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Sexual Reward and Depression

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SEXUAL REWARD AND DEPRESSION

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ABSTRACT

Sexual behavior in male rats is a complex rewarding behavior and many neurotransmitters and neuropeptides play an important role in mediation of sexual performance, motivation and reward. The hypothalamic neuropeptide orexin has been shown play a key role in reward associated with food and drugs of abuse, but the role of this neuropeptide in control of sexual performance, motivation and reward is currently unclear. First, it was shown that orexin neurons in the hypothalamus are activated during sexual performance and reward. Next, using cell specific lesions of orexin neurons it was demonstrated that orexin is involved in arousal and anxiety, but is not critical for sexual performance or motivation. Moreover, orexin was shown to play a critical role in control of sexual reward. Thus, these studies provided novel information regarding a role for orexin in this natural reward behavior. Recent studies have shown that sexual behavior in male rats causes neuroplasticity in the mesolimbic system and enhanced psychostimulant-induced locomotor activity and drug craving. The latter of which is dependent on a period of abstinence from sexual behavior, suggesting an increased vulnerability for addiction following loss of sexual reward. Thus, the next goal of this thesis determined if abstinence from sexual behavior also leads increased vulnerability for other disorders related to reward processing, specifically depression-like behavior. It was demonstrated that a prolonged (28 day) but not short (1 or 7 day) period of abstinence causes depression-like behavior in sexually experienced male rats seen as increased passive stress coping behaviors and anhedonia. Development of depression-like behavior was associated with increased levels of corticotropin releasing factor mRNA in the paraventricular nucleus of the hypothalamus and increased hypothalamic-pituitary-

adrenal axis activity in response to an acute stressor. Thus, these studies provide novel information on behavioral and neuroplastic alterations observed following a prolonged period of abstinence from mating and suggest that loss of sexual reward in male rats may be a paradigm to study depression following loss of social reward in humans.

Keywords: orexin, hypocretin, sexual behavior, neural activation, motivation, reward, hypothalamus, conditioned placed preference, anxiety, depression, abstinence, passive stress coping, anhedonia, corticotropin releasing factor, hypothalamic-pituitary adrenal axis, corticosterone, adrenocorticotropic hormone

CO-AUTHORSHIP

Sections 1.2 -1.5 of Chapter 1 entitled “Orexin and Natural Reward: Feeding, Maternal and Male Sexual Behavior” was written by Andrea R. Di Sebastiano with inputs by Lique M. Coolen. Chapter 2 entitled “Orexin mediates initiation of sexual behavior in sexually naive male rats, but is not critical for sexual performance.” was written by Andrea R. Di Sebastiano with inputs by Lique M. Coolen, and Michael N. Lehman. Study design was by Andrea R. Di Sebastiano and Lique M. Coolen, with intellectual inputs from Michael N. Lehman. Experimental procedures and data analysis were performed by Andrea R. Di Sebastiano, Sabrina Yong-Yow and Lauren Wagner. Chapter 3 entitled “Lesions of orexin neurons block conditioned place preference for sexual behavior in male rats” was written by Andrea R. Di Sebastiano with inputs by Dr. Lique M. Coolen and Michael N. Lehman. Study design was by Andrea R. Di Sebastiano and Lique M. Coolen, with intellectual inputs from Michael N. Lehman. Experimental procedures and data analysis were performed was by Andrea R. Di Sebastiano and Hilary E. Wilson-Perez. Chapter 4 entitled “Loss of sexual reward causes depression-like behavior in male rats” was written by Andrea R. Di Sebastiano with inputs by Dr. Lique M. Coolen, experimental procedures and data analysis were performed was by Andrea R. Di Sebastiano. Drs. Michael N. Lehman, Steven R. Laviolette, and Lique Coolen provided intellectual input. Chapter 5 entitled “Loss of Sexual Reward Causes Enhanced Stress Reactivity in Male Rats” was written by Andrea R. Di Sebastiano with inputs by Dr. Lique M. Coolen, experimental procedures and data analysis were performed was by Andrea R. Di Sebastiano. Drs. Michael N. Lehman, Steven R. Laviolette, James P. Herman and Lique M. Coolen provided intellectual input.

