, estradiol prevents baclofen-induced hyperpolarization of cells prior to the luteinizing hormone surge, whereas, during the luteinizing hormone surge, estrogen treatment decreases GAD-67 immunoreactivity (Wagner et al., 2001). Additionally, since almost all mPOA neurons are sensitive to GABA (Mayer, 1981) and GABA release readily modulates the activity of mPOA

neurons (Malinina et al., 2005), it is also possible that increased estradiol alters the sensitivity of GABAergic projections neurons as well. This would include glutamatergic neurons (Lee et al., 2008) as well as neurons that express neruotensin (Geisler and Zahm, 2006) and galanin (Whitelaw et al., 2012) (Cheung et al., 2001), excitatory and inhibitory neuropeptides respectively. Taken together with the results described above, estrogen may attenuate inhibitory VTA inputs from mPOA neurons by increasing the sensitivity of GABAergic mPOA neurons to inhibitory input from other brain regions.

It is worth noting that subjects that were pretreated with systemic estradiol exhibited a greater increase in cocaine-induced dopamine release 15-minutes after cocaine administration. This suggests that, while estradiol regulates cocaine induced dopamine release by acting in the mPOA, estradiol also facilitates cocaine-induced dopamine release by acting elsewhere in the brain. These results are not surprising given that estradiol modulates dopamine activity within the NAc (Becker, 1990b). Specifically, basal levels of striatal dopamine vary across the estrous cycle (Becker and Hu, 2008). Additionally, OVX attenuates, and estradiol restores, amphetamine-induced dopamine release (Becker and Hu, 2008). However, most of the direct effects of estradiol on NAc dopamine activity occur rapidly (within 30 minutes of estradiol administration) (Becker and Hu, 2008), whereas the present study found an increased dopamine response five days after the last systemic dose of estradiol. A significant decrease in circulating sex steroid hormones following OVX alters synaptic connectivity in other brain regions such as the hippocampus (Day and Good, 2005), increases GFAP-ir, a protein found in

astrocytes, in the VTA and prefrontal cortex, but not the NAc, four weeks after OVX (Flores et al., 1999). Additionally, OVX decreases the firing rate of VTA dopamine neurons (Zhang et al., 2008). Thus, it is possible that a 3.5-week absence of E2 altered the connectivity between the NAc and other estrogen-sensitive brain regions while estrogen replacement every four days was sufficient to maintain some of this connectivity.

Subjects treated with systemic estradiol prior to microinjections also exhibited a greater percent change in dopamine in response to a saline injection. This effect was not statistically significant, but there was a visual difference between these groups. These results suggest that systemic estradiol might also be modulating dopaminergic response to an acute stressor. While an IP injection is a stressor sufficient to increases corticosterone by itself (Ryabinin et al., 1999), it is not generally considered a major stressor (Meijer et al., 2006). However, the process of microinjecting an animal tethered for microdialysis is awkward and may be significantly more stressful for the animal and is significantly more stressful for the experimenter. Stress increases dopamine levels and dopamine metabolism in the NAc (Fadda et al., 1978; Abercrombie et al., 1989; Cabib and Puglisi-Allegra, 2012). Social defeat stress increases dopamine levels in the NAc(Holly et al., 2015) as well as potentiating cocaine self-administration and amphetamine induced dopamine release in the NAc (Han et al., 2015). If, as hypothesized above, estradiol in the mPOA is modulating NAc via inhibition of inhibitory projections to VTA dopamine neurons, then VTA dopamine neurons should be less inhibited in

estradiol treated animals. Acute stress increases both the firing frequency and bursting of VTA neurons (Valenti et al., 2011), although these neurons may represent a sub population of VTA dopamine neurons (Mirenowicz and Schultz, 1996; Bromberg-Martin et al., 2010). Additionally, an increase in the rate of bursting persists for at least 24 hours following exposure to acute stress (Anstrom and Woodward, 2005). However, this proposed mechanism is equivocal, as others have questioned whether VTA neurons that exhibit increased responding to aversive stimuli are dopamine neurons (Ungless et al., 2004). While sex differences in response to stress in humans are well documented (Kajantie and Phillips, 2006) less is known about the role estrogens play in response to stressors. Estrogens protect against the deleterious effects of chronic stress (Wei et al., 2014) and acute stress can increase estrogen levels in females (Shors et al., 1999). To the best of our knowledge, there are no studies examining the interaction between acute stress and estradiol administration on accumbal dopamine levels. However, it is worth noting that female rats in proestrus have a more robust hypothalamus-adrenal-pituitary (HPA) axis response to stress, increased corticosterone release, than estrus and diestrus females (Viau and Meaney, 1991). Glucocorticoids, such as corticosterone, facilitate stressed induced dopamine release in the NAc (Barrot et al., 2000). Thus it is possible that this increase in NAc dopamine in response to saline could be a function estradiol regulation of glucocorticoid signaling which might have been attenuated in animals that received systemic vehicle treatment.

Estradiol modulates behavior via both rapid molecular cascades and classical long-lasting genomic actions (McEwen and Alves, 1999). Estrogen-sensitive behaviors regulated by hypothalamic nuclei such as eating, drinking, maternal behavior, and female sexual behavior are typically displayed one to two days after estradiol administration, suggesting they are regulated by classical estrogen signaling (Graves et al., 2011; Stolzenberg and Numan, 2011; Hull and Dominguez, 2012). In the present study, we chose to examine cocaine-induced dopamine release 24-hours after the administration of estradiol, as previous literature suggests that feeding behavior is modulated by estradiol microinjections into the mPOA 24-hours post-injection (Santollo et al., 2011). Additionally, female rats are most sensitive to cocaine on the day of estrus (Sell et al., 2005), hours after the natural estrogen surge (McEwen and Alves, 1999) suggesting that facilitation of response to cocaine by estradiol is occurring via a long-lasting genomic mechanism. While, estradiol microinjections into the mPOA were sufficient increases cocaine induced dopamine in the NAc, other studies have shown estradiol is also able to modulate dopamine activity in a rapid manner (Di Paolo et al., 1985; Becker, 1990a; Bazzett and Becker, 1994). Whether or not estradiol administration into the mPOA immediately prior to cocaine administration would also be sufficient to modulate NAc dopamine levels is currently not clear and was a question beyond the scope of the present study.

In conclusion, the results of this chapter demonstrate that estradiol in the mPOA influences cocaine-induced dopamine release in the NAc. Moreover, our data supports

studies demonstrating estradiol -mediated enhancement of cocaine response (Hu et al., 2004; Jackson et al., 2006; Evans and Foltin, 2010). This data builds upon and provides a mechanism from previous results obtained in our lab. Specifically, previous studies have demonstrated the mPOA is involved in the behavioral and neuronal regulation of response to cocaine in female rats and that projections from the mPOA to the VTA are estrogen sensitive (Tobiansky et al., 2013a) (Tobiansky et al., 2015). The present results provide a mechanism through which the mPOA modulates neuronal response to cocaine, namely estradiol signaling in the mPOA. Finally, these results underline the importance of the mPOA as an integral region involved in the processing of reinforcing stimuli, both natural and drug-related.

Chapter 3: The medial preoptic area modulates the behavioral response to cocaine in male rats

Locomotion data presented in this chapter was published in (Will et al., 2016)

3.1 ABSTRACT

Drugs of abuse produce rewarding effects, at least in part, by modulating activity of the mesolimbic dopamine system. Our lab has demonstrated that the medial preoptic area (mPOA), a brain region that regulates naturally rewarding behaviors and interacts with the mesolimbic system, facilitates the behavioral and neurological response to cocaine in female rats. Given that the mPOA also plays a role in sexual response and motivation in males, the goal of the present study was to determine whether this sexually dimorphic brain region is also involved in the regulation of the behavioral response to cocaine in male rats. To this end we measured cocaine-induced locomotion and conditioned place preference (CPP) in male rats with lesions of the mPOA. Subjects with lesions of the mPOA exhibited an increase in locomotor response to cocaine as well as a greater cocaine-induced shift in place preference compared to sham-treated conspecifics. These results suggest that the mPOA plays a role in the modulation of both the locomotive and rewarding aspects of cocaine in male rats.

3.2 INTRODUCTION

Cocaine exerts its rewarding effects by modulating activity in the mesolimbic dopamine system (Koob et al., 1998; Wise, 2002; Koob and Volkow, 2010). The mesolimbic dopamine system consists primarily of dopamine neurons that reside in the ventral tegmental area (VTA), which project to the nucleus accumbens (NAc), but also the hippocampus, amygdala, and prefrontal cortex (Pierce and Kumaresan, 2006).

Manipulations to this system influence drug approach and response behaviors. For example, dopamine-depleting lesions of the NAc attenuate cocaine self-administration (Caine and Koob, 1994a; Gerrits and Van Ree, 1996). Conversely, cocaine administration increases levels of dopamine in the NAc (Hurd et al., 1989). Inhibition of neural activity in the VTA attenuates cocaine self-administration on a progressive ratio schedule (Brebner et al., 2000a) and blocks reinstatement of cocaine seeking behavior (Shen et al., 2014). Taken together the results of these studies demonstrate the mesolimbic dopamine system regulates the behavioral response to cocaine, an artificially reinforcing stimulus.

The mesolimbic system is also involved in the regulation of natural reward, independent of drug administration. Stimulation of the medial forebrain bundle, which sends dopaminergic projections from the VTA to the NAc as well as other forebrain structures (Fibiger et al., 1987; Kuhr et al., 1987; Ikemoto and Panksepp, 1999; Cheer et al., 2005), promotes feeding, drinking, and reproductive behaviors (Glickman and Schiff, 1967). Food and food-related cues increase dopaminergic response in the NAc (Hernandez and Hoebel, 1988; Bassareo and Di Chiara, 1999), while consumption of palatable food is disrupted by dopamine blockage or depletion in the NAc (Salamone et al., 1991). Reproductive behaviors increase dopamine activity in the NAc in both males (Damsma et al., 1992) and females (Meisel et al., 1993). Furthermore, electrical (Eibergen and Caggiula, 1973) or pharmacological (Mitchell and Stewart, 1990) stimulation of the VTA enhances male sexual behavior. Finally, maternal care increases dopamine activity in the NAc (Champagne et al., 2004), whereas inhibition of the VTA impairs pup grooming

(Seip and Morrell, 2009). Thus, both drugs of abuse and naturally rewarding behaviors activate and are in turn modulated by similar neural loci, namely the mesolimbic system.

The mPOA regulates the expression of naturally rewarding behaviors and has reciprocal connections with the mesolimbic dopamine system. Specifically, the mPOA sends projections to and is innervated by the VTA (Ikemoto, 2007) (Yetnikoff et al., 2015), but not the NAc (Simerly and Swanson, 1988). The mPOA is a sexually-dimorphic brain area that regulates sexual (Hull and Dominguez, 2012) (Whitney, 1986) and maternal (Stolzenberg and Numan, 2011) behaviors. Lesions of the mPOA attenuate male sexual behavior in a variety of species (Hillarp et al., 1954; Larsson and Heimer, 1964; Hull et al., 2006) whereas electrical stimulation of the mPOA facilitates male sexual response (Malsbury, 1971). In female rats, unilateral lesions of the mPOA attenuate pup-induced cellular activity in the ipsilateral NAc (Stack et al., 2002), and the combination of unilateral mPOA denervation and a contralateral VTA lesion impairs maternal behaviors (Numan and Smith, 1984). Finally, recent work in our lab demonstrated that the mPOA also regulates response to cocaine. Specifically, lesions of the mPOA enhanced cocaine-induced CPP and c-Fos immunoreactivity in the NAc of female rats (Tobiansky et al., 2013a). Finally, work presented above in chapter 2 demonstrates that estradiol acts in the mPOA to modulate cocaine induced dopamine response in the NAc. Taken together these studies demonstrate that the mPOA is involved in the regulation of naturally rewarding behaviors in males and females, response to cocaine in females, and that the mPOA presumably modulates these behaviors via projections to the VTA.

Given the role of the mPOA in the regulation of sexual motivation and reproductive behaviors in males, it is possible that the mPOA also modulates behavioral response to cocaine in males as well as females. In order to address this possibility, the present study was designed to determine whether the mPOA regulates the psychomotor and rewarding aspects of cocaine response in male rats. To this end, male rats received lesions of the mPOA and were then tested for cocaine-induced changes in locomotion or CPP.

3.3 MATERIALS AND METHODS

3.3.1Subjects

Male Sprague Dawley rats (Harlan, Indianapolis), weighing 300-400 g, were double housed in large plastic cages on a 12/12 h light-dark cycle, lights off at 10am, with access to food and water *ad libitum*. The room temperature was set at 22 ± 1 °C. Subjects were randomly assigned into surgical condition (sham or lesion) as well as drug administration order (saline first or cocaine first), and were then housed based on treatment group such that cage mates received all the same treatments (surgery and injections). All procedures were in accordance with the NIH Guidelines for the Use of Animals and were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

3.3.2 Surgery

Three weeks before behavioral testing subjects received bilateral radiofrequency (n=56), neurotoxic (n=32), sham radiofrequency (n=43), or sham neurotoxic (n =18) lesions of the mPOA. All surgeries were performed using a stereotaxic apparatus under isofluorane

(2-5%) anesthesia. For both radiofrequency and neurotoxic lesions bilateral boreholes were drilled in the skull above the central region of the mPOA (AP, −0.4 mm; ML, ±0.5 mm; DV, −8.2 mm; according to coordinates from (Swanson, 2004)). To produce radiofrequency lesions a TCZ thermo-coupled electrode (0.25 mm exposed tip) was lowered into the mPOA and a Radionics radiofrequency lesion generator was used to heat the electrode to 80°C (±3°C) for twenty seconds. Sham radiofrequency animals underwent the same procedure without the electrode being heated. For neurotoxic lesions, injections of N-methyl-D-aspartate (NMDA; 25µg/µL) in 0.1M PBS in each hemisphere of the mPOA produced neurotoxic lesions. NMDA was injected into the mPOA with a 1ul Hamilton syringe at 0.1ul/min for two minutes per hemisphere. The syringe was then left in the mPOA for five minutes before being removed. The same procedure was followed for sham NMDA lesions except vehicle (0.1 M PBS) was infused instead of NMDA. Lesion placement was verified histologically after behavioral testing was complete; methyl green was used for subjects with radiofrequency lesions whereas an antibody against hexaribonucleotide binding protein-3 (NeuN), a histological marker of neurons (Mullen et al., 1992), was used to evaluate NMDA lesions.

Among the animals receiving radiofrequency lesions, two subjects had to be euthanized due to tumor growth before the end of the behavioral component of the study. Additionally, eight lesion subjects were excluded from analysis due to lesions outside of the mPOA, two sham subjects had infections and three animals were excluded due to

cocaine-induced seizures: locomotion (radiofrequency lesion (n=13), sham lesion (n=15)), CPP (radiofrequency lesion (n=32), sham lesion (n=24)).

There was more variability in lesion placement in neurotoxic lesioned subjects. Specifically, seven subjects had bilateral lesions, thirteen subjects had unilateral lesions, and twelve subjects had lesions that did not contain the mPOA (excluded from analysis). Thus, bilateral lesions and unilateral lesions of the mPOA were treated as separate levels of surgery during analysis.

3.3.3 Behavioral apparatus

3.3.3.1 Locomotion

Two Med Associates locomotion boxes (44cm x 44cm x 30cm), equipped with photodiodes (2.5cm apart on each side of the box; 16 per side) were used to measure locomotor behavior in this study. Locomotion was operationalized as beam breaks with a beam break defined as breaking every 5th photodiode. Locomotor activity was recorded in 5-minute bins using Activity Monitor 5 software (Med Associates Inc.) and then exported from Microsoft Access to Microsoft excel using python (2.7) to manipulate relational tables.