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LIST OF ABBREVIATIONS

5HT, serotonin

8-OH-DPAT, 8-Hydroxy-N,N-dipropyl-2-aminotetralin

ABC, avidin-biotin–horseradish peroxidase complex

ACTH, adrenocorticotropic hormone

AD, antidepressant

AF, anestrous female

AMPA, 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid

BDNF, brain derived neurotrophic factor

BLA, basolateral amygdala

BnST, bed nucleus of the stria terminalis

CeA, central amygdala

CPA, conditioned place aversion

CPP, conditioned place preference

CRF, corticotropin releasing factor

CRFR1, corticotropin releasing factor receptor 1

CRFR2, corticotropin releasing factor receptor 2

cort, corticosterone

DAB, diaminobenzidene

DAMGO, D-Ala2, N-MePhe4,Gly-ol-enkephalin

DPX, dibutyl phthalate xylene

E, Ejaculation

EPM, elevated plus maze

EF, estrous female

Exp, experienced

GABA, gamma-aminobutyric acid

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

GR, glucocorticoid receptor

HPA, hypothalamic-pituitary-adrenal

ICV, Intracerebroventricular

IP, intraperitoneal

IM, intromission

IP, intraperitoneal

LiCl, lithium chloride

LHA, lateral hypothalamic area

MEA medial amygdala

MCH, melanocyte concentrating hormone

MDMA, 3,4-methylenedioxy-methamphetamine

MOR, mu-opioid receptor

M, mount

MeA, medial amygdala

mPFC, medial prefrontal cortex

mPOA, medial preoptic are

NAc, nucleus Accumbens

NMDA, N-methyl-D-aspartate

OxR1, orexin receptor 1

OxR2, orexin receptor 2

PB, phosphate buffer

PBS, phosphate buffered saline

PEI, post ejaculatory interval

PFA, paraformaldehyde

PFA-DMH , perifornical dorsomedial hypothalamus

PVN, paraventricular nucleus of the hypothamalus

qRT-PCR, quantitative real time polymerase chain reaction

SON, supraoptic nucleus

SPFp, subparafascicular thalamic nucleus

SSRI, selective serotonin reuptake inhibitor

TNF-alpha, tumor necrosis factor alpha

VTA, ventral tegmental area

CHAPTER 1: GENERAL INTRODUCTION

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1.1 Introduction – Part 1

1.1.1 Natural Reward Behaviors

An individual's behavior is altered by experience (Schultz, 2006). If a behavior is followed by reward, this will increase frequency of the behavior (i.e. reinforcement), however if a behavior is followed by punishment, frequency of the behavior will be decreased (Schultz, 2006). The brain system that is critical for mediation of reward behaviors is the mesolimbic system, which consists of interconnected brain regions including the ventral tegmental area (VTA), nucleus accumbens (NAc) and medial prefrontal cortex (mPFC) (Morgane et al., 2005). The mesolimbic system is important for mediation of natural rewards including feeding (Hernandez and Hoebel, 1988, Martel and Fantino, 1996, Avena et al., 2008, Vucetic and Reyes, 2010), drinking (Yoshida et al., 1992), maternal behavior (Numan, 2007), social bonding (Young et al., 2001, Young et al., 2011) and sexual behavior (Pfaus et al., 1990a, Balfour et al., 2004, Frohmader et al., 2010, Pitchers et al., 2010).

There is great interest in understanding the neurobiology of natural reward behaviors and how these behaviors change the brain to alter subsequent behavior. As such, studies in this thesis will examine the neurobiology of sexual behavior and neuroplasticity related to sexual behavior, an extremely motivated and rewarding behavior in mammals (Paredes, 2009).

1.1.2. Male Rat Sexual Behavior

The model animal for studies in this thesis is the male rat. In male rats, sexual behavior is a complex interaction which involves pursuit and investigation of the female,

mounts, intromissions, culminating in ejaculation (Hull et al., 2002). Following investigation the male will mount the female and perform rapid pelvic thrusts, without penetration (Agmo, 1997). An intromission begins with a mount and continues until the male performs a deeper pelvic thrust that results in vaginal penetration (Agmo, 1997). Culmination of this sexual activity is ejaculation, which in turn is followed by a 4-7 minute period of sexual inactivity, the post-ejaculatory interval, after which the male reinitiates sexual behavior. Male rats will perform numerous copulatory series prior to reaching sexual satiety (Agmo, 1997).

1.1.3 Sexual Motivation and Performance

In male rats, approach and investigation of the receptive female is indicative of sexual motivation. Latencies to first mount and intromission are also considered to be measures of sexual motivation. Sexual behavior is highly motivating in male rats, as males spend increased time investigating a receptive female presented behind a barrier vs. a nonreceptive female, show increased speeds to reach a sexually receptive female in a straight runway test (Lopez et al., 1999) and change levels more frequently in the bilevel chamber test in search of a sexually receptive female (Mendelson and Pfaus, 1989). Males also show decreased latencies to mount and intromission with repeated sexual experience, indicating increased sexual motivation (Pfaus et al., 1990b, Agmo, 1997). Sexual performance in male rats is indicated by the frequency of mounts and intromissions an animal performs in a copulatory series, and also by latencies to ejaculation (Agmo, 1997). Sexual performance is enhanced by sexual experience, as copulation efficiency is increased (increased numbers of intromissions relative to number

of mounts) (Agmo, 1997), and latencies to ejaculation are shortened (Pitchers et al., 2012). The neural systems mediating sexual motivation and performance begin with chemosensory input from the olfactory bulb that project to the medial amygdala (MeA), which sends these signals to the medial preoptic area (mPOA), both directly and indirectly, via the bed nucleus of the stria terminalis (BnST). Genitosensory inputs are relayed through the spinal cord to the subparafascicular thalamic nucleus (SPFp) which projects to the MeA and mPOA. Furthermore the mPOA, MeA, BnST and SPFp all show cFos activation following display of sexual behavior (Coolen et al., 1996, 1997b), indicating neural activation by mating. The mPOA is a major integrative site for control of sexual behavior and lesions of this brain region cause impairments in both sexual motivation (Paredes et al., 1993, Edwards et al., 1996) and performance (Larsson and Heimer, 1964, Arendash and Gorski, 1983).

1.1.4 Sexual Reward

In humans, ejaculation is associated with the pleasurable feeling of orgasm. Similarly in rats, ejaculation is associated with reward and is the most rewarding component of sexual behavior (Tenk et al., 2009). Sexual behavior in male rats has been shown to be a rewarding and reinforcing behavior as males will perform operant tasks, (Everitt et al., 1987, Everitt and Stacey, 1987) climb barriers (Sheffield et al., 1951) or cross electrified grids (Moss, 1924) to gain access to a sexually receptive female. Moreover, male rats facilitate their sexual performance with repeated sexual experience (Pitchers et al., 2012) and form a conditioned place preference for sexual behavior (Agmo and Berenfeld, 1990, Tenk et al., 2009). The mesolimbic system, which is critical

for mediation of reward, is activated during sexual behavior. It has been shown that both sexual behavior and exposure to cues associated with sexual reward lead to cFos activation in the NAc and increased cFos activation of dopamine neurons in the VTA (Balfour et al., 2004), suggesting an important role of these brain regions in mediation of sexual reward.

1.1.5. Neuropeptides in Sexual Motivation, Performance and Reward

As mentioned above, sexual behavior is a complex behavior that involves integration of many different brain regions. As such, there are also many neurotransmitters and neuropeptides involved in mediation of this behavior that will be discussed below.

Glutamate

Glutamate is the main excitatory neurotransmitter in the central nervous system and is released in the mPOA during sexual behavior (Dominguez et al., 2006). NMDA receptors also become activated in the mPOA following copulation (Dominguez et al., 2007). Both systemic (Powell et al., 2003) and intra-mPOA (Dominguez et al., 2007) administration of glutamate antagonists also have been shown to lead to impairments in sexual performance. Thus, glutamate appears to play a facilitatory role in sexual performance. Moreover, blockade of N-methyl-D-aspartate (NMDA) receptors decreased mating induced cFos as well as mating induced phosphorylation of the NR1 subunit of the NMDA receptor in the mPOA (Dominguez et al., 2007), suggesting that facilitatory effects of glutamate on sexual performance may be via NMDA receptors in the mPOA.

Dopamine

Dopamine has also been shown to play a role in sexual behavior. Systemically administered dopamine agonists facilitate male sexual performance (Ahlenius and Larsson, 1990, Pfaus and Phillips, 1991, Melis and Argiolas, 1995, Olivier et al., 2007), while systemic administration of dopamine antagonists impair sexual performance (Ahlenius and Larsson, 1990). Dopamine is released into the mPOA with presentation of a receptive female and remains elevated throughout display of sexual behavior (Hull et al., 1995), indicating a role for mPOA dopamine in sexual motivation and performance. Additionally, intra-mPOA administration of dopamine receptor antagonists has been shown to lead to impairments in both sexual motivation and performance (Pfaus and Phillips, 1991). During sexual behavior dopamine is also released into the NAc with presentation of a receptive female, and remains elevated during the entire display of copulation (Pfaus et al., 1990a, Pfaus and Phillips, 1991, Damsma et al., 1992, Wenkstern et al., 1993), suggesting dopamine may be important for mediation of sexual reward. However, there have been many studies demonstrating that dopamine neurotransmission does not appear to be critical for mating-induced conditioned place preference (Agmo and Berenfeld, 1990, Garcia Horsman and Paredes, 2004, Paredes and Agmo, 2004, Ismail et al., 2009), a well established model for measuring sexual reward (Tzschentke, 2007). Instead, it has been proposed that dopamine in the NAc may play a role in the prediction of reward upon presentation of a reward-associated cue (Schultz, 2006) or be important for mediation of incentive motivation for sexual behavior (Robinson and Berridge, 1993).

Endogenous Opioids

Opioids have been shown to be inhibitory to sexual performance as infusion of morphine, heroin and methadone all lead to impairments in sexual behavior (Pfaus and Gorzalka, 1987). Similarly, infusion of opioid peptides such as β-endorphin impairs sexual performance (Hughes et al., 1987). Infusion of μ-opioid receptor antagonists such as naloxone and natrexone facilitate sexual performance in sexually naïve (Pfaus and Wilkins, 1995) and sexually experienced male rats (Myers and Baum, 1979). However, there are also studies that have shown that opioids may have facilitative effects on sexual behavior. Increasing enkephalin levels ICV using enkephalinase inhibitors that block degradation of endogenous enkephalin, facilitated sexual behavior and decreased latency to ejaculate in male rats (Agmo et al., 1994), while intra-VTA morphine or dynorphin led to improvements in copulatory performance and increased dopamine release in the NAc (Mitchell and Stewart, 1990). Furthermore, opioid receptors are activated by sexual behavior as mating induces internalization of μ-opioid receptors in the mPOA (Coolen et al., 2004b) and VTA (Balfour et al., 2004). Endogenous opioids also appear to be critical for mediation of sexual motivation, as infusion of naloxone in the VTA prevented the increase in level changes in the bilevel chamber in anticipation of a sexual receptive female (van Furth and van Ree, 1996). Endogenous opioids have also been shown to be activated by sexual reward, as μ-opioid receptors in the VTA are activated by exposure to conditioned contextual cues associated with prior sexual reward (Balfour et al., 2004). Furthermore, opioids play a critical role in mediation of sexual reward as both systemic and intra-mPOA infusion of naloxone blocks development and expression sexual reward

induced conditioned place preference (Agmo and Berenfeld, 1990, Mehrara and Baum, 1990, Agmo and Gomez, 1993, Paredes and Martinez, 2001, Ismail et al., 2009).