3.3.3.2 Conditioned place preference

A two-chambered CPP apparatuses from San Diego Instruments was used to assess cocaine-induced place preference. The inner dimensions of each conditioning chamber were 35cm x 21cm x 34.5cm. The left chamber featured a rough (haircell textured) black

acrylic floor whereas the right chamber featured a smooth black acrylic floor. Each individual chamber featured an overhead LED (red/white). The red LED was also illuminated over the left chamber while the right chamber was dark. Each apparatus contained a 16×4 photobeam array for recording time spent in each chamber (sec). An acrylic black guillotine door separated the two chambers during conditioning and was raised (~6.5cm) during the posttest to allow free movement between chambers.

3.3.4 Behavioral procedure

3.3.4.1 Locomotion

All tests were carried out under red illumination during the dark cycle. Subjects were removed from the colony in their home cage and brought to the behavioral testing room; during transportation a dark sheet was draped over the home cage. On the first day of testing all subjects were allowed twenty-five minutes to habituate to the locomotion test chamber before being returned to the colony. The following day, subjects were placed in the chamber for five (radiofrequency) or ten (neurotoxic) minutes to establish baseline locomotion and then given an intraperitoneal (IP) injection of either saline (0.9% NaCl) 0.5mL/kg or cocaine 15mg/kg/0.5mL. After 20 minutes subjects were removed from the test chamber and returned to the colony. Six days later all subjects underwent a second twenty-five minute habituation session and the following day experienced the counterbalanced test session: subjects that received cocaine first received saline and vice versa (Figure 3.1).

There was a difference in the time before saline/cocaine administration on tests days between lesion types because the radiofrequency lesion experiment was performed before the neurotoxic lesion experiment. In the radiofrequency experiment we noticed a burst of locomotion during the first five minutes fallowed by a precipitous decline in locomotion over the next several 5-minute intervals. In the neurotoxic lesion study (a replication of the radiofrequency lesion study) we allowed for a ten minute baseline period and averaged across these two five minute intervals to establish a more accurate measure of baseline locomotion. However, we also analyzed locomotion from the neurotoxic lesion subjects using only the first five-minute interval as a measure of baseline locomotion and this data is presented in the results.

3.3.4.2 Conditioned place preface

For CPP rats were randomly divided into four groups; lesion cocaine (LC), lesion saline (LS), sham cocaine (SC), and sham saline (SS). Rats were tested two at a time in two adjacent CPP boxes and were always tested together as cage mate pairs. CPP was conducted between one and five hours after the initiation of the dark phase and rats were trained and tested at the same time every day. Since the colony room and behavior rooms were on different floors of the vivarium, subjects were brought to the behavior room immediately prior to conditioning or testing in plastic black painted containers under a black sheet to minimize light induced circadian disturbance. Transfer chambers were cleaned with 70% ethanol between rats. White noise at approximately 85dB (parameters: https://simplynoise.com/, white noise 50%, computer speaker 100%, external speaker 50%) was constantly on in the behavior room to minimize the effect of outside noise on

conditioning and testing. However, two animals were removed from analysis due to excessive noise from floor cleaning on the testing day.

On the first day of CPP all subjects were given a fifteen-minute pretest where they were allowed to explore both sides of the CPP apparatus. Rats were placed in the middle of each CPP box such that they were not facing either conditioning chamber in order to minimize initial bias. Following the pretest all rats received four pairings of saline (IP; 0.5mL/kg) in their initially preferred chamber and four pairings of either cocaine (IP; 15mg/kg/0.5mL) or saline (IP; 0.5mL/kg) in their initially non-preferred chamber. Following the last day of conditioning subjects underwent a posttest where the guillotine door separating the two chambers was raised to allow access to both compartments. During the post test rats were placed in their initially preferred chamber. After each conditioning or testing session the Plexiglas walls of each chamber were cleaned with deionized water and the textured floors were cleaned with a 70% ethanol solution before the next rats underwent conditioning or testing. In previous studies we cleaned the chambers with 5% bleach between each animal, but noticed animals were forming a strong aversion to the haircell textured floor which appeared to retain the bleach scent better than the smooth floor. At the end of each day Plexiglas walls were cleaned with a 5% bleach solution and then deionized water the following morning.

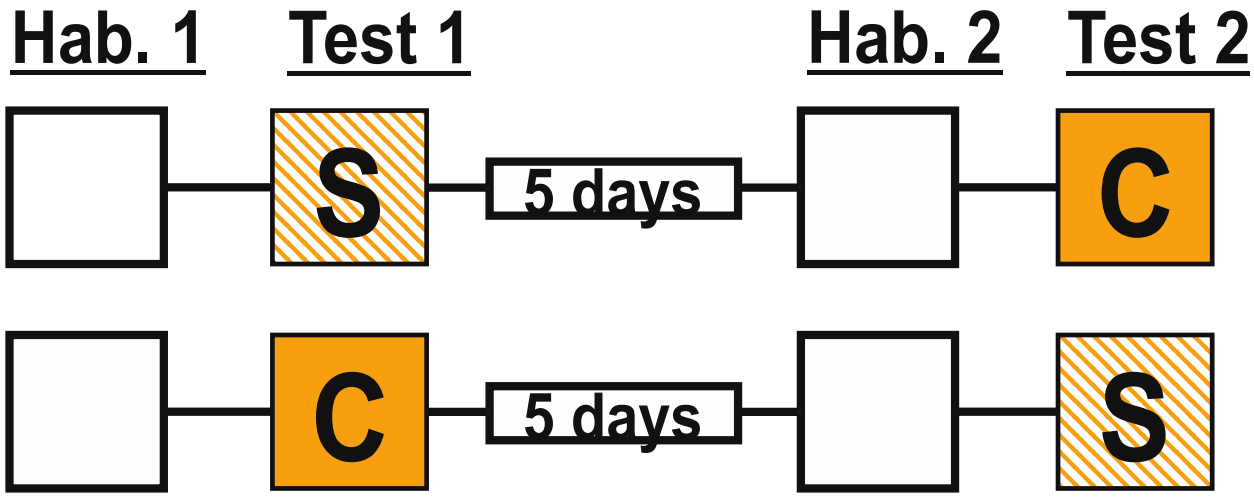

Figure 3.1 Timeline of events for locomotion experiments

On day one all subjects were placed in locomotion chamber of 25 minutes to habituate to the testing apparatus. The next day subjects received either saline or cocaine. Five days later subjects were allowed to explore the chamber once again to insure prior drug exposure had no lasting effects and then received the opposite compound the following day. S=Saline, C=Cocaine, Hab.=Habituation

3.3.5 Histology for lesion placement

All subjects received a lethal dose of Euthasol (130mg/kg) and were transcardially perfused with 50 mL of PBS and 250 mL of 4% paraformaldehyde. Brains were removed and stored in 4% paraformaldehyde for one hour before being transferred to 30% sucrose and stored at 4° C overnight; after 24-hours brains were transferred to another 30% sucrose solution and stored at 4° C until sectioning. Brains were sliced at 40 μm and stored in cryoprotectant (30% ethylene glycol, 30% sucrose, and 0.0002% sodium azide in 0.1 M phosphate buffer (PB)) at -20° C.

In order to determine neurotoxic lesion placement immunohistochemistry was performed to visualize NeuN, a neuronal marker. Brain slices containing the mPOA were washed in 0.1M PB and incubated in a monoclonal mouse anti-NeuN antibody (1:15000, Millipore, MAB337) overnight. Tissues were further incubated in an anti-mouse biotinylated antibody (1:1000, Vector) for 1-hour and enhanced with avidin/biotin (1:1000, ABC kit, Vector). NeuN immunoreactivity was visualized by incubating tissues in a 3,3'Diaminobenzidine (DAB) for 10 minutes. Between all incubations tissue was washed in 0.1 M PB 4x5 min. Slices were then mounted, dehydrated, and cover slipped in preparation for bright field microscopy.

Radiofrequency lesion placement was determined by staining tissue with methyl green. Brain slices containing the mPOA were mounted on charged slides and soaked in methyl

green solution for 5 minutes. Slides were then dehydrated and cover slipped in preparation for bright field microscopy.

3.3.6 Statistical analysis

In order to account for within subject variability all locomotion data was analyzed as a percent change from baseline (time point prior to the administration of saline/cocaine). For radiofrequency lesioned subjects, a 4x2x2 mixed factorial ANOVA was performed with surgery (sham/lesion) as a between subject factor and drug (saline/cocaine) and time (four 5-minute intervals after drug administration) as within subject factors. A two-way interaction between surgery and drug was then decomposed using Welch's two sample t-tests (holding drug constant) and paired t-tests (holding surgery constant). For neurotoxic lesioned subjects, a 4x3x2 mixed factorial ANOVA was performed with surgery (sham, bilateral, or unilateral lesion) as a between subject factor and drug (saline/cocaine) and time (four 5-minute intervals after drug administration) as within subject factors. A two-way interaction between surgery and drug was then decomposed using one-way ANOVAs (holding drug constant) followed by analysis of Tukey's corrected contrasts and paired t-tests (holding surgery constant). For both radio frequency and neurotoxic lesioned subjects a two-way interaction between time and drug was decomposed using a repeated measures ANOVA (holding drug constant) followed by analysis of Tukey's corrected contrasts and a series of paired t-tests (holding time constant). Differences in baseline locomotor scores as a function of surgery were assessed using a one-way ANOVA (neurotoxic lesions) or Welch's two-sample t-test (radiofrequency lesions). Cocaine-induced CPP was assessed by difference between the time spent in the drug-

paired chamber on the posttest and the time spent in the drug-paired chamber during the pretest. To analyze the effect of mPOA lesions on cocaine-induced CPP a 2x2 factorial ANOVA was performed with surgery (sham/lesion) and drug (saline/cocaine) as between subject factors. A two-way interaction between surgery and drug was then decomposed using Welch's two sample t-tests (holding drug or surgery constant). Marginal effects are discussed in the process of decomposing interactions. All statistics were performed using R version 2.15.1 "Roasted Marshmallows" and repeated in 3.2.2 "Fire Safety" due a bug that effected the calculation of degrees of freedom in 2.15.1.

3.4 RESULTS

3.4.1Radiofrequency lesion of the mPOA enhance cocaine-induced locomotion

A 4x2x2 mixed factorial ANOVA performed on subjects receiving radiofrequency or sham lesions revealed there was not a significant three-way interaction between drug, lesion, and time ($F_{(3,182)} = 0.159$, $p > 0.05$). The three-way interaction was then dropped from the model and two-way interactions were assessed. There was a significant interaction between surgery and drug ($F_{(1,185)} = 40.958$, $p < 0.05$), however the time by drug ($F_{(3,185)} = 1.809$, $p > 0.05$) and surgery by time interactions ($F_{(3,185)} = 0.228$, $p > 0.05$) were not statistically significant (Figure 3.2). Post hoc decomposition of the surgery by drug interaction revealed that subjects with radiofrequency lesions exhibited greater cocaine-induced locomotion ($t_{(21.368)} = 3.19$, $p < 0.05$), but less locomotion when exposed to saline ($t_{(23.383)} = -2.29$, $p < 0.05$) compared to sham operated subjects. Cocaine facilitated locomotion in both sham and radiofrequency lesioned subjects.

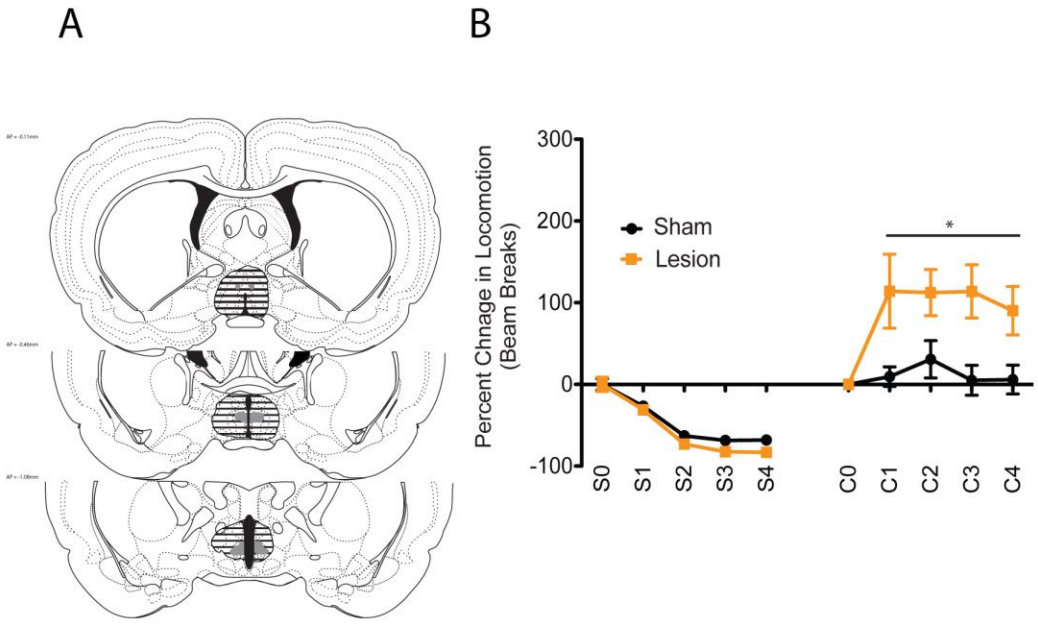

Figure 3.2 Radiofrequency lesions of the mPOA enhanced cocaine-induced locomotion. (A) Representative perimeters of smallest (solid gray) and largest (dashed black lines) radiofrequency lesion of the mPOA from rostral to caudal; drawn from (Swanson, 2004). (B) Graphical depiction of percent change in locomotion in response to an IP injection of either saline or cocaine. Saline and cocaine administration occurred one week apart. S0 and C0 are baseline locomotion measurement prior to injection where as S1-4 and C1-4 are five-minute locomotion intervals after injection. Subjects with radiofrequency lesions had a greater overall increase in cocaine-induced locomotion compared to sham treated subjects (* $p <$ 0.05). All subjects exhibited cocaine-induced increase in locomotion compared to saline treatment.

3.4.2 Neurotoxic lesions of the mPOA enhance cocaine-induced locomotion

A 4x3x2 mixed factorial ANOVA performed on subjects receiving neurotoxic or sham lesions, using the average of the two five-minute locomotion intervals as a baseline, revealed there was not a three-way interaction between drug, lesion, and time ($F_{(6,245)}$ = 0.519, $p > 0.05$). The three-way interaction was then dropped from the model and two-way interactions were assessed. There was interaction between surgery and drug ($F_{(2,251)}$ = 20.541, $p < 0.05$) as well as an interaction between time and drug ($F_{(3,251)}$ = 3.329, $p <$ 0.05), whereas there was not a surgery by time interaction ($F_{(6,251)}$ = 0.589, $p > 0.05$) (Figure 3.3). Post hoc decomposition of the time by drug interaction revealed percent change in locomotion decreased over time in response to saline (F (3,111) = 61.59, $p <$ 0.05) but did not change over time in response to cocaine (F (3,111) = 1.47, $p > 0.05$). Specifically, Tukey corrected contrasts revealed percent change in locomotion was significantly lower at the last three time points compared to the first for saline treatment. When compared to saline, cocaine-induced locomotion was higher across all time points. Post hoc decomposition of the surgery by drug interaction revealed that lesion type modulated cocaine (F (2,35) = 5.048, $p < 0.05$) induced locomotion but not the response to saline (F (2,35) = 0.041, $p > 0.05$). Specifically, Tukey correct post hoc contrasts revealed subjects with bilateral neurotoxic lesions exhibited a greater increase in cocaine-induced locomotion than subjects with unilateral (z = -2.929, $p < 0.05$) or sham lesions (z = -2.902, $p < 0.05$). All three groups exhibited greater locomotor response to cocaine than saline.