Serotonin

Serotonin (5HT) is generally regarded as being inhibitory to sexual behavior (Hull et al., 2004) as a common side effect of selective serotonin reuptake inhibitor (SSRI) antidepressants, which increase extracellular 5HT release, is impaired sexual behavior in both humans (Rosen et al., 1999) and rats (Mos et al., 1999). There are fourteen known G-protein-coupled receptors for serotonin that are divided into seven classes; however only a few thus far have been shown to be related to sexual behavior. The inhibitory actions of 5HT appear to be mediated by the $5HT_{1B}$ and the $5HT_2$ receptors. Systemic or intracranial administration of a $5HT_{1B}$ (Fernandez-Guasti et al., 1989a, Fernandez-Guasti et al., 1992) or 5HT2 (Foreman et al., 1989, Watson and Gorzalka, 1991) agonists impair copulatory performance, while $5HT_2$ antagonists facilitate sexual behavior (Gonzalez et al., 1994). Furthermore, the mPOA and NAc appear to be brain regions important for 5HT's inhibitory effect on sexual behavior, as 5HT injected into these brain regions impairs copulatory performance (Verma et al., 1989, Fernandez-Guasti et al., 1992). Serotonin has also been shown to have facilitatory effects on sexual behavior and these effects have been shown to be mediated by the $5HT_{1A}$ receptor, as systemic (Ahlenius and Larsson, 1990, Haensel et al., 1991, Fernandez-Guasti et al., 1992, Coolen et al., 1997a), intra-mPOA (Fernandez-Guasti et al., 1992), intra-NAc (Matuszewich et al., 1999), and intra-MeA (de Castilhos et al., 2006) administration of the $5HT_{1A}$ agonist, 8-OH-DPAT facilitates sexual performance. There have been fewer studies on the role of 5HT in

sexual reward, however it has been shown that systemic administration of 3,4-methylenedioxy-methamphetamine (MDMA,"ecstasy") that leads to a 40-55% depletion of 5HT in brain regions such as the striatum, hypothalamus, cortex, hippocampus and NAc blocks formation of conditioned place preference for sexual behavior (Straiko et al., 2007), suggesting that serotonin may also play an important role in mediation of sexual reward.

Oxytocin

Oxytocin is a hypothalamic neuropeptide produced in the paraventricular nucleus of the hypothalamus (PVN) and supraoptic nucleus (SON) (Bargmann and Scharrer, 1951). Oxytocin is generally regarded as facilitatory to sexual performance as intra-PVN infusion of oxytocin stimulates penile erections (Kita et al., 2006), and systemic, ICV, or intra-mPOA administration of oxytocin facilitates sexual performance in male rats (Argiolas and Melis, 2005, Gil et al., 2011). Conversely, oxytocin antagonists have been shown to impair sexual performance (Argiolas and Melis, 2005). Furthermore, sexual interaction has been shown to increase cFos in oxytocin neurons of the PVN that have projections to the spinal cord and are involved in control of penile erection (Witt and Insel, 1994). Low levels of oxytocin mRNA in the PVN are associated with sexual impotence in rats (inability to copulate with a sexually receptive female) (Arletti et al., 1997), further supporting a role for oxytocin in sexual performance. Oxytocin may also play a role in sexual motivation and reward as oxytocin has been shown to facilitate sexually conditioned partner preference in male rats (Pfaus, 2009), and interaction with a sexually receptive female leads to oxytocin release in male rats (Hillegaart et al., 1998).

Furthermore, neutral odors associated with prior sexual reward have been shown to activate oxytocin in the PVN (Ménard et al., 2005).

Therefore, it is evident that many neurotransmitters and neuropeptides play an important role in sexual performance, motivation and reward. As such, studies in Chapters 2 and 3 of this thesis will focus on a more recently discovered neuropeptide, the hypothalamic neuropeptide, orexin. Orexin has been implicated to play a key role in reward associated with food and drugs, but the role of this neuropeptide in mediation of sexual performance, motivation or reward is currently unclear.

1.2 Orexin and Natural Reward Behavior: Feeding, Maternal and Male Sexual Behavior

Orexin-A and orexin-B, also known as hypocretin-1 and hypocretin-2, are hypothalamic neuropeptides produced exclusively in the perifornical dosomedial hypothalamus (PFA-DMH) and lateral hypothalamic area (LHA), that arise from the precursor peptide prepro-orexin (de Lecea et al., 1998, Sakurai et al., 1998). The orexins bind to two G-protein coupled receptors, orexin receptor 1 (OxR1) and orexin receptor 2 (OxR2), with OxR1 being selective for orexin-A and OxR2 being equally selective for both orexin-A and orexin-B (Sakurai et al., 1998). Orexin neurons project their axons broadly throughout the brain and spinal cord to regions important for arousal and sleep, feeding behavior, and reward and motivation (Peyron et al., 1998). OxR1 and OxR2 are also widely distributed throughout the brain and spinal cord (Trivedi et al., 1998, Marcus et al., 2001). The discovery of orexins was accompanied with great interest in orexin's role in arousal and sleep (Paredes et al., 1997, Chemelli et al., 1999, Lin et al., 1999,

Furlong and Carrive, 2007, Sakurai, 2007) and in feeding behavior (de Lecea et al., 1998, Sakurai et al., 1998, Sakurai, 2006). Orexin neuronal dysfunction leads to narcolepsy in mice (Chemelli et al., 1999), dogs (Lin et al., 1999) and humans (Siegel, 1999, Nishino et al., 2000, Thannickal et al., 2000), suggesting that orexin is critically involved in arousal and maintenance of waking state. Orexin neurons indeed project to arousal associate brain regions such as the locus coeruleus (Peyron et al., 1998). Furthermore, intracerebroventricular (ICV) administration of orexin-A and orexin-B causes increased food intake in rats, and fasting leads to upregulation of prepro-orexin mRNA (Sakurai et al., 1998). With a role established for orexin in feeding it was next hypothesized that these peptides are involved in mediation of reward and motivation. Indeed, there is now ample evidence that orexin plays a critical role in reward related to drugs of abuse and addiction behaviors.

1.3 Orexin, Feeding and Energy Homeostasis

Orexin neurons are localized in brain regions important for regulation of feeding behavior and energy homeostasis, the PFA-DMH and LHA (Elmquist et al., 1999). Furthermore ICV infusions of orexin-A and orexin-B stimulate food intake in rats and mice (Sakurai et al., 1998, Edwards et al., 1999). Thus orexin likely plays a role in feeding and energy balance. Indeed, human narcoleptics that have orexin neuron deficiency (Peyron et al., 2000, Thannickal et al., 2000) and decreased orexin-A levels in CSF (Nishino et al., 2000, Schuld et al., 2002, Scammell, 2003) show decreased food intake (Lammers et al., 1996) with an increased body mass index (Nishino et al., 2000, Schuld et al., 2000, Nishino et al., 2001), indicating metabolic abnormalities. Mice with

genetic ablation of orexin and that display narcoleptic symptoms have significantly decreased food intake, and eventually develop late-onset obesity (Hara et al., 2001), suggesting that orexin is an important mediator of energy homeostasis and metabolism. Additionally, increasing extracellular glucose and leptin levels leads to hyperpolarization of orexin neurons, while decreasing extracellular glucose and leptin levels leads to depolarization of orexin neurons, indicating that orexin neurons are glucose and leptin sensitive (Yamanaka et al., 2003). Orexin neurons are also sensitive to triglycerides, as intraperitoneal (IP) injection of lipids increases orexin mRNA levels in the hypothalamus (Chang et al., 2004) and are sensitive to dietary amino acids, as oral gavage of nutritionally relevant levels of amino acids leads to increased cFos expression in orexin neurons (Karnani et al., 2011). Furthermore, orexin has been suggested to act as a sensor for nutritional state as prepro-orexin mRNA is upregulated by fasting (Sakurai et al., 1998) and hypoglycemia (Griffond et al., 1999). The findings that orexin neurons respond to glucose, leptin, triglycerides and dietary amino acids further implicate a role for orexin in energy homeostasis. Orexin neurons are innervated by both the appetite suppressing peptide, proopiomelanocortin and the appetite stimulatory peptide neuropeptide-Y (Elias et al., 1998). Furthermore, ICV infusion of agouti related peptide, which is co-expressed in the same neurons as neuropeptide-Y and stimulates appetite, leads to increased cFos expression in orexin neurons (Zheng et al., 2002). Thus there is clear evidence that orexin interacts with the hypothalamic peptides that regulate food intake and energy homeostasis.

1.4 Orexin and Food Motivation and Reward

In addition to mediating energy homeostasis, orexin is an important mediator of motivation and reward associated with feeding. ICV orexin-A and orexin-B infusion stimulates feeding (Sakurai et al., 1998) and IP infusion of an OxR1 specific antagonist, SB-334867 reduces food intake in both male and female rats (Haynes et al., 2000). Moreover, IP pre-treatment with SB-334867 attenuates orexin-A induced feeding behavior, indicating the actions of orexin-A on feeding are via OrXR1 (Rodgers et al., 2001). Mice with a genetic ablation of orexin have decreased intake of food (Hara et al., 2001) and orexin knockout mice show decreased intake of sucrose (Matsuo et al., 2011). These effects of orexin or orexin receptor antagonism appear to be caused by alterations in motivation to eat or reward associated with feeding. Orexin-A stimulates feeding when infused into the nucleus accumbens shell (Thorpe and Kotz, 2005), an area important for goal-directed behaviors and hedonia related to feeding (Berridge, 1996, Zhang et al., 1998, Pecina and Berridge, 2000, Pecina et al., 2006). In self administration studies which assess motivation for food, animals learn to lever press to receive food reward. ICV administration of SB-334867 attenuates lever pressing on a fixed ratio schedule for food (Borgland et al., 2009), sucrose pellets (Thorpe et al., 2005) and high fat chocolate pellets (Nair et al., 2008). SB-334867 also attenuates the number of sucrose reinforcers obtained in a fixed ratio self administration paradigm in both ad libitum fed and food restricted rats (Cason et al., 2010). Studies using a progressive ratio self administration procedure in which the animal must press a lever progressively more times to obtain the same reinforcer are also used to examine the role of orexin in food motivation. Motivation in the progressive ratio procedure is determined by the maximum number of times an animal will lever press to receive the reinforcer, called the breakpoint