Since the baseline lengths differed for the neurotoxic and radiofrequency lesioned subjects, we also analyzed locomotion data from the neurotoxic lesioned subjects using the first five-minute interval as a baseline period; the results were effectively the same. A 4x3x2 mixed factorial ANOVA performed on subjects receiving neurotoxic or sham lesions, using the average of the two five-minute locomotion intervals as a baseline, revealed there was not a three-way interaction between drug, lesion, and time ($F_{(6,245)} = 0.460$, $p > 0.05$). The three-way interaction was then dropped from the model and two-way interactions were assessed. There was interaction between surgery and drug ($F_{(2,251)} = 22.682$, $p < 0.05$) as well as an interaction between time and drug ($F_{(3,251)} = 3.526$, $p < 0.05$), whereas there was not a surgery by time interaction ($F_{(6,251)} = 0.579$, $p > 0.05$). Post hoc decomposition of the time by drug interaction revealed percent change in locomotion decreased over time in response to saline ($F_{(3,111)} = 58.345$, $p < 0.05$) but did not change over time in response to cocaine ($F_{(3,111)} = 1.216$, $p > 0.05$). Specifically, Tukey corrected contrasts revealed percent change in locomotion was significantly lower at the last three time points compared to the first for saline treatment. When compared to saline, cocaine-induced locomotion was higher at all time points. Post hoc decomposition of the surgery by drug interaction revealed that lesion type modulated cocaine ($F_{(2,35)} = 6.032$, $p < 0.05$) induced locomotion but not response to saline ($F_{(2,35)} = 0.279$, $p > 0.05$). Specifically, Tukey correct post hoc contrasts revealed subjects with bilateral neurotoxic lesions exhibited a greater increase in cocaine-induced locomotion than subjects with unilateral ($z = -3.354$, $p < 0.05$) or sham lesions ($z = -2.954$, $p < 0.05$). All three groups exhibited greater locomotor response to cocaine than saline.

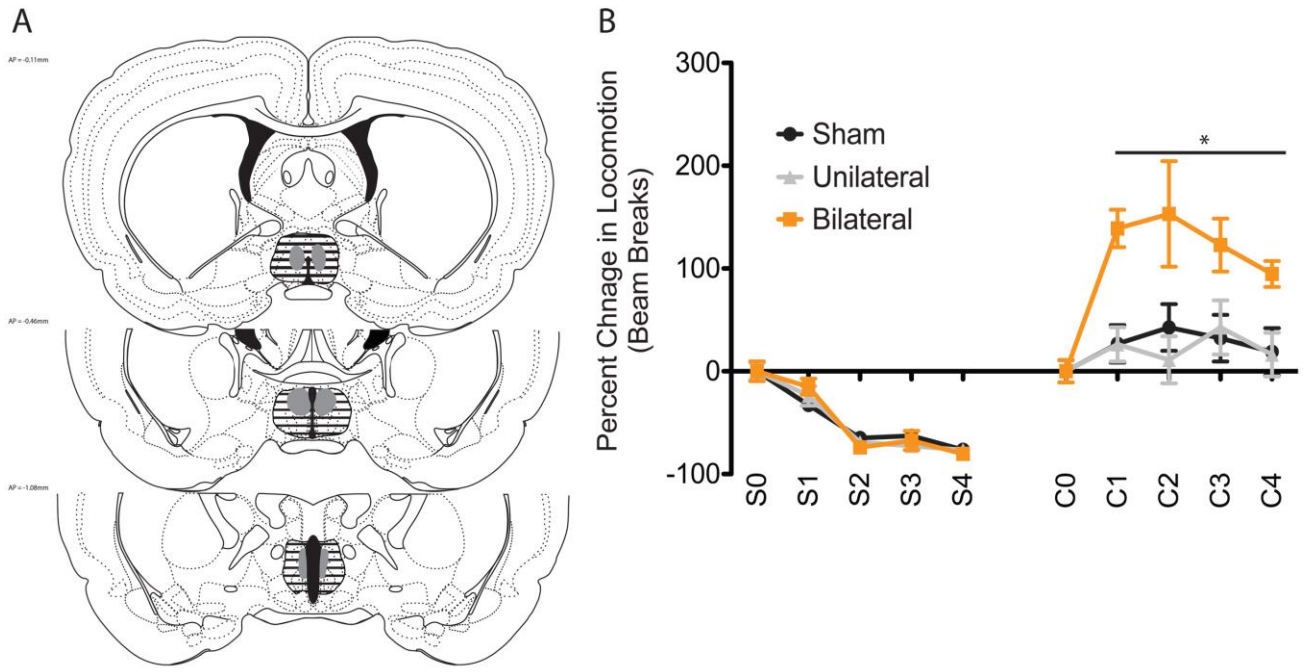

Figure 3.3 Neurotoxic lesions of the mPOA enhanced cocaine-induced locomotion. (A) Representative perimeters of smallest (solid gray) and largest (dashed black lines) radiofrequency lesion of the mPOA from rostral to caudal; drawn from (Swanson, 2004). (B) Graphical depiction of percent change in locomotion in response to an IP injection of either saline or cocaine. Saline and cocaine administration occurred one week apart. S0 and C0 are baseline locomotion measurement prior to injection and represent the average of two 5-min periods whereas S1-4 and C1-4 are five-minute locomotion intervals after injection. Subjects with bilateral neurotoxic lesions had a greater overall increase in cocaine-induced locomotion compared to sham and unilateral lesion subjects (* $p < 0.05$). All subjects exhibited cocaine-induced increase in locomotion compared to saline treatment.

3.4.3 Analysis of baseline locomotion

Locomotion was analyzed as a percent change from baseline to help control for individual differences. However, basal differences in locomotion were also assessed. Baseline locomotion was higher for subjects with sham lesions compared to those with radiofrequency lesions ($t_{(25.661)} = 2.81$, $p < 0.05$). In spite of this baseline difference, there is still a significant drug by lesion interaction when the raw scores are used in the analysis instead ($F_{(1,185)} = 24.903$, $p < 0.001$). However there was not a statistical difference in baseline locomotion in the neurotoxic group with either baseline measure: average of 2 five-minute intervals ($F_{(2,35)} = 2.671$, $p > 0.05$), first five-minute interval ($F_{(2,35)} = 1.606$, $p > 0.05$).

3.4.4 Radiofrequency lesions of the mPOA enhance cocaine-induced conditioned place preference

A 2x2 factorial ANOVA performed on CPP subjects revealed an interaction between surgery and drug ($F_{(1,52)} = 4.102$, $p < 0.05$). Decomposition of this interaction revealed animals that received cocaine had a significantly higher drug shift than animals that received saline regardless or surgical condition: sham ($t_{(21.601)} = 2.237$, $p < 0.05$), lesion ($t_{(26.613)} = 5.155$, $p < 0.05$). There was no difference in drug shift between surgery groups that received saline during conditioning ($t_{(23.020)} = -1.125$, $p > 0.05$), but there was a marginal effect of surgery for subjects that received cocaine during conditioning ($t_{(27.997)} = -1.897$, $p = 0.068$) where lesion subjects exhibited a more robust drug shift than sham subjects (Figure 3.4). Thus the significant interaction is likely being driven by the difference between sham and lesion cocaine treated subjects.

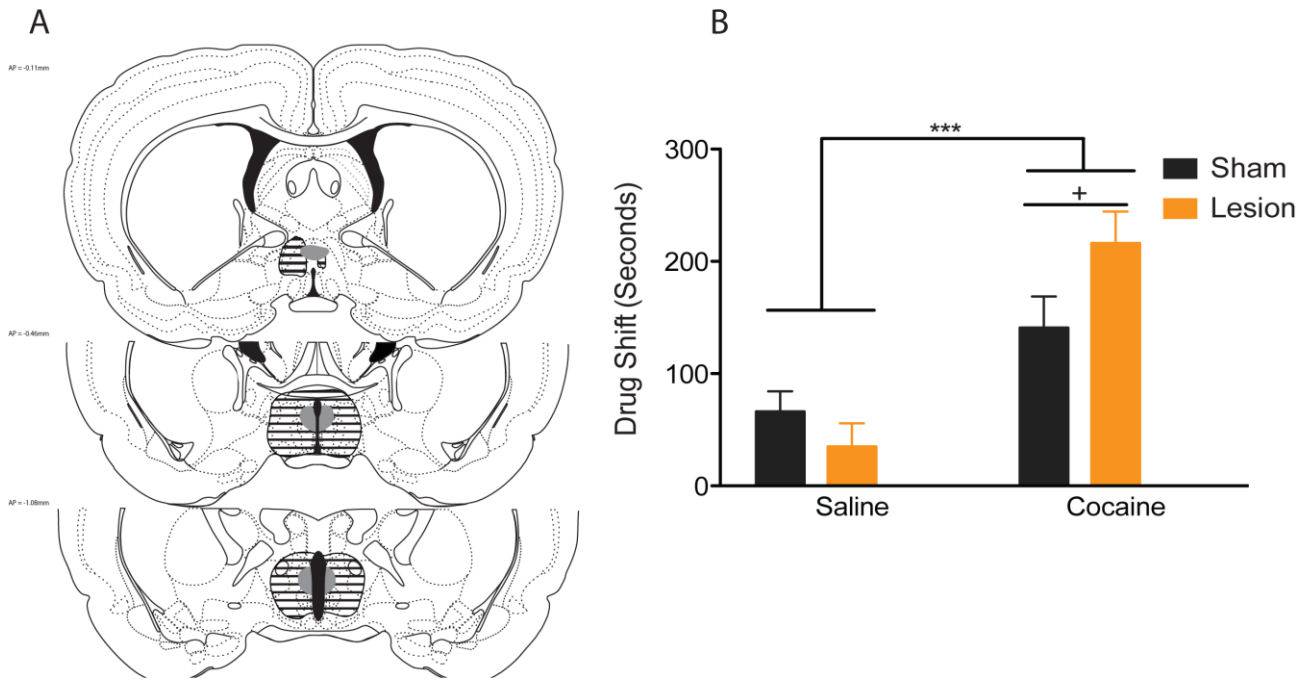

Figure 3.4 Lesions of the mPOA facilitated cocaine-induced place preference.

(A) Representative perimeters of smallest (solid gray) and largest (dashed black lines) radiofrequency lesion of the mPOA; drawn from (Swanson, 2004). (B) Graphical depiction of the effects of radiofrequency lesions on cocaine-induced CPP. Both sham and lesion treated individuals spent more time in the cocaine-paired chamber after 4 conditioning sessions than did saline treated subjects (* $p < 0.05$). Lesions of the mPOA increased the magnitude of cocaine conditioned compared to sham treated subjects but did not effect responding to saline (+ = 0.068).

3.5 DISCUSSION

In this study we demonstrated that lesions of the mPOA enhanced both physiological and rewarding aspects of response to cocaine in male rats. Both radiofrequency and neurotoxic lesions produced similar elevations in cocaine-induced locomotion suggesting the mPOA, and not merely fibers passing thorough the mPOA, is involved in the regulation of the stimulant properties of cocaine. This effect appears to be dependent on both hemispheres of the mPOA, since cocaine-induced locomotion in subjects with unilateral neurotoxic lesions did not differ from sham operated individuals. Lesions of the mPOA also enhanced the rewarding aspects of cocaine as lesioned subjects exhibited a greater post conditioning shift towards the drug-paired chamber than did sham treated subjects. These results are in line with previous work from our lab where mPOA lesions increased cocaine-induced CPP in female rats (Tobiansky et al., 2013a). Taken together these two studies suggest the regulation of cocaine reward by the mPOA is occurs in males as well as females.

The mPOA is essential for sexual (Hull and Rodriguez-Manzo, 2009) and maternal behaviors (Lonstein et al., 2015), and sends projections to the mesolimbic dopamine system (Simerly and Swanson, 1988). Maternal care and male sexual behavior also induce dopamine release in the NAc (Damsma et al., 1992; Champagne et al., 2004), suggesting the connection between the mPOA and mesolimbic system is behaviorally relevant. Dopamine in the NAc increases locomotion (Costall et al., 1979; Costall et al., 1984) and cocaine-induced CPP is at least partially modulated by NAc dopamine (Baker

et al., 1998; Liao et al., 2000). However, it is worth noting that dopamine-depleting lesions of the NAc attenuated IV but not IP cocaine-induced-CPP, whereas as cocaine-induced locomotion was attenuated for both routes of administration (Sellings et al., 2006). Given these findings, the present behavioral results suggest that the mPOA sends inhibitory projections to the mesolimbic system. Thus removing a source of inhibition into the mesolimbic dopamine system, should facilitate behavioral responses to cocaine (Lavezzi et al., 2015), as is evidenced in our present findings. However, how the mPOA modulates behavioral response to cocaine is not entirely clear.

Given the present findings, the most parsimonious circuit would be that the mPOA directly modulates the activity of cells in the NAc. However, anatomical studies suggest few neurons project directly from the mPOA to the NAc (Simerly and Swanson, 1988). The VTA, which sends dopaminergic projections to the NAc (Ikemoto, 2007), and modulates both CPP (Phillips et al., 1983; Harris and Aston-Jones, 2003; Galaj et al., 2014) and locomotion (Swanson and Kalivas, 2000), is innervated by the mPOA (Simerly and Swanson, 1988) (Tobiansky et al., 2013a). This suggests that the mPOA may modulate activity of the NAc via projections to the VTA. Similarly, manipulations of the VTA modulate mPOA-dependent behaviors. Electrical stimulation (Eibergen and Caggiula, 1973), as well as pharmacological manipulation (Mitchell and Stewart, 1990), of the VTA facilitates male sexual behavior. Unilateral lesions of the mPOA paired with contralateral lesions of the VTA impair copulation in a manner similar to bilateral lesions of the mPOA (Brackett and Edwards, 1984). Pharmacological depression of the VTA

impairs CPP for pups in female rats as well as pup directed maternal care (Seip and Morrell, 2009). Additionally, unilateral lesions of the mPOA and contralateral VTA lesions abolish pup retrieval but not nursing behavior (Numan and Smith, 1984). Together these studies suggest there is a behaviorally relevant connection between the mPOA and VTA.

In the present study, removal of the mPOA increased cocaine-induced locomotion and CPP, suggesting that the mPOA may inhibit dopamine activity in the NAc of male rats. This inhibition is likely the result of GABAergic projections from the mPOA to the VTA. The majority of the neurons within the mPOA are GABAergic (Leranth et al., 1985; Herbison et al., 1992; Wagner et al., 2001; Tsuneoka et al., 2013; Zhao et al., 2013). Additionally, pharmacological inactivation of the mPOA with Muscimol increases locomotion, as well as dopamine levels in the NAc (Osborne et al., 1993). Furthermore, a substantial number of mPOA-VTA neurons exhibit GABA immunoreactivity (Tobiansky et al., 2013a). Thus, it is possible that the mPOA sends GABAergic projections to the VTA that inhibit dopamine neurons, which in turn reduces dopamine release in the NAc. However, if this were the case lesioned subjects should have higher baseline locomotion, yet we observed a decrease in baseline locomotion in animals with radiofrequency lesions whereas there were no group differences with neurotoxic lesions.