(Richardson and Roberts, 1996). The effects of orexin in the progressive ratio schedule appears to be dependent on the satiety state of the animal as intra-LHA administration of orexin-A increased breakpoint responding for sucrose pellets (Thorpe et al., 2005, Choi et al., 2010), and this breakpoint was increased further following 24 hours of food deprivation (Thorpe et al., 2005). Further supporting this, IP administration of SB-334867 decreased breakpoint for sucrose pellets in sated but not food-restricted rats (Espana et al., 2010). IP injection of SB-334867 also reduces consumption of high fat, palatable food in both a fixed ratio schedule (Nair et al., 2008, Choi et al., 2010) and a progressive ratio schedule (Borgland et al., 2009). The latter study also showed that the same dose of SB-334867 did not affect intake of normal chow, suggesting orexin-A is important for mediation of motivation for positive, highly salient reinforcers (Borgland et al., 2009). In mice, pharmacological blockade of OxR1 using SB-334867 or viral vector mediated knockdown of prepro-orexin decreases responding for food in a progressive ratio schedule (Sharf et al., 2010). In contrast, orexin knockout mice do not show any differences in responses for food on a progressive ratio schedule, suggesting developmental compensation in orexin knockout mice (Sharf et al., 2010). Another method of measuring food motivation is an effort based feeding paradigm in which rats are given a choice between exerting a small amount of effort to obtain regular food pellets, or jump over a barrier to receive high fat chocolate pellets in a T-maze. Rats will willingly jump the barrier to obtain the palatable food pellets (Salamone et al., 1994). Systemic or intra-VTA administration of SB-334867 significantly decreases number of entries into the high fat-containing arms (Borgland et al., 2009), demonstrating that OxR1 blockade decreases motivation for palatable food. Orexin is also important for cue-

induced reinstatement of sucrose seeking, as IP SB-334867 significantly attenuates lever pressing in reinstatement trials in food restricted, but not in ad libitum fed rats (Cason et al., 2010). Furthermore, SB -334867 pre-treatment also significantly attenuates yohimbine-induced reinstatement of sucrose seeking, further implicating a role for orexin-A and OxR1 in food seeking and motivation for palatable food (Richards et al., 2008).

Orexin is an important mediator of food motivation and is also important for the rewarding aspects of feeding behavior. Orexin neurons in the LHA show significant cFos induction following exposure to a conditioned place preference (CPP) apparatus they had previously learned to associate with food reward (Harris et al., 2005) and the degree of cFos induction is correlated with the degree of preference animals display for the food reward paired chamber, thus suggesting that orexin is activated by food reward associated conditioned cues (Harris et al., 2005). Orexin is also involved in reward based feeding behavior, a paradigm in which sated animals will over-consume a high fat diet. This is referred to as non-homeostatic feeding and in humans is thought to be a major contributor to the obesity epidemic (Berthoud, 2004). IP administration of SB-334867 in sated rats decreased intake of high fat diet (Choi et al., 2010) and IP administration of SB-334867 attenuates ghrelin enhanced conditioned place preference for high fat diet in ad libitum fed rats that were not sated (Perello et al., 2010). Finally, orexin-A expressing neurons are significantly activated in animals expecting a daily meal of regular chow or high fat chocolate (Choi et al., 2010), suggesting a role for orexin in food anticipation.

In summary, orexin clearly plays a role in mediating reward and motivation related to food intake. Outstanding questions remain regarding the areas in the brain

where orexin may be acting to mediate these effects, and the roles of orexin-B and OxR2 as studies described above primarily examine the role of orexin-A and OxR1 in food motivation and reward.

1.5 Orexin and other naturally rewarding behaviors

Even though a role for orexin in feeding behavior, food motivation and reward is well documented, a role for orexin in other naturally rewarding behaviors has not been extensively studied. Studies that examined orexin's role in the natural rewarding behaviors maternal behavior and male sexual behavior will be discussed below.

1.5.1 Orexin and maternal behavior

Orexin receptors are found in brain regions shown to be important for mediation of maternal behavior and aggression (Trivedi et al., 1998) including the paraventricular nucleus of the hypothalamus (Numan and Corodimas, 1985, Insel and Harbaugh, 1989, Giovenardi et al., 1997, 1998), nucleus accumbens (Hansen, 1994, Keer and Stern, 1999), medial preoptic area (Numan, 1974, Numan et al., 1977) and bed nucleus of the stria terminalis (Terkel et al., 1979). Additionally, it has been shown that lactating female rats have significantly higher levels of prepro-orexin and OxR1 mRNA in the lateral hypothalamus compared to pregnant females (Wang et al., 2003), suggesting orexin may be involved in maternal behavior. D'Anna and Gammie tested this hypothesis and show that an intermediate doses of orexin-A (0.06 – 1 μg) ICV increases licking and grooming behavior of the mothers towards the pups, and increases numbers of nursing bouts. In addition, this intermediate dose of orexin-A induces cFos activation in brain areas

involved in maternal behavior including the ventral tegmental area, bed nucleus of the stria terminalis, central and medial amygdala (D'Anna and Gammie, 2006). In contrast, a higher dose of ICV orexin-A (3µg) impairs maternal behavior by delaying latency to nurse, decreasing total time nursing and increasing time away from the nest (D'Anna and Gammie, 2006). Furthermore, this higher dose of orexin-A also decreases maternal aggression in the mothers towards an intruder male. However, IP injection of SB-334867 (10 or 30 mg/kg), does not significantly affect any aspect of maternal behavior or aggression, although there were trends towards impaired maternal care (D'Anna and Gammie, 2006). These findings suggest that intermediate levels of orexin-A may be important for maintaining proper maternal behavior such as licking and grooming, however, when levels of orexin are too high maternal care is impaired. Future studies are needed to further elucidate the role of orexin in maternal behavior and aggression, in particular to determine where in the brain orexin may be acting to mediate maternal behavior and aggression.

1.5.2 Orexin and sexual performance & motivation

Several studies have demonstrated a role for orexin in sexual performance, reward and motivation. It has been long known that electrical stimulation of the lateral hypothalamus facilitates sexual behavior in male rats (Vaughan and Fisher, 1962, Caggiula and Hoebel, 1966). Since the lateral hypothalamus contains orexin neurons it was thus hypothesized that orexin may be important for sexual behavior in male rats. Sexual behavior culminating in ejaculation in male rats leads to cFos induction in orexin neurons of the PFA-DMH and LHA (Muschamp et al., 2007). These results suggest that

orexin may play an important role in the initiation of, or motivation for mating as well as sexual performance.

Several studies have used pharmacological manipulations to test whether orexin is critically involved in mediation of sexual motivation and performance. Orexin-A administration into the medial preoptic area (mPOA), a brain region that is important for sexual performance (Hansen et al., 1982, Coolen et al., 2004a) facilitates sexual motivation and performance in male rats. Orexin-A in the mPOA increases sexual motivation, by decreasing latencies to mount, intromit, and pursue a receptive female (Gulia et al., 2003). Intra-mPOA orexin-A also increases sexual performance, by decreasing latency to ejaculate and increasing frequency of mounts and intromissions (Gulia et al., 2003). These studies suggest that orexin-A may act in the mPOA to facilitate sexual motivation and performance. In contrast to these findings ICV administration of orexin-A attenuates sexual motivation, but only in highly sexually motivated male rats (Bai et al., 2009b). This discrepancy in findings may suggest that orexin-A has opposing effects on sexual behavior by acting in multiple brain regions, with facilitatory effects in mPOA, but inhibitory effects in other areas. Studies testing effects of antagonists however, have also yielded puzzling results. In one study ICV administration of SB-334867 had no effect on sexual motivation or arousal (Bai et al., 2009b), and in another study systemic injection of SB-334867 caused only a slight impairment in sexual performance seen as longer latencies to intromission and decreases in ejaculation frequency (Muschamp et al., 2007). Thus, with numerous conflicting studies, the role of orexin in mediation of sexual behavior and performance is unclear. Moreover, a role for orexin in sexual reward has yet to be tested.

1.6 Rationale and Objectives: Part 1

In chapters 2 and 3, I set out to test the hypothesis that orexin is a critical mediator of sexual performance, motivation, and reward.

1.6.1 Objectives

1. Demonstrate that exposure to different parameters of sexual motivation and performance cause activation of orexin neurons seen as increased expression of the immediate early gene cFos in orexin neurons. Establish that orexin is a critical mediator of sexual motivation and performance utilizing cell-specific lesions of orexin neurons in the hypothalamus of male rats.

2. Show that orexin is activated by conditioned cues related to sexual reward using cFos expression as marker of neural activity. Establish that orexin is critically involved in sexual reward by demonstrating that cell-specific lesions of orexin neurons block conditioned place preference for sexual reward.