One possible explanation for lack of enhanced basal locomotion is a portion of putative GABA efferents from the mPOA to the VTA innervates GABA interneurons in the VTA

(Johnson and North, 1992; Tan et al., 2012) or the rostromedial tegmental nucleus (RMTg)(also called the GABAergic tail of the VTA) (Kaufling et al., 2009; Bourdy and Barrot, 2012). Track tracing reveals that the mPOA projects to both the VTA and RMTg (Kaufling et al., 2009; Ikemoto and Bonci, 2014). Projections from the mPOA contain GABA or glutamate (Kallo et al., 2015), and these projections appose tyrosine hydroxylase positive and negative cells within the VTA in approximately equal number (Tobiansky et al., 2015). Thus, given the abundance of GABA immunoreactivity in the mPOA(Herbison et al., 1992), it is possible some of these projections are GABAergic. Lesions of the mPOA would then remove this inhibitory input on the RMTg, which, in turn could lead to an increased inhibition of dopamine neurons in the VTA, since the RMTg inhibits dopamine neurons in the VTA (Bourdy and Barrot, 2012). In this case there would also be a decrease in dopamine release in the NAc and it is possible that this decrease in dopaminergic tone in the NAc could sensitize dopamine receptors. As evidence of this, destruction of presynaptic dopamine terminals in the NAc with 6-hydroxydopamine lesions increases locomotion produced by the dopamine agonist apomorphine(Kelly and Iversen, 1976). Additionally, long-term treatment with dopamine receptor antagonists such as anti-psychotics sensitizes animals to the behavioral effects of cocaine. Specifically, locomotion rebounds upon abrupt anti-psychotic withdrawal (Bernardi and Neto, 1979) and rats treated chronically with haloperidol show increased locomotion in response to cocaine compared to vehicle treated subjects (LeDuc and Mittleman, 1993). Long-term haloperidol treatment also enhances cocaine-induced place preference sensitivity (Kosten et al., 1996) and there is an increase in sensitivity to

cocaine self-administration following two weeks of continuous dopamine antagonism (Howell and Byrd, 1992). It is thus possible that mPOA lesions may increase inhibition of the VTA by disinhibiting the rMTG, this in turn would decrease dopamine release in the NAc and sensitize dopamine receptors ultimately leading to a sensitized response to cocaine. However, given the projections to the VTA contain GABA, glutamate, or neither a similar effect would theoretically be observed if the mPOA sent glutamatergic projections to the VTA (Kallo et al., 2015). Additional studies are needed to concretely determine the neurochemical relationship between the mPOA and VTA. Specifically, temporary inactivating the mPOA, with pharmacology, optogenetics, or chemogentics, and then recording the activity of GABA and dopamine neurons in the VTA would help better elucidate the nature of this circuit.

One concern with radiofrequency lesions is that they destroy neural projections passing through a brain region along with cell bodies in the brain region of interest. In the present study we first performed radiofrequency lesions to determine if the mPOA was involved in the physiological response to cocaine. We then replicated this experiment with tract sparing NMDA lesions that destroyed only cell bodies within the mPOA and found in both cases the mPOA lesions increase cocaine-induced locomotion. However, the NMDA lesions were less reliable than the radiofrequency lesions with only 25% complete hits. While, 25% success is disheartening this is not totally unexpected, as numerous others have reported the preoptic area is resistant to neurotoxic lesions (Hu et al., 1992; Nishino et al., 1995; Ebling et al., 1998). Thus when we performed the CPP study we employed

radiofrequency lesions to minimize animal waste. While it is possible that different circuits underlie mPOA modulation of cocaine-induced locomotion and CPP, given the neural overlap between these two behaviors (Le Moal and Simon, 1991; Baker et al., 1998) and the similarity in the locomotion results, it seems likely that cells in the mPOA modulate cocaine-induced CPP.

As mentioned above, there was a significant difference in baseline locomotion between the sham and radiofrequency lesion animals. This makes the percent change data somewhat difficult to interpret as the radiofrequency animals exhibited significantly lower locomotion at baseline. Thus it is possible that the magnitude of cocaine's effect on locomotion did not change as a function of mPOA lesions, and that these results are merely the product of the mPOA lesion animals starting at a lower baseline. We do not think this is the case for two reasons. First, the lesion effect on locomotion was also present in the neurotoxic lesion study and there were no baseline group differences between these animals. Second, after detecting this baseline difference we ran a secondary analysis examine the effect of lesion on raw cocaine-induced locomotion scores and found analogous results. Taken together these results suggest the significant effect of mPOA radiofrequency lesion on cocaine-induced locomotion is not merely a mathematical artifact due to baseline differences in locomotion.

In conclusion, we observed an increase in cocaine-induced locomotion in male rats following neurotoxic or radiofrequency lesions of the mPOA as well as an increase in

cocaine-induced CPP following radiofrequency lesions of the mPOA. The potentiation of locomotion required lesions of both hemispheres of the mPOA, as this effect was not observed with unilateral lesions alone. The mPOA may modulate cocaine-induced behavioral responses through interactions with the VTA, as the mPOA projects directly to the VTA. However, given that the mPOA sends both GABA and glutamate projections to the VTA and mPOA projections appose dopamine and none dopamine neurons in the VTA, the exact mechanism through which the mPOA modulates behavioral responses to cocaine in male rats is still unclear.

Chapter 4: Activation of the lateral preoptic area modulates reinstatement of cocaine seeking but not cocaine taking

4.1 Abstract

The lateral preoptic area (LPO) is a hypothalamic brain region involved in the regulation of sleep, thirst, and locomotion. The LPO also sends projects to the ventral tegmental area (VTA). Activity of neurons in the VTA is important for addiction-related behaviors such as cocaine taking and seeking. Finally, previous work has demonstrated that optogenetic activation of LPO projections to the VTA decreased the firing rate of GABAergic neurons and increased the firing rate of dopaminergic neurons in the VTA. The goal of this study was to determine how LPO activation modulates dopamine responsive behaviors such as cocaine taking and reinstatement of seeking. Disinhibition of the LPO neurons via administration of bicuculline into the LPO or activation of the LPO via an excitatory designer receptor exclusively activated by designer drugs (DREADD) facilitated cocaine-seeking behavior but had no effect on cocaine taking during self-administration. Our results demonstrate activation of the LPO increases cocaine seeking; suggesting projections from the LPO to VTA modulate behavioral responsiveness to cocaine. This data supports the role of LPO as a novel structure involved in cocaine-seeking behavior.

4.2 Introduction

The LPO is a hypothalamic brain region located lateral of the medial preoptic area, medial of the ventral pallidum and extends caudally into the lateral hypothalamus (LH), with an ambiguous LPO-LH transition zone between the two regions (Swanson et al., 1984). Functionally, the LPO is implicated in the regulation of sleep (Nauta, 1946;

McGinty and Sterman, 1968; Sherin et al., 1996), thirst (Peck and Blass, 1975; Buggy and Jonhson, 1977; Saad et al., 1996), and locomotion (Mogenson et al., 1983; Swerdlow and Koob, 1984; Sinnamon, 1993; Lavezzi et al., 2015). Additionally, stimulation of the LPO supports intracranial self-stimulation (Elder and Work, 1965). A sub-nucleus of the LPO, the ventral lateral preoptic area, regulates the shift between slow wave and REM (rapid eye movement) sleep by sending inhibitory projections to the reticular activating system (Sherin et al., 1998; Gaus et al., 2002; Saper et al., 2005). The LPO regulates locomotion at least in part by sending projections to the VTA and the pedunculopontine nucleus. The pedunculopontine nucleus is part of the mesencephalic locomotor region and there is considerable overlap between LPO projections to the pedunculopontine nucleus and descending projections in this nucleus to the spinal cord. Additionally, stimulation of the LPO inhibits and excites different populations of neurons in this region (Swanson et al., 1984). These results support the observation that the LPO contains both GABAergic and glutamatergic neurons (Kallo et al., 2015). The LPO also sends projections to the VTA (Ikemoto and Bonci, 2014; Yetnikoff et al., 2015) though whether or not these projections modulate locomotion is less clear (Lavezzi et al., 2015). Thus it is possible that the LPO is involved in the regulation of the neural reward circuit given its sensitivity to intracranial self-stimulation and the large number of projections it sends to the VTA.

Previous, though currently unpublished, work in our lab using optogenetics and pharmacology has demonstrated that the LPO modulates activity of neurons in the VTA.

For optogenetics, rats received microinjections of channelrhodopsin into the LPO and then received optical stimulation of either the whole LPO or fibers from the LPO that project to the VTA. In both cases activation of the LPO increased the firing of dopaminergic neurons and decreased the firing of GABAergic neurons. The LPO was also stimulated with the GABA-A receptor antagonist bicuculline; bicuculline also blocks calcium sensitive potassium channels (Khawaled et al., 1999). Pharmacological activation of the LPO with bicuculline increased the firing rate of VTA dopaminergic neurons and decreased the firing rate of GABAergic neurons in a manner similar to that observed in the optogenetic study. Together these electrophysiological results suggest the LPO modulates the activity of VTA neurons, however whether or not this functional change is behaviorally relevant is currently unknown.

The activity of the VTA regulates the behavioral response to drug of abuse such as cocaine or heroin. Dopamine depleting lesions of the VTA with 6-Hydroxydopamine decreased cocaine self-administration in trained rats (Roberts and Koob, 1982). Either a systemic or intracerebral injection of baclofen, a GABA-B receptor agonist, at a dose that inhibits the activity of dopamine neurons decreased cocaine self-administration (Brebner et al., 2002). Direct administration of baclofen into the VTA attenuates the maintenance of cocaine self-administration (Shoaib et al., 1998), suppress self-administration of a low (0.75mg/kg) but not high (1.5mg/kg) dose of cocaine (Brebner et al., 2000b), and decreases the motivation to self-administer cocaine on a progressive ratio schedule (Brebner et al., 2000a). Administration of NMDA receptor antagonists such as ketamine

or dizocilpine directly into the VTA increases heroin self-administration (Xi and Stein, 2002). Amphetamine microinjections into the VTA prior to acquisition of cocaine self-administration potentiate self-administration, while pretreatment with a combination of amphetamine and a variety of glutamate receptor antagonists blocks this effect (CPP, CNQX, or MCPG) (Suto et al., 2003). Electrophysiological activity of putative dopamine neurons in the VTA increases prior to heroin self-administration (Kiyatkin and Rebec, 1997, 2001). Additionally, brain temperature, a correlate of neural activity (Kiyatkin et al., 2002), in the VTA increases in prior to cocaine self-administration suggesting that both heroin and cocaine self-administration increases the activity of neurons in the VTA (Kiyatkin and Brown, 2003, 2004). The results from studies examining the role of VTA activity with regards to cocaine CPP are mixed. Pharmacological inactivation of the VTA with bupivacaine does not interfere with cocaine-induced CPP (Seip and Morrell, 2009) whereas the administration of the D1 receptor antagonist SCH 23390 into the VTA blocks cocaine-induced CPP (Galaj et al., 2014). Microinjections of SCH 23390 into the VTA also increase and decrease cocaine-self administration on a fixed and progressive ratio schedule respectively (Ranaldi and Wise, 2001). Finally, reinstatement of drug seeking following extinction is regulated by the activity of VTA dopamine neurons. Dopamine neurons that project from the VTA to the NAc modulate reinstatement of cocaine seeking and dopamine administration in the NAc potentiates reinstatement of cocaine seeking (Anderson et al., 2003; Bachtell et al., 2005; Schmidt et al., 2005). Administration of morphine into the VTA but not adjacent nuclei facilitates reinstatement of both heroin and cocaine seeking following extinction (Stewart, 1984). Furthermore,

activation of the VTA with NMDA is also sufficient to produce reinstatement of cocaine seeking (Vorel et al., 2001). Activation of dopamine autoreceptors with quinpirole or systemic administration of baclofen attenuates reinstatement of cocaine seeking (Marinelli et al., 2003) (Di Ciano and Everitt, 2003). Additionally, microinjections of baclofen and muscimol (McFarland and Kalivas, 2001; Di Ciano and Everitt, 2004) inhibit reinstatement of cocaine seeking, whereas microinjections of a group II metabotropic glutamate receptor agonist, LY379268 ((-)-2-oxa-4-aminobicylco hexane-4,6-dicarboxylic acid), into the VTA blocks cue-induced reinstatement of heroin seeking (Bossert et al., 2004). Taken together these studies demonstrate that VTA activity is involved in the behavioral response to drugs of abuse.

Given previous observations in out lab that projections from the LPO to the VTA modulate the activity of neurons in the VTA, the goal of the present study was to test if activation of the LPO modulates the behavioral response to cocaine using pharmacological and chemogenetic methods. To accomplish this we tested whether unilateral activation of the LPO with bicuculline or an excitatory DREADD modulated cocaine seeking as well as reinstatement of cocaine seeking following extinction or prolonged withdrawal.

4.3 MATERIALS AND METHODS

4.3.1 Subjects

Male Sprague Dawley rats (Harlan, Indianapolis), weighing 300-450g (PN 59-63 at arrival), were double housed in large plastic cages on a 12/12 h reverse light-dark cycle,

with access to food and water *ad libitum*. The room temperature was set at 22 ± 2 °C. Rats were weighted 2-3 times per week for the duration of the study. All procedures were in accordance with the NIH Guidelines for the Use of Animals and were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

4.3.2 Surgery

All rats were anesthetized with isofluorane (2-5%) and underwent two surgical procedures. Catheterization of the jugular vein occurred for all rats while some rats also received unilateral cannulation of the LPO and a different subset received a microinjection of either a control virus containing green fluorescent protein (GFP) or a virus containing an excitatory DREADD. Following surgery all rats were individually housed and placed on a heating pad for one hour in the surgery room and then returned to the colony room. The day after surgery all subjects were rehoused with their pre-surgery cage mate. Health was monitored daily for one week post surgery. All rats were given flunixin at a dose of (5mg/kg/mL) prior to surgery and (2.5mg/kg/mL) of flunixin the day following surgery. The jugular catheter was flushed with cefazolin (50mg/kg/0.5mL), to prevent the development of infection, on the day of surgery and the six days following surgery. Aseptic conditions were maintained throughout surgery and while catheters were flushed.

4.3.2.1 Jugular catheter

Intravenous catheters were custom-made using sylastic tubing (0.30 mm inner diameter, 0.64mm outer diameter, 11.5 cm long according to the size of the rat, 10 µL dead volume) and a backmount guide cannula (Plastics One). All catheters were autoclaved

prior to insertion. To implant catheters in the jugular vein, a 1-2cm incision is made in the mid-scapular region and in the area overlaying the right jugular vein. The external end of the catheter was placed in the mid scapular region, and the bottom end was passed under the skin, to exit in the jugular vein area. The jugular vein is isolated with forceps, and the catheter is inserted in the vein by producing a small hole in the vein with a 23G needle. The catheter was secured to the vein with sterile suture, and then flushed with sterile saline and capped. The incision above the jugular vein was closed with suture while the back incision was closed with surgical staples. Both front and back sutures are sprayed with Solarcaine (Bayer AG), a non-prescription external analgesic, and covered with triple antibiotic ointment.

4.3.2.2 Cannulation of the LPO

For cannulation of the LPO all subjects were mounted in a stereotaxic apparatus with non-piercing earbars (Kopf) and the skull was leveled based on the bregma and lambda. The skin above the skull was cut with a single incision and connective tissue beneath was cleared. A guide cannula (23-gauge thin wall hypodermic tubing, 15mm length; Vita Needle, or 22-gauge, 8mm length and 8mm pedestal; Plastics One) was lowered to 2mm above the LPO (AP, -0.3mm; ML, +4mm; DV, -6.3mm; angle 18 degrees (Paxinos and Watson, 2007)). The cannula was placed at an angle to avoid the lateral ventricle. Results from a pilot study suggested there were no left right differences so all cannula were placed in the left LPO to minimize surgical error. Prior to implantation each guide cannula was sterilized with 70% ethanol to prevent infection. Three small screws were inserted in the skull and then cannula was fixed in place with dental resin (Hygenic

Corporation, Cuyahoga Falls, OH). A dummy cannula cut to the length of the guide cannula was then inserted into the guide cannula in order to maintain patency. The posterior portion the incision was closed with surgical staples while the portion of the incision around the guide cannula was left open. Triple antibiotic was then spread over the site of the incision.

4.3.2.3 Microinjection of excitatory DREADD

For microinjections of excitatory DREADD (AAV-hSyn-HA-hM3D(Gq)-IRIS-mCitrine) or control (AAV-hSyn-EGFP) virus into the LPO all subjects were mounted in a stereotaxic apparatus with non-piercing earbars (Kopf) and the skull was leveled based on the bregma and lambda. The skin above the skull was cur with a single incision and connective tissue beneath was cleared. A microinjector (30-gauge thin wall hypodermic tubing, 15mm length; Vita Needle) was the mounted on a stereotaxic arm and attached to a 5ul Hamilton syringe, on a microinfusion syringe pump (Pump 11 elite, Harvard Apparatus), with PE20 tubing filled with double distilled H_20. A bubble was then created by withdrawing 180nL of air at 2µL/min followed by 900nL of virus. A borehole was drilled above the LPO (AP, -0.3mm; ML, +4mm; angle 18 degrees (Paxinos and Watson, 2007)) and the dura was removed with a 25-gage needle. The microinjector was then lowered into the LPO (DV, -8.3mm) and 400nL of virus were injected at a rate of 100nL/min. The injector was then left in place for another 5 minutes to allow for diffusion of the virus. After each microinjection 2µL of H_20 were flushed through the microinjector to remove excess virus and any remaining blood at the tip of the microinjector and the microinjector was cleaned with 70% ethanol. Separate

microinjectors were used for DREADD and GFP control viruses. Bone wax was placed over the hole, the incision was closed with surgical staples, and triple antibiotic ointment was applied.

4.3.3 Microinjections

Immediately prior to the last cocaine self-administration session or the test of reinstatement of cocaine seeking, 84ng of bicuculline (Sigma-Aldrich St. Louis, Mo), dissolved in artificial cerebrospinal fluid (aCSF (Harvard Apparatus, Holliston, MA.)), or equal volume vehicle was microinjected into the LPO. All microinjections were performed under red light in a room adjacent to the self-administration and colony rooms. The dummy cannula was removed and a 30-gauge microinjector that extended 2.0 mm past the guide in the LPO was inserted. After forty-five seconds of equilibration, 0.3 μL of solution was injected over the course of one min using a microinfusion pump (Pump 11 elite, Harvard Apparatus). The injection volume was chosen based in part on a previous study that examined the role of LPO in locomotion using bicuculline microinjections (Lavezzi et al., 2015). The injector was left in place for ninety seconds after the infusion was finished to allow for diffusion before it was removed and the dummy cannula reinserted. All subjects were then place in the self-administration chambers for cocaine-self administration or reinstatement of cocaine seeking.

4.3.4 Behavioral procedures

4.3.4.1 Self administration

Subjects were allowed to recover from surgery for two weeks prior to self-administration. Catheter patency was maintained by daily flushes with cefazolin (50mg/kg/0.5ml) for the first week post-surgery and then with 0.9% NaCl (0.1cc) every other day until self-administration began. Once self-administration began catheters were flushed with 0.1cc of 0.9% NaCl directly before and after self-administration. Catheter patency was assessed following the last day of self-administration by a sodium brevital (5mg/kg/0.5ml) infusion. All rats were trained to self-administer cocaine on a fixed-ratio 1 (FR1) schedule for ninety minutes a day using nose pokes as the operant response. A nose poke in the active hole resulted in an infusion of cocaine (600µg/kg/100µL/infusion) and the presentation of a light in the active hole for ten seconds, whereas a nose poke in the inactive hole resulted in nothing. Both the active and inactive holes were on the same side of the self-administration chamber. Post infusion timeouts, to prevent cocaine overdose, were structured such that the length of the time out increased with the number of infusions (1-20 infusions =10 second timeout, 21-30 infusions =20 second timeout, 31+ infusions =30 second time out), however the active light was always be on for ten seconds following an infusion. One group of rats was maintained on a FR1 schedule for eight days of self-administration before undergoing extinction. Two other groups of rats underwent training to increase the effort required to obtain cocaine (increased fixed ratio). These subjects underwent four days of training on an FR1 followed by three days of training on an FR3 and were finally moved up to a FR5 for four days before cocaine

taking was tested. For all subjects the criteria for inclusion in the study were at least ten infusions over the last three days of self-administration prior to testing and no more than 30% variation in responding over during this time.

4.3.4.2 Extinction and reinstatement of seeking

Following cocaine self-administration and the test of cocaine taking, subjects underwent either extinction or home cage withdrawal. For extinction, subjects were placed in the self-administration chambers for ninety minutes once a day for 21-25 days at the same time of day they had undergone cocaine self-administration, however they were not tethered to the infusion pump during exaction. Nose pokes on the active hole still yielded the light and the sound of the pump turning but cocaine was not delivered. The nose poke requirement for the light on the active hole was the same as it was on the last day of self-administration (one or five). Once nose poking had been extinguished, bicuculline or aCSF was microinjected into the LPO on the day following the last extinction session. Immediately after microinjections subjects were placed back in the self-administration chambers to determine if activation of the LPO modulated reinstatement of cocaine seeking. All chamber conditions were identical to an extinction session during the reinstatement test. One group of subjects underwent home cage withdrawal for 23-25 days, instead of extinction, followed by a within session test of extinction/reinstatement. For within session extinction/reinstatement subjects underwent one extinction session as described above with the exception that the session was only sixty minutes. Immediately after extinction ended subjects received a microinjection of either bicuculline or aCSF and were then placed in the self-administration boxes for another sixty minutes to test

reinstatement of cocaine seeking. Inclusion criteria varied based on self-administration FR and extinction type and are discussed below in the procedural relationship section.

4.3.5 Tissue collection and injector placement

After the test for reinstatement of cocaine seeking subjects were euthanized and brains were removed. Brains were stored in 20 μL of 10% Formalin for one week at 4°C. Fifty micron coronal section of the LPO were then cut with a cryostat to assess injector placement. The LPO does not have clear anatomical boundaries, so the optic chiasm, anterior commissure, and lateral ventricle were used as landmarks. Several subjects showed signs of infection at the site of injection or along the cannula track. These individuals were excluded from analysis.

4.3.6 Procedural relationship

The present study contains a variety of self-administration and extinction-reinstatement paradigms. For pharmacology experiments, subjects were assigned to treatment groups based on the last three days of self-administration prior to microinjections. Blocking was employed to insure baseline self-administration behavior variability was consistent across treatment groups. Subjects in the DREADD experiment were randomly assigned to a treatment group prior to surgery.

4.3.6.1 Bicuculline induced excitation of the LPO, FR1 self-administration, and between session extinction

Following surgery, subjects underwent seven days of self-administration of FR1 schedule. On the eighth day of self-administration all subjects that met the criteria for inclusion (discussed in self-administration section above) received a unilateral

microinjection of either bicuculline or aCSF immediately prior to self-administration. Subjects then underwent twenty-one days of extinction where no cocaine was available in response to a nose poke in the active hole. On the twenty-second day of extinction subjects received a microinjection of bicuculline or aCSF and underwent a reinstatement test. The criterion for extinction was an average of no more than 20 nose pokes on the active hole of the last three days of extinction.

4.3.6.2 Bicuculline induced excitation of the LPO, FR5 self-administration, and within session reinstatement

Following surgery, subjects underwent ten days of self-administration on a schedule that progressed from FR1 up to FR5 on the last four days. On the eleventh day of self-administration all subjects that met the criteria for inclusion received a unilateral microinjection of either bicuculline or aCSF immediately prior to self-administration. Subjects then underwent 23-25 days of home cage withdrawal where cocaine was not available. Following withdrawal all subjects underwent a within session extinction/reinstatement test. Subjects that exhibited more than twenty nose pokes in the active hole during the last twenty minutes of extinction were excluded from analysis.

4.3.6.3 DREADD induced excitation of the LPO, FR5 self-administration, and between session extinction

Following surgery, subjects underwent eleven days of self-administration on a schedule that progressed from FR1 up to FR5 on the last five days. Subjects then underwent extinction where no cocaine was available in response to a nose poke in the active hole for 23-25 days. The effects of LPO activation via DREADDs on cocaine taking were not tested in this experiment. Following the last day of extinction subjects received an

intraperitoneal injection of clozapine-N-oxide (CNO), an inert molecule that acts only on DREADDs (Armbruster et al., 2007), and underwent a reinstatement test. The criterion for extinction was an average of no more than 70 nose pokes on the active hole of the last three days of extinction. This criterion is higher than the one described above for extinction following an FR1 schedule. However, these subjects underwent extinction after responding on a FR5 schedule for five days and as such their responding on the active lever was far higher.

4.3.7 Statistics

Welch's two-sample t-tests were employed in order to determine if activation of the LPO modulated cocaine taking on a FR1 or FR5 schedule. A mixed ANOVA with treatment (bicuculline/aCSF) as between subject factor and hole (active hole/inactive hole) as a within subject factor was performed in order to determine if activation of the LPO modulates active hole responding during cocaine taking on an FR1 or FR5 schedule. A mixed ANOVA with treatment (bicuculline/aCSF or excitatory DREADD/control DREADD) as between subject factor and hole (active hole/inactive hole) as a within subject factor was performed in order to determine if excitation of the LPO modulates cocaine reinstatement of cocaine seeking following extinction. It would also have been possible to analyze this data with an ANCOVA treating inactive hole responding as a covariate. However, we elected not to do this given the possibility that activation of the LPO may also effect responding on the inactive hole, which would complicate the interpretation of such an analysis. Significant two-way interactions were decomposed by comparing simple main effects of one factor at the specific level of the other factor. As a

secondary analysis the effects of unilateral LPO activation on general locomotion were also analyzed using Welch's two-sample t-test. Locomotion was only analyzed during reinstatement of seeking since cocaine was not also administered during these tests. All statistics were performed using R version 3.2.2 "Fire Safety".

4.4 RESULTS

4.4.1 Pharmacological activation of the LPO enhances reinstatement of cocaine seeking but not cocaine taking under an FR1 schedule

Activation of the LPO with bicuculline did not affect cocaine taking on a FR1 schedule ($t_{(20.169)} = 0.149$, $p = 0.883$; Infusions: bicuculline (20.50) vs. aCSF (19.84)) (Figure 4.1A). Analysis of responding during cocaine self-administration with a mixed factorial ANOVA relieved there was not an interaction between hole and treatment ($F_{(1,21)} = 0.091$, $p = 0.763$), nor was there main effect of treatment ($F_{(1,21)} = 0.0004$, $p = 0.983$). However, there was a main effect of hole with subjects having significantly more response on the active hole ($F_{(1,21)} = 10.073$, $p < 0.01$; Responses: active hole (33.39) vs. inactive hole (7.869)) (Figure 4.1B). There were no baseline group differences on average active hole responding over the three days prior to reinstatement of seeking ($t_{(7.776)} = 1.084$, $p = 0.311$; responses: bicuculline (20.27) vs. aCSF (14.70)). Analysis of the effects of LPO activation on reinstatement of cocaine seeking revealed there was a significant treatment by hole interaction ($F_{(1,14)} = 20.964$, $p < 0.001$)(Figure 4.2). Decomposition of the interaction revealed bicuculline treated animals made significantly more responses on the active hole (t (5.58) = 3.284, $p < 0.05$; 41.5 vs. 9.8) while there was no significant difference in the number of response made on the inactive hole

between groups (t ($_{5.062}$)=1.72, p = 0.145; 12.0 vs. 1.9). For both Bic (t ($_5$)= 5.700, < 0.01) and aCSF (t ($_9$) = 3.9, p < 0.01 animals made significant more responses in the active hole than the inactive hole. Finally, activation of the LPO in the absence of cocaine during the test of reinstatement of seeking did not significantly modulate general locomotion (t ($_{5.660}$) = 2.322, p = 0.062; photo beam breaks: bicuculline (1383.33) vs. aCSF (623.30)), however it does appear there may be a statistical trend.

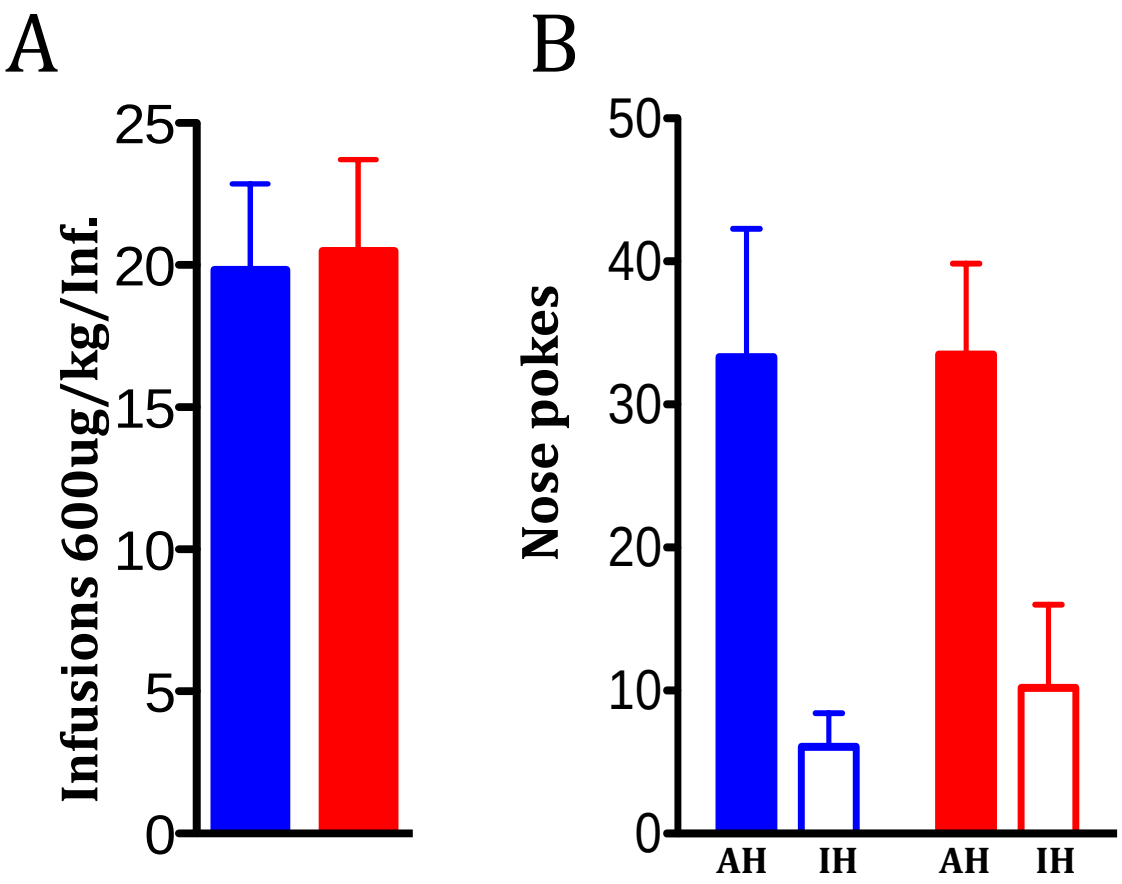

Figure 4.1 Activation of the LPO with bicuculline does not modulate cocaine taking or responding for cocaine during self-administration on an FR1 schedule.

(A) There was no difference in the number of infusions earned in a 90-minute self-administration session between animals that received unilateral microinjection of bicuculline (red) or aCSF (blue) into the LPO. (B) Responding on neither the active hole (AH) nor the inactive hole (IH) was modulated by microinjection of bicuculline (red) into the LPO when compared to aCSF (blue). However, responding on the active was significantly higher across treatment groups.

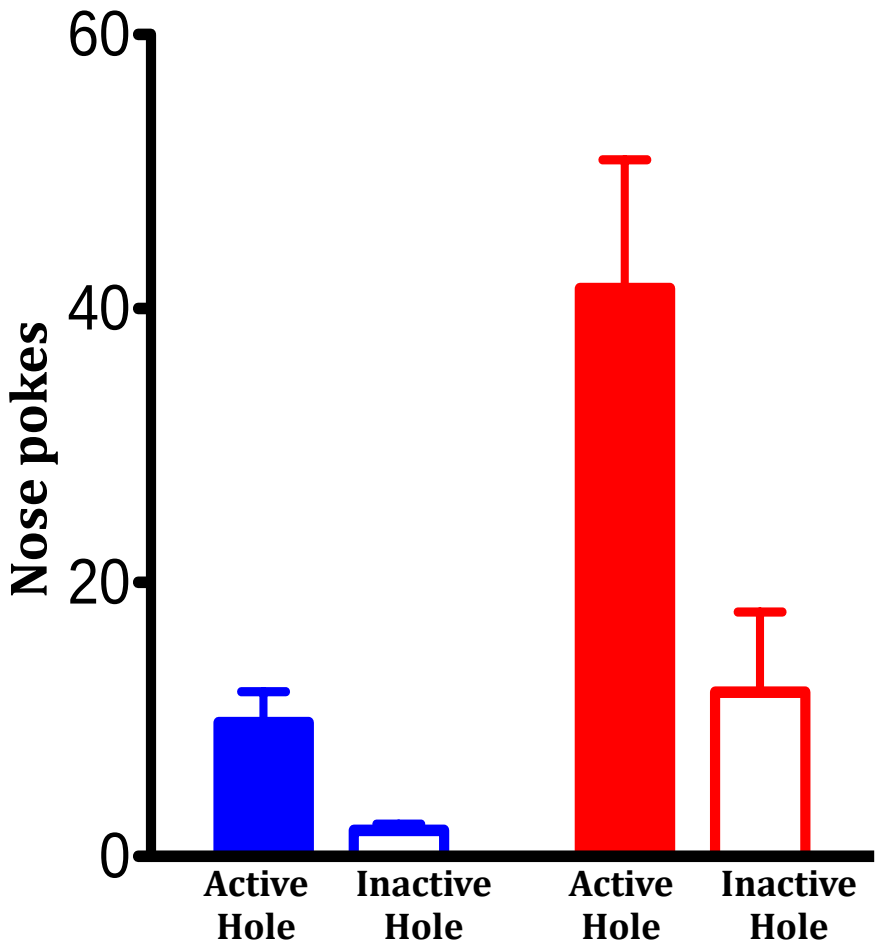

Figure 4.2 Activation of the LPO with bicuculline reinstates cocaine seeking for rats trained on a FR1 schedule following extinction.

A unilateral microinjection of bicuculline (red) increased responding on the active hole, previously resulted in cocaine administration, compared to a unilateral microinjection of aCSF (blue).

4.4.2 Pharmacological activation of the LPO enhances reinstatement of cocaine seeking but not cocaine taking under an FR5 schedule

Activation of the LPO with bicuculline did not affect cocaine taking on a FR5 schedule ($t_{(15.144)} = 0.208$, $p = 0.8379$; Infusions: bicuculline (19.6) vs. aCSF (20.1))(Figure 4.3A). Analysis of responding during cocaine self-administration with a mixed factorial ANOVA relieved there was not an interaction between hole and treatment ($F_{(1,17)} = 0.067$, $p = 0.799$), nor was there main effect of treatment ($F_{(1,17)} = 0.015$, $p = 0.904$). However, there was a main effect of hole with subjects having significantly more response on the active hole ($F_{(1,17)} = 86.463$, $p < 0.001$; Responses: active hole (120.42) vs. inactive hole (2.8)) (Figure 4.3B). There were no group differences in active hole responding over the last twenty minutes of extinction prior to reinstatement of seeking ($t_{(9.080)} = 0.324$, $p = 0.753$; responses: bicuculline (14.71) vs. aCSF (12.50)). Analysis of the effects of LPO activation on reinstatement of cocaine seeking revealed there was a significant treatment by hole interaction ($F_{(1,11)} = 8.144$, $p < 0.01$) (Figure 4.4). Decomposition of the interaction revealed bicuculline treated animals made significantly more response on the active hole ($t_{(6.734)} = 3.056$, $p < 0.05$; responses: bicuculline (36.29) vs. aCSF (9.67)) while there was no significant difference in the number of response made on the inactive hole between groups ($t_{(10.223)} = 1.72$, $p = 0.733$; responses: bicuculline (3.28) vs. aCSF (2.66)). For both bicuculline ($t_{(6)} = 4.042$, $p < 0.01$) and aCSF ($t_{(5)} = 3.130$, $p < 0.05$) animals made significantly more responses in the active hole than the inactive hole. Finally, activation of the LPO in the absence of cocaine during the test of reinstatement of seeking did not modulate general locomotion ($t_{(7.475)} = 0.976$, $p = 0.359$; photo beam breaks: bicuculline (631.29) vs. aCSF (496.50)).

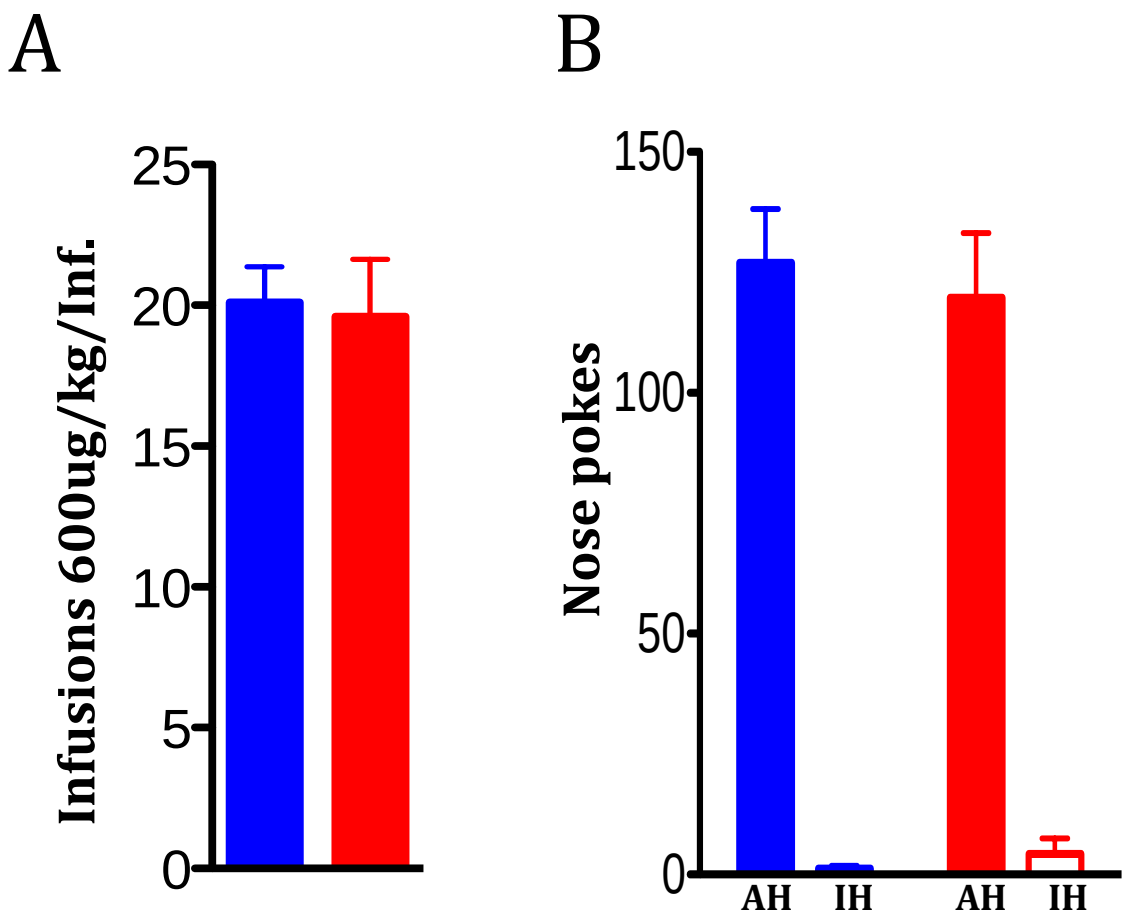

Figure 4.3 Activation of the LPO with bicuculline does not modulate cocaine taking or responding for cocaine during self-administration on an FR5 schedule.

(A) There was no difference in the number of infusions earned in a 90-minute self-administration session between animals that received unilateral microinjection of bicuculline (red) or aCSF (blue) into the LPO. (B) Responding on neither the active hole (AH) nor the inactive hole (IH) was modulated by microinjection of bicuculline (red) into the LPO when compared to aCSF (blue). However, responding on the active was significantly higher across treatment groups.

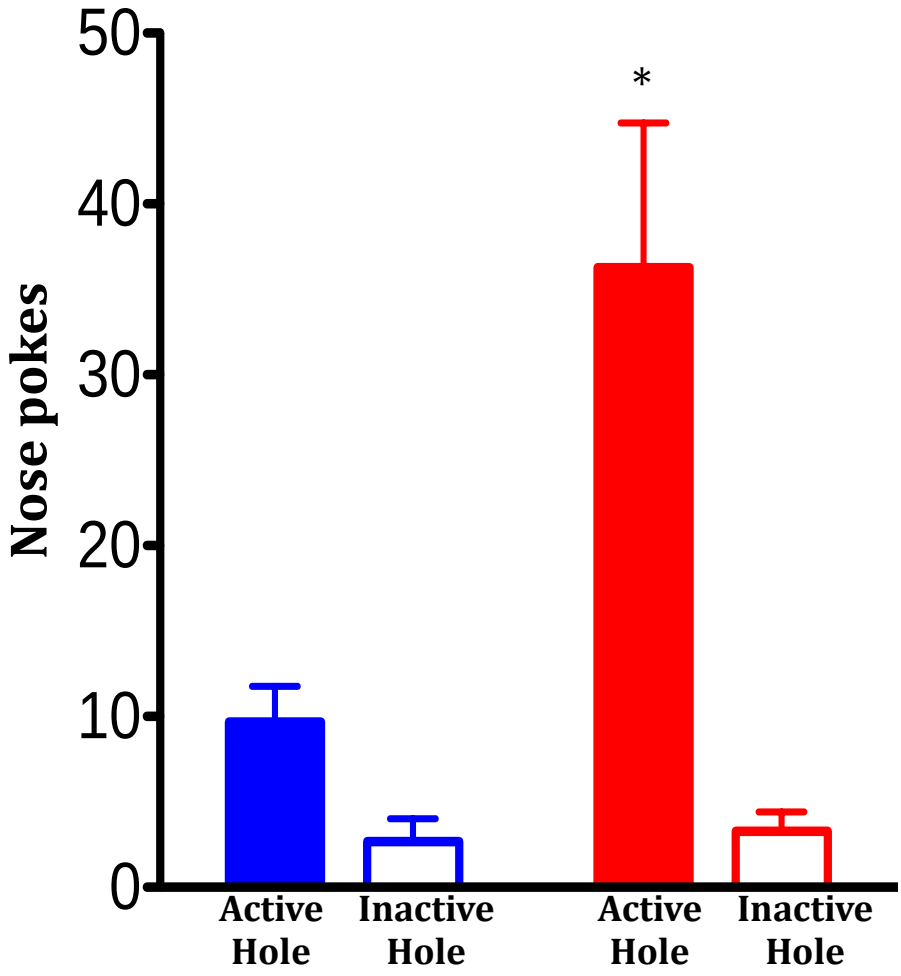

Figure 4.4 Activation of the LPO with bicuculline reinstates cocaine seeking for rats trained on a FR5 schedule following extinction.

A unilateral microinjection of bicuculline (red) increased responding on the active hole, previously resulted in cocaine administration, compared to a unilateral microinjection of aCSF (blue).

4.4.3 Chemogenetic activation of the LPO enhances reinstatement of cocaine seeking

There were no differences in the average number of nose pokes in the active hole over the last three days of extinction (t ($_{20.765}$)=0.382, p = 0.706; responses: excitatory DREADD (44.06) vs. control (49.47)). Analysis of the effects of LPO activation, with excitatory DREADDs, on reinstatement of cocaine seeking revealed there was a significant treatment by hole interaction (F ($_{1,17}$) = 4.158, p < 0.05)(Figure 4.5). Decomposition of the interaction revealed animals with excitatory DREADDs made more response on the active hole (t ($_{16.938}$) = 2.368, p < 0.05; responses: excitatory DREADDs (78.11) vs. control (45.8)) while there was no significant difference in the number of response made on the inactive hole between groups (t ($_{15.646}$)=0.841, p = 0.413; responses: excitatory DREADDs (10.889) vs. control (7.4)). For both excitatory DREADDs (t ($_{8}$) = 6.977, p < 0.01) and control (t ($_{9}$) = 3.800, p < 0.01) animals made significant more responses in the active hole than the inactive hole. Finally, activation of the LPO in the absence of cocaine during the test of reinstatement of seeking did not significantly modulate general locomotion (t ($_{13.323}$) = 1.115, p = 0.285; photo beam breaks: excitatory DREADDs (1670.30) vs. control (1333.40)).

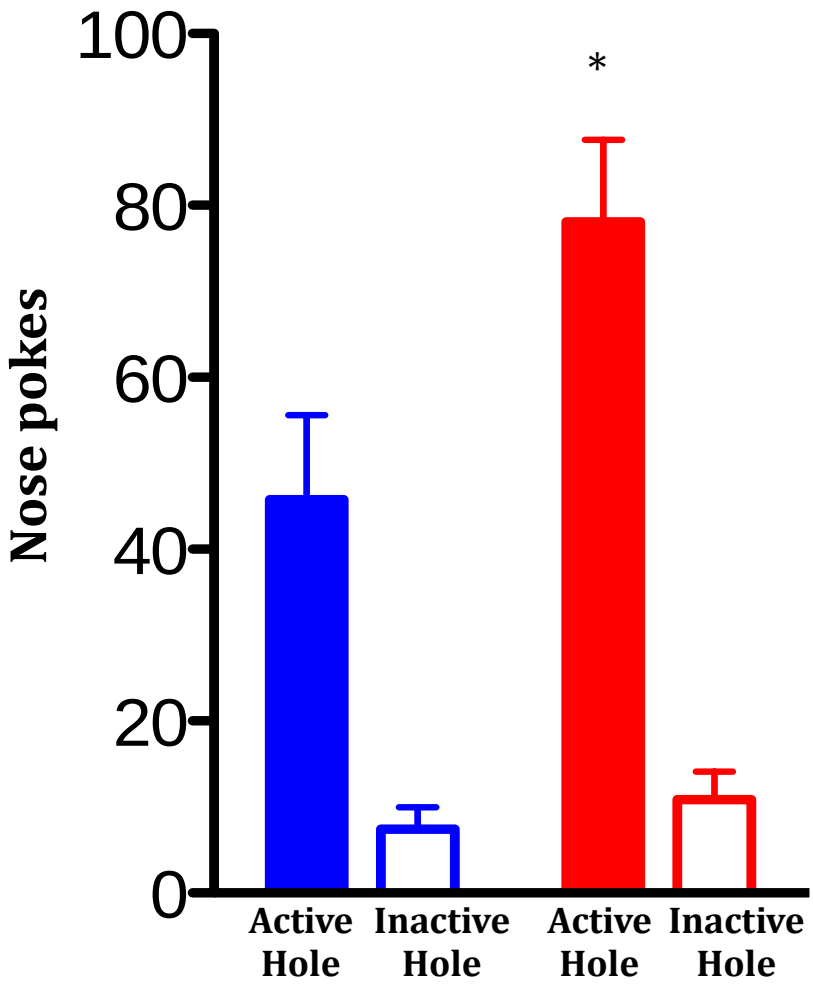

Figure 4.5 Activation of the LPO with an excitatory DREADD reinstates cocaine seeking for rats trained on a FR5 schedule following extinction.

Systemic administration of CNO increased responding on the active hole, previously resulted in cocaine administration in animals previously treated with an excitatory DREADD compared to control animals (blue).

4.5 DISCUSSION

The results of the present study demonstrate that unilateral activation of the LPO, with either pharmacology or chemogentics, is sufficient to reinstate cocaine-seeking behavior following a period of either prolonged extinction or withdrawal. A similar pattern of results was observed in animals that underwent self-administration training on either an FR1 or FR5 schedule. However, it is worth noting, that while not statistically significant, there did appear to be a LPO activation-induced increase in responding on the inactive hole during reinstatement for animals that were trained on a FR1 but not a FR5 schedule. Unilateral activation of the LPO with bicuculline did not affect cocaine taking nor responding on either a FR1 or FR5 schedule. These results are inline with previous anatomical results that demonstrate the LPO sends projections to the VTA that inhibit the activity of GABAergic neurons and excite dopaminergic neurons, as the activity of VTA dopamine neurons modulates reinstatement of drug seeking (Stewart, 1984; Neisewander et al., 1996; Bossert et al., 2013). Taken together these studies point to the LPO as a novel structure involved in the regulation of cocaine seeking behavior: an animal model of drug relapse.

The present results clearly demonstrate that activation of the LPO is sufficient to modulate behavioral seeking of cocaine following extinction. Given the LPO projects heavily to the VTA (Ikemoto and Bonci, 2014; Yetnikoff et al., 2015) and excitation of the LPO also modulates the activity of VTA dopamine neurons, it is likely that the LPO modulates cocaine seeking via the VTA. However, it is worth noting that the LPO

projects heavily to other brain regions that may also contribute to this reinstatement of seeking network. Specifically, the LPO sends projections to the lateral habenula (LH) and the rostromedial tegmental nucleus (RMTg)(Yetnikoff et al., 2015), both of which are behaviorally functional (Lavezzi et al., 2015).

The RMTg is a GABAergic nucleus caudal to the VTA and is sometimes referred to as the GABAergic tail of the VTA (Jhou et al., 2009b; Kaufling et al., 2009). Functionally, the RMTg sends inhibitory projections predominantly to VTA dopamine neurons thereby acting as a break for the VTA (Jhou et al., 2009a; Jhou et al., 2009b). Specifically, ~83% RMTg to VTA projects are to tyrosine hydroxylase immunereactive cells (Barrot et al., 2012). These projections have been implicated in responding to aversive stimuli, as foot shock, reward omission and food deprivation increase activity of these neurons whereas reward or reward cue presentation inhibit firing (Jhou et al., 2009a). Recently it was shown that the LPO sends projections to the RMTg that modulate locomotion. Specifically, pharmacological activation of the LPO with bicuculline increased locomotion (Lavezzi et al., 2015). However, microinjections of bicuculline into the RMTg concurrent with LPO activation had no effect on locomotion suggesting that LPO sends inhibitory projections to the RMTg, which in turn disinhibits RMTg inhibition of VTA dopamine neurons, ultimately leading to increased locomotion (Swanson and Kalivas, 2000; Lavezzi et al., 2015). Inactivation of the RMTg prior to cue-induced reinstatement facilitates reinstatement of cocaine seeking (Huff and LaLumiere, 2015). Thus it is possible that the LPO sends inhibitory projections to the RMTg, which

disinhibits inhibitory projections to VTA dopamine neurons, thereby increasing VTA dopamine activity and facilitating reinstatement of cocaine seeking (Stewart, 1984).

The LPO also projects heavily to the LH and sends significantly more projections to the LH than the RMTg (Yetnikoff et al., 2015). The LH is involved in the regulation of dopamine and serotonin activity in the brain (Hikosaka, 2010). Stimulation of the LH inhibits the activity of VTA dopamine neurons (Christoph et al., 1986) via GABAergic neurotransmission (Ji and Shepard, 2007). Conversely, lesions of the LH increase mesolimbic dopamine activity as well as locomotor behavior (Lisoprawski et al., 1980) (Lee and Huang, 1988). LH neurons are also implicated in the coding of negative events. Specifically, in both humans and macaques LH neurons show increased activity in response to a smaller than predicted reward and decreased activity in response to a larger than expected reward (Ullsperger and von Cramon, 2003; Matsumoto and Hikosaka, 2007). This is in direct opposition to VTA dopamine neurons that exhibit increased firing in response to an unexpected reward and inhibition in response to reward omission (Schultz, 1998; Waelti et al., 2001; Schultz, 2002). Neurons in the LH are also excited by omission of reward, punishment, or stimuli associated with punishment (Matsumoto and Hikosaka, 2009). Descending projections from the LH to the VTA are primarily glutamatergic (Geisler et al., 2007; Omelchenko et al., 2009). This observation seems somewhat contradictory given the opponent nature between activity of the LH and VTA. However, the RMTg also receives projections from the LH (Lavezzi and Zahm, 2011; Goncalves et al., 2012) that are primarily glutamatergic (Brinschwitz et al., 2010), and

lesions of the RMTg prevent LH induced depression of VTA dopamine neurons (Ji and Shepard, 2007). Thus it is possible that the LPO sends inhibitory projections to the LH, which inhibits glutamate neurons in the LH. This in turn would decrease excitation of RMTg GABA neurons and therefore potentiate VTA dopamine activity. However, this is not likely the case as LH inhibition did not affect cue-induced reinstatement of cocaine seeking and reduced yohimbine induced reinstatement (Gill et al., 2013). While the exact neurochemical composition of LPO-LH projections is currently unknown, reward relevant projections from a neighbor structure, the lateral hypothalamus, to the LH are glutamatergic (Stamatakis et al., 2016). Thus, it is also possible that the LPO sends excitatory projections to the LH but again this is unlikely given the present results, as electrical stimulation of the LH attenuates cocaine seeking (Friedman et al., 2010).

It seems unlikely that the LPO modulates cocaine seeking via the LH whereas the interaction between the LPO and RMTg in the regulation of cocaine seeking is still unknown. One way to concretely determine if LPO activation modulates cocaine seeking, via projections to the VTA, would be to inject excitatory DREADDs into the LPO and then microinject CNO into the VTA thus activating only projections from the LPO to the VTA (Mahler et al., 2014; Vazey and Aston-Jones, 2014). However, that experiment is beyond the scope of the present dissertation.

Stress, drug administration, and drug related cues are all capable of reinstating drug seeking (Shalev et al., 2002) (Bossert et al., 2013). Furthermore, three circuits have been

reported to regulate reinstatement of drug seeking in response to these three stimuli (McFarland and Kalivas, 2001; Mantsch et al., 2016). Where the LPO fits in this neural circuitry is less clear. Given that the LPO modulates the activity of VTA dopamine neurons and that changes in activity of mesolimbic dopamine circuit modulate drug induced reinstatement of seeking (Anderson et al., 2003; Bachtell et al., 2005; Schmidt et al., 2005), the most parsimonious explanation is that the LPO is part of the drug induced reinstatement circuitry. However, stressors also modulate activity of mesolimbic dopamine neurons (Shaham and Stewart, 1995; Cabib and Puglisi-Allegra, 1996; Shaham and Stewart, 1996), and given the LPO projects to both the LH and RMTg (Yetnikoff et al., 2015), two brain regions implicated in responding to aversive events (Jhou et al., 2009a; Hikosaka, 2010), it is also possible that the LPO is involved in stress induced reinstatement of cocaine seeking. To the best of our knowledge no one has determined if the LPO is involved in response to stress or the regulation of the hypothalamic-pituitary axis, although the adjacent medial preoptic area is at least indirectly involved via regulation of gonadal hormones (Viau, 2002; Williamson and Viau, 2008). In order to fully address if activation of the LPO is reinforcing or aversive future experiments would have to be conducted.

While the results of the present study convincingly demonstrate the activation the LPO modulates cocaine seeking, there are a few limitations that should be discussed.
It is possible that results observed as a function of pharmacological manipulation of the LPO may be due to overflow into the ventral pallidum or the lateral hypothalamus, brain

regions that boarder the LPO and have been implicated in behavioral response to cocaine (Harris et al., 2005; Tang et al., 2005; Mahler and Aston-Jones, 2012; Mahler et al., 2014). To address this possibility we replicated the reinstatement study using chemogenetic excitation of the LPO by microinjecting an excitatory DREADD. The virus that contains the DREADD was conjugated with a green fluorescent protein (GFP) reporter gene. The expression of GFP allowed us to determine if DREADD expression was limited LPO and then include only those animals in analysis. Thus while some of the pharmacology results might be due to stimulation of the ventral pallidum or lateral hypothalamus the DREADD experiment demonstrates that excitation of the LPO is sufficient to reinstate cocaine seeking.

Animals in the DREADD study never extinguished, in terms of number of active responses, to the level of the animals in the bicuculline study that underwent a similar extinction paradigm. However, subjects in the DREADD experiment displayed, on average, a 75% decreases in responding on the active lever over the course of extinction. The administration of the DREADD alone is unlikely the cause of this difference as control animals in the DREADD study also displayed resistance to extinction. The difference in extinction responding between studies may be due to the partial reinforcement extinction effect (Domjan, 2010). The partial reinforcement extinction effect occurs when animals trained under intermittent reinforcement exhibit slower and less robust extinction than animals trained under a continuous reinforcement paradigm (Chen and Amsel, 1980). Since the DREADD animals were trained on an FR5 schedule

whereas the bicuculline animals were trained on an FR1 schedule, and training on intermitted FR schedules increases resistance to extinction after cocaine self-administration (Valles et al., 2006), it is likely that differences in responding during extinction were due to differences reinforcement schedules during training.

Finally, bicuculline induced activation of the LPO did not promote locomotion as it has previously been shown to in the past (Lavezzi et al., 2015). It is unlikely that this difference the result of a dose effect as the dose of bicuculline was the same between studies. However, in (Lavezzi et al., 2015) locomotion was tested in an open field where as locomotion was assessed with three photo beams in a self-administration apparatus during a test of reinstatement of seeking. Thus it is possible that the target stimuli, active hole, biased locomotor responding; animals spent more time around the active hole than exploring the small box. Additionally, by the time locomotion was measured in this study, subjects had at least eleven exposures to the self-administration chamber.

In conclusion the present results demonstrate that unilateral activation of the LPO reinstates cocaine seeking following either an extended period of extinction or home cage withdrawal and a single extinction session. Similar results were observed with both pharmacological and chemogenetic activation of the LPO suggesting these results are not due to activation of adjacent structures such as the lateral hypothalamus or ventral pallidum. Taken together with previous anatomical data collect by our lab it is likely the

case that the LPO sends inhibitory projections to VTA GABA neurons which in turn disinhibits VTA dopamine neurons ultimately leading to reinstatement behavior.

Chapter 5: General discussion

5.1 Overview of results

The results presented in this dissertation expand on previous work by demonstrating the preoptic area modulates of the behavioral and neurological response to cocaine. Previous studies have demonstrated that the medial preoptic area (mPOA) modulates the behavioral and neuronal response to cocaine in female rats (Tobiansky et al., 2013b; Tobiansky et al., 2015). Here we expand upon those results and show that mPOA facilitation of cocaine-induced dopamine levels in the nucleus accumbens (NAc) is estrogen sensitive. Specifically, microinjections of estradiol directly into the mPOA one day prior to systemic cocaine administration facilitate cocaine-induced dopamine in the NAc. Given that unilateral lesions of the mPOA have a similar effect (Tobiansky et al., 2015) it is likely the case that estradiol in the mPOA is decreasing activity of the mPOA and/or the projections from the mPOA to the mesolimbic dopamine system (Simerly and Swanson, 1988; Ikemoto and Bonci, 2014). These results are also in line with clinical and preclinical findings that estradiol enhances the response to cocaine in females (Lynch et al., 2001; Becker and Hu, 2008; Bobzean et al., 2014; Becker and Koob, 2016). Additionally, the results presented here demonstrate the role the mPOA plays in the modulation of the behavioral response to cocaine is not restricted to females. Bilateral lesions of the mPOA increased both cocaine-induced locomotion and cocaine-induced conditioned place preference (CPP) in male rats. These results are similar to the previous CPP results reported in females whereby radiofrequency lesions of the mPOA increased cocaine-induced CPP (Tobiansky et al., 2013b). While the effects of mPOA lesions on

locomotion were not previously reported in females, locomotion was measured but not reported in a cocaine free condition during the CPP post-test. Females with radiofrequency lesions of the mPOA had lower locomotion during the CPP post-test than sham operated subjects ($t_{(62.994)} = 4.36$, $p < 0.01$, $r^2 = .23$)(unpublished analysis). Even though these two studies are not directly comparable for several reasons, different measure of locomotion and conducted at different times, the similarities are worth considering. The present dissertation also examined how the adjacent lateral preoptic area (LPO) modulated cocaine-induced behavioral responding in male rats. Activation of the LPO with either pharmacology or chemogenetics facilitated reinstatement of cocaine seeking: an analog to relapse in humans (Shalev et al., 2002; Bossert et al., 2005). However, pharmacological activation of the LPO did not modulate responding during cocaine-self administration regardless of response requirement. Taken together these results demonstrate the preoptic area, as a whole, is involved in the behavioral response to cocaine.

5.2 THE REGULATION OF COCAINE REWARD BY THE MEDIAL PREOPTIC AREA AND LATERAL PREOPTIC AREA: SIMILARITIES AND DIFFERENCES

The present dissertation examined how different regions of the preoptic area modulate the behavioral and neuronal response to cocaine. These results extend a degree of functional similarity to regions that appear more different than similar, at least as far as regulation of behavior is concerned. The mPOA is involved in the regulation of male sexual behavior (Hull and Dominguez, 2006; Hull and Dominguez, 2012) as well as female sexual behavior: though to a lesser extent (Graham and Pfaus, 2013; Pfaus et al., 2015).

Maternal care is also regulated by the mPOA (Numan et al., 1977; Stolzenberg and Numan, 2011). Given that gonadal hormones act in the mPOA to modulate these three behaviors it is not surprising that the mPOA also modulates activity of hypothalamic–pituitary–gonadal (HPG) axis. Specifically, the mPOA contains neurons that express gonadotropin releasing hormone (GnRH)(Caligioni et al., 2007), lesions of the mPOA disrupt the HPG axis (Bravo et al., 1987; Ma et al., 1990), the mPOA projects to median eminence (Conrad and Pfaff, 1976; Yin and Gore, 2010) the site where GnRH modulates the HPG axis (Gore, 2008), and inhibition of the mPOA with muscimol attenuates the release of GnRH (Tomaszewska-Zaremba et al., 2002). The mPOA has also been implicated in the regulation of feeding behavior as microinjection of galanin enhance food intake (Patterson et al., 2006). In addition to modulation of hormonally sensitive and rewarding behaviors the mPOA is also involved in the regulation of thermoregulation (Roberts and Martin, 1977), locomotor behavior (Osborne et al., 1993; Sinnamon, 1993; Reynolds et al., 2006), and slow wave sleep (Kaushik et al., 2011). It is in these last two behaviors where there is functional overlap between the mPOA and LPO. Specifically, the LPO also modulates locomotion (Swerdlow and Koob, 1985; Sinnamon, 1993; Lavezzi et al., 2015) and the ventral portion of the LPO has been intensely studied for its role in the regulation of REM sleep (Swerdlow and Koob, 1985; Alam et al., 2014). The only other behavior the LPO has been implicated in, prior to the study presented above, is osmoregulation/thirst (Osaka et al., 1993; Saad et al., 1996). Thus the major functional differences between the mPOA and LPO are that the mPOA is sexually dimorphic and well suited to modulate sex differences in reinforcing behaviors whereas there is little

behavioral evidence, prior to the results presented within, that the LPO modulates rewarding behaviors.

Both the mPOA and the LPO appear to be well suited to modulate rewarding behavior based on neural circuitry. Specifically, there is ample evidence that both structure project to the ventral tegmental area (VTA)(Ikemoto and Bonci, 2014; Kallo et al., 2015; Yetnikoff et al., 2015), the source of dopamine for the mesolimbic dopamine system (Volkow and Morales, 2015). Of the projections the VTA receives from other brain regions, the LPO accounts for approximately 5.24% of these projections, whereas the mPOA accounts for approximately 2.68% of these projections (Yetnikoff et al., 2015). Both the mPOA and LPO contain cells that express glutamate, GABA, and neruotensin (Geisler and Zahm, 2006; Kallo et al., 2015). Anatomically, there does not seem to be a discernable boundary between the two structures, although it would be possible to histologically delineate the mPOA from the LPO given that galanin and estrogen receptor alpha are predominantly found within the mPOA (Cheung et al., 2001; Perez et al., 2003).

Activation of the LPO with optical or pharmacological stimulation robustly decreases the firing rate of GABA neurons in the VTA while increasing the firing rate of VTA dopamine neurons (Will et al., 2015). Optical stimulation of fibers that project to the VTA from the LPO confirmed that these changes in firing rate could be sufficiently explained by monosynaptic projections (Will et al., 2015). Based on these observations

and the behavioral results above, the most persimmons LPO-VTA circuitry would be that the LPO sends inhibitory (GABA) projections to VTA GABA-interneurons, which project to VTA dopamine neurons (Johnson and North, 1992). Thus stimulation of the LPO would disinhibit VTA dopamine neurons. This hypothesis is in line with behavioral data presented above as activation of the mesolimbic dopamine system potentiates reinstatement of drug seeking (Stewart, 1984; Bossert et al., 2005; Bossert et al., 2013). However, it is worth noting projections from the LPO to VTA are an equal mix of glutamate and GABA containing neurons (Kallo et al., 2015). Thus it is possible that glutamate neurons project to VTA dopamine neurons or that GABA and glutamate LPO neurons project to VTA GABA and dopamine neurons in a heterogeneous manner where the net effect of LPO stimulation is an increase in VTA dopamine activity. To further complicate this circuit, it is also worth noting that the LPO sends neruotensin projections to VTA neurons (Zahm et al., 2001; Geisler and Zahm, 2006) and neruotensin increases activity of VTA neurons (Seutin et al., 1989; Farkas et al., 1996). Based on the available data, stimulation of the LPO enhances activity of VTA dopamine neurons. I am speculating in this dissertation that is accomplished via inhibitory projections from the LPO to VTA GABA neurons; however the exact neurochemical profile of this circuit is a matter of on going research.

The mPOA also projects to the VTA. However, given the examination of this circuit has been predominantly restricted to tract tracing and immunohistological colocalization studies, the manner in which the mPOA modulates activity of the VTA is less clear. The

mPOA sends monosynaptic projections to the VTA (Simerly and Swanson, 1988; Ikemoto and Bonci, 2014), but also sends projections to the rostromedial tegmental nucleus (RMTg) (Jhou et al., 2009b; Tobiansky et al., 2013b); a GABAergic nucleus that modulates the activity of VTA dopamine neurons (Jhou et al., 2009a; Barrot et al., 2012). Given that the mPOA has been reported to be a primarily GABAergic brain region (Tsuneoka et al., 2013; Zhao et al., 2013), it stand to reason that projections form the mPOA to the VTA should be primarily GABAergic. In female rats, approximately 67% of mPOA-VTA neurons contain immunohistochemical markers for GABA (Tobiansky et al., 2013b). Based on the results described here, it seems likely that the mPOA sends inhibitory projections to the VTA that inhibit the activity of VTA dopamine neurons. Thus lesions of the mPOA would disinhibit VTA dopamine neurons, thereby facilitating the behavioral and neurological response to cocaine.

This circuit would be inline with an electrophysiology study of mPOA-VTA projections that demonstrated mPOA activation decreased activity of VTA neurons (Maeda and Mogenson, 1980). However, it is worth noting the phenotype of VTA neurons was not reported in this study and to the best of my knowledge this is the only electrophysiology study examining mPOA-VTA projections. There are a few caveats to this circuit that are worth discussing. First, as with the LPO, the neurons that project to the VTA express GABA, glutamate, or other neurotransmitters/peptides (Kallo et al., 2015). While, the GABA/glutamate ratio in the LPO as approximately equal, in the mPOA-VTA glutamate projection out number GABA projections 3:1 in male rats (Kallo et al., 2015). While

these differences could be attributed to mechanistic sex difference (Loyd and Murphy, 2006; McCarthy et al., 2012; Bangasser and Valentino, 2014), it is important to note these sex differences were observed in different studies using different methods of GABA detection; immunohistochemistry for GABA (Tobiansky et al., 2013b) vs. *in situ* hybridization for GAD65 (Kallo et al., 2015). However, the distribution of GAD65 and GAD67 is similar in the hypothalamus (Urbanski et al., 1998) suggesting the observed differences are not due to probe selection. If mPOA-VTA neurons contain primarily glutamate then we would expect the mPOA neurons to project to VTA GABA neurons based on the behavioral and neurochemical data presented in this dissertation. It is also possible that there are sex differences in projection targets, whereby there are glutamatergic projections to VTA GABA neurons in males and GABAergic projections to VTA dopamine in females. However, this is unlikely given mPOA neurons that project to the VTA in females appose GABA and dopamine neurons in approximately equal number (Tobiansky et al., 2015). Finally, it is also possible that the mPOA indirectly influences activity of the mesolimbic system via projections to the RMTg. Excitatory projections from the mPOA to the RMTg would increase inhibition of VTA dopamine and lesions of the mPOA would remove this circuit. We are currently unsure of the exact mechanism through which the mPOA and VTA interact, but given the behavioral and neurochemical results presented here and by Dr. Tobiansky (Tobiansky et al., 2013b; Tobiansky et al., 2015) it seems likely that the mPOA inhibits VTA dopamine activity.

The mPOA and LPO both project to the VTA and manipulation of both structures impact the behavioral response to cocaine. However, these conclusions are based on independent studies using different behavioral measures: a Pavlovian task for the mPOA and an operant task for the LPO. Therefore it would be interesting to examine the role of the mPOA and LPO simultaneously on the same behavioral measure of response to cocaine. It is also possible that there may be some degree of interconnection between the LPO and mPOA, as tract-tracing studies suggest the mPOA sends fibers through the LPO to the medial forebrain bundle (Simerly and Swanson, 1988). Whether or not some of these fibers form synapses in the LPO is currently unclear. Thus future studies detailing anatomical interactions between the mPOA and LPO would expand upon the results presented here, as would studies examining LPO and mPOA manipulation on the same behavioral outcome.

In conclusion, it is not surprising that both the mPOA and LPO modulate response to cocaine, given that activity of the mesolimbic dopamine system strongly correlates with both drug and natural reward (Di Chiara and Imperato, 1988; Wise, 2002, 2004). While the mPOA is more heavily implicated in the regulation of natural reward, both regions send ample projections to the mesolimbic dopamine system by way of the VTA. Although the exact profile of these circuits is still being actively examined, the behavioral data suggests opposing roles for the LPO and mPOA as activation of the LPO, or removal of the mPOA potentiates the behavioral response to cocaine.

5.3 THE MPOA AND SEX DIFFERENCES IN RESPONSE TO COCAINE

The mPOA is a likely candidate to modulate sex differences in cocaine response given the role of the mPOA in the regulation of sexually dimorphic natural rewards and the concentration of gonadal hormone receptors within (Simerly et al., 1990; Stolzenberg and Numan, 2011; Hull and Dominguez, 2012). Females are more sensitive to drugs of abuse such as cocaine, and this sensitivity is modulated in part by gonadal hormones (Becker and Hu, 2008; Becker and Koob, 2016). Specifically, estradiol potentiates the behavioral and neuronal response to cocaine whereas progesterone attenuates these responses (Hu et al., 2004; Jackson et al., 2006; Zhang et al., 2008; Quinones-Jenab and Jenab, 2010). There is limited expression of estrogen receptors in the mesolimbic system suggesting gonadal hormones may act elsewhere to modulate response to cocaine (Perez et al., 2003). Here we demonstrated that estradiol acts in the mPOA to facilitate neuronal response to cocaine, as estradiol microinjections into the mPOA enhanced cocaine-induced dopamine in the NAc. Based on this data it seems as though the mPOA may be involved in the modulation of sex differences in response to cocaine. However, data presented here also demonstrates the mPOA modulates the behavioral response to cocaine in male rats in a manner strikingly similar to that observed in female rats (Tobiansky et al., 2013b). Thus, clarification is need as to the role the mPOA plays in sex dependent response to cocaine.

It is possible that estrogens also act in the mPOA of male rats to modulate response to cocaine. Estrogens are colloquially considered to be a female typical hormone, however

males are also sensitive to the physiological effects of estrogens such as estradiol (Sharpe, 1998) and produce estradiol in the gonads and adrenal (Gillies and McArthur, 2010). Males also derive estradiol locally, in tissue, from testosterone, as testosterone is metabolized into estradiol by aromatase (Simpson et al., 2002; Bulun et al., 2004). Aromatase is expressed throughout the hypothalamus and in the mPOA in male rodents suggesting that estradiol may regulate the activity of the mPOA in males (Wagner and Morrell, 1997; Lephart et al., 2001). It is possible that estradiol merely acts in the mPOA in an organizational manner to promote survival of neurons during a developmentally critical period (Dohler et al., 1984; Dohler et al., 1986). However, one-year-old transgenic aromatase knockout mice exhibit reduced number of tyrosine hydroxylase containing cells as well as an increase in pro-apoptotic gene expression in the mPOA (Hill et al., 2004), and eight week old aromatase knockout mice exhibited impaired copulation (Robertson et al., 2001). Taken together these studies suggest estradiol affects the mPOA throughout the life cycle. Furthermore estradiol modulates rodent sexual behavior when administered after puberty. Specifically, castrated rats that received systemic estradiol replacement demonstrated an increase in but not a complete restoration of reproductive behavior (Putnam et al., 2003), whereas non-copulating rats that received estradiol implants in the mPOA exhibited the full range of male sexual behavior sixteen weeks after implant insertion (Antonio-Cabrera and Paredes, 2014). Similar results are observed in Japanese quail where systemic estradiol restores some aspects of male sexual behavior (Schumacher et al., 1983; Watson and Adkins-Regan, 1989). Finally, estradiol administration increases basal extracellular dopamine levels in the mPOA but does not

promote female invoked dopamine release (Putnam et al., 2003). Taken together these studies demonstrate that estradiol acts in the mPOA to modulate the behavioral and neurological response to a natural reward. Thus it is possible that estradiol may also modulate the activity of the mesolimbic circuit in males by acting in the mPOA. However, this is not likely the case, as hormonal manipulation of male rats does not alter the behavioral response to cocaine (Russo et al., 2003a; Hu et al., 2004; Jackson et al., 2006; Minerly et al., 2008). In either case an experiment directly comparing the role of estradiol in hormonally controlled males and females would be needed to definitively address this question.

Another possibility is that there are mechanistic sex differences in a circuitry through which the mPOA modulates the activity of mesolimbic system with the behavioral end point being the same. There are clear cases of neural sex differences directly modulating behavior, but most of these definitive examples are limited to structural differences in hindbrain regions that innervate muscles used for courtship or reproductive behaviors (Nottebohm and Arnold, 1976; Breedlove and Arnold, 1980; Kelley et al., 1988; Wade, 1998). However, there are many cases where sex differences in nervous system do not translate into overt functional sex differences in behavior. For example the sexual dimorphic nucleus (SDN) of the mPOA is five-seven times larger in males than it is in females (Gorski et al., 1978; Arendash and Gorski, 1983). Given the mPOA has been heavily implicated in male reproductive behavior across taxa (Hull et al., 2006) it seems logical that sex differences in the SDN my potentiate male reproductive behavior, yet

lesions of the SDN effect reproductive behavior only in sexually naïve rats suggesting a more nuanced role in behavior (Arendash and Gorski, 1983; De Jonge et al., 1989). Both male and female prairie voles exhibit parental behavior with few discernable differences save female nursing behavior (Thomas and Birney, 1979; Lonstein and De Vries, 1999). In females hormonal changes during pregnancy alter brain activity to precipitate paternal care (Lonstein et al., 2002) whereas vasopressin signaling in the lateral septum has been implicated in the initiation of parental care in males but not females (Bamshad et al., 1994; Wang et al., 1994). Thus similar behavioral results are observed via activation of different neural circuitry. In humans there exist sex differences in how the brain processes language (Shaywitz et al., 1995), object identification (Grabowski et al., 2003), as well as memory and emotion perception (Piefke et al., 2005). However, in none of these cases did sex effect behavioral performance on the studied tasks, suggesting that in some cases neural sex differences may serve to compensate for biological sex differences by bring males and females to functionally the same behavioral endpoint (De Vries, 2004) (de Vries and Sodersten, 2009).

If the mPOA does modulate the activity of the mesolimbic systemic in a sexually dimorphic yet functionally similar manner, then opioid signaling in the mPOA may be a candidate for males. The mPOA contains a large number of cells that express mu and kappa opioid peptides as well as receptors suggesting opioid signaling modulates activity of the mPOA (Le Merrer et al., 2009). Administration of opioid receptor agonists into the mPOA inhibit male sexual behavior (Matuszewich et al., 1995; van Furth et al., 1995;

Paredes, 2014) whereas blocking opioid receptors in the mPOA enhances male sexual behavior in some cases (Hughes et al., 1987; van Furth et al., 1995) but not others (Agmo and Paredes, 1988). Given that opioid receptors are G_i/G_o coupled protein receptors, activation of opioid receptors decrease neuronal activity (Waldhoer et al., 2004; Al-Hasani and Bruchas, 2011). Thus opioid acting in the mPOA should inhibit activity of neurons.

In order to better address these issues further experiments are needed, as all of the above discussion is ultimately speculative. The conclusion that the mPOA may be serving similar behavioral role in males in females is based on two lesion studies demonstrating mPOA lesions increases cocaine-induced CPP in both males and females. While the pattern of results is similar between studies, different individuals at different time points obtained these results. Thus more nuanced sex differences may arise if a direct sex comparison is performed. However, given the role of systemic estrogens in the behavioral response to cocaine (Russo et al., 2003b; Jackson et al., 2006) and present finding that estradiol administration into the mPOA enhances the neuronal response to cocaine, careful consideration would need to be given to the hormonal state of the subjects in any sex difference comparison. This is especially true for females, as lesions of the mPOA disrupt the estrous cycle in a highly variable manner (Nance et al., 1977; Ma et al., 1990). Nevertheless, a true behavioral comparison of the mPOA's role in the regulation of the behavioral response to cocaine is needed to determine if functional sex differences exist.

This information would inform future studies probing potential structural sex difference in mPOA regulation of the mesolimbic circuit.

5.4 CONCLUSION

The data presented in this dissertation demonstrates that the preoptic are modulates the behavioral response to cocaine. Within in the preoptic area estradiol acts in the mPOA to facilitate cocaine-induced dopamine in the NAc of female rats. These results provide a mechanism for neuronal and behavioral results obtained via mPOA lesions in previous studies (Tobiansky et al., 2013b; Tobiansky et al., 2015). The mPOA was also shown to play a role in the behavioral response to cocaine in male rats, as lesions of the mPOA increased both cocaine-induced locomotion and CPP, two behaviors regulated by the mesolimbic dopamine system. As with the mPOA, the adjacent LPO also sends projections to VTA, in fact the LPO sends more than twice as many neural projections to the VTA (Yetnikoff et al., 2015). These projections appear to be functionally relevant as stimulation of the LPO via pharmacology or chemogentics potentiated reinstatement of cocaine seeking. The data presented here expands upon the role of the preoptic area as a modulator of rewarding behaviors as well as providing evidence for an expanded neural circuit of brain reward. It is my sincere hope that others will expand on this work to elucidate the circuitry through which the preoptic area modulates activity of the mesolimbic system that ultimately modulates the behaviors described here.

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