1.7 Introduction: Part 2

1.7.1 Drug Induced Neuroplasticity in the Mesolimbic Sytem

The mesolimbic system, consisting of the interconnected brain regions of the ventral tegmental area (VTA), nucleus accumbens (NAc) and medial prefrontal cortex (mPFC) is critical for mediation of natural reward behaviors (Hyman et al., 2006). Drugs of abuse have been shown to converge upon the mesolimbic system and specifically, repeated exposure to drugs of abuse causes neural alterations at the levels of gene expression, synaptic strength and neuron morphology within the mesolimbic system (Hyman et al., 2006; Kalivas et al., 2009). These alterations subsequently lead to the development of drug addiction and cause increased susceptibility for relapse (Kalivas et al., 2009). In rodents, repeated exposure to drugs of abuse leads to sensitization of the locomotor response to opiates and psychostimulants (Segal and Mandell, 1974, Post and Rose, 1976, Kalivas and Stewart, 1991), enhanced conditioned drug reward (Lett, 1989, Shippenberg et al., 1996) and increased lever pressing for conditioned cues associated with prior drug reward (Crombag et al., 2008). Repeated exposure to drugs of abuse causes long lasting alterations in numbers of dendritic spines and spine density within the NAc (Robinson and Kolb, 1997, 1999, Robinson et al., 2002, Li et al., 2003, Li et al., 2004, Robinson and Kolb, 2004) and VTA (Sarti et al., 2007). Moreover, repeated exposure to drugs of abuse causes alterations in synaptic strength in VTA dopamine neurons (Kauer, 2003, Liu et al., 2005) and neurons in the NAc (Thomas et al., 2000, Thomas and Malenka, 2003). Thus, is clear that repeated exposure to drugs of abuse leads to neuroplasticity in the mesolimbic system. Until recently, it was unclear as to

whether natural rewarding behaviors, such as sexual behavior lead to similar neuroplasticity.

1.8 Sexual Reward and Neuroplasticity

Studies from our lab have recently shown that repeated exposure to sexual behavior also causes neuroplasticity in the mesolimbic system, similar to that seen following repeated exposure to drugs of abuse. Repeated sexual experience has been shown to cause sensitized responses to sexual behavior, seen as facilitation of sexual performance with experience (Pitchers et al., 2012), as well as cross-sensitization to the psychostimulant amphetamine (Frohmader et al., 2010, Pitchers et al., 2010). It has also been demonstrated that these changes persist following a period of abstinence from mating (Pitchers et al., 2012), as facilitation of sexual behavior and sensitization to amphetamine are both maintained one week and one month following last sexual experience (Pitchers et al., 2012). Repeated sexual experience also causes electrophysiological changes observed as long-term depression in the NAc that persists after a one week period of abstinence from mating (Pitchers et al., 2012). Furthermore, some of the neuroplastic changes seen following repeated sexual experience appear to be dependent on this period of abstinence from mating, consistent with findings that some neuroplastic alterations seen following repeated exposure to drugs of abuse are dependent on a period of abstinence from drug taking (Nestler, 2001, Wolf et al., 2004, Kauer and Malenka, 2007). Repeated sexual experience in male rats, followed by a seven-ten day period of abstinence from mating causes enhanced drug reward seen as formation of a conditioned place preference for subthreshold doses of amphetamine (Pitchers et al.,

2010). This effect is only seen following a period of mating abstinence, and is not observed one day following last sexual experience (Pitchers et al., 2010). Moreover, morphological changes in the NAc including increased dendritic branching and increased numbers of dendritic spines are observed seven days following last mating, but not at earlier timepoints (Pitchers et al., 2010). It is therefore evident that natural reward causes neuroplasticity in the mesolimbic system, similar to that caused by repeated exposure to drugs of abuse. Neuroplasticity induced by sexual behavior appears to regulate the reinforcing components of sexual behavior and may regulate reward behavior in general. Yet, abstinence from sexual reward appears to induce a state of increased reward-seeking and potential vulnerability to the addictive properties of drugs of abuse, which may mimic the effects of abstinence from drugs of abuse on the "incubation of craving" (Lu et al., 2004, Lu et al., 2005). Thus, this neuroplasticity following abstinence from sexual behavior that causes increased reward seeking and vulnerability for drugs of abuse, may also cause increased susceptibility for other disorders related to reward, including mood disorders such as depression.

1.9 Drug Addiction and Depression

Drug addiction is often comorbid with other neuropsychiatric disorders, including mood disorders such as depression (Kessler et al., 2003, Volkow, 2004, DuPont, 1995). It is estimated that the comorbidity of drug abuse and mood disorders is 19.4%, and drug abuse increases risk for depression by a factor of 5 (Regier et al., 1990). Withdrawal and subsequent abstinence from drugs of abuse in human addicts leads to feelings of anxiety and depressed mood (Koob, 2009). Similarly, studies in rats and mice have shown that

withdrawal and abstinence from drugs of abuse cause anxiety and depression-like phenotypes (Perrine et al., 2008).

1.10 Depression

Depression is a mood disorder in which feelings of sadness, loss, anger, or frustration interfere with everyday life for a prolonged period of time (Krishnan and Nestler, 2008). It is estimated that 16.2% of adults in the United States will experience a major depressive episode at some point in their lifetime (Kessler et al., 2003). Depression is often triggered by stressful life events such as childhood abuse or neglect, chronic stress, chronic abuse or loss of rewarding social interactions such as death of partner, friend or relative, or loss of job (Newport et al., 2002, Henn and Vollmayr, 2005, Krishnan and Nestler, 2008, Nestler and Hyman, 2010, Krishnan and Nestler, 2011). However, some individuals are more susceptible to develop depression due to genetic and environmental vulnerability (Krishnan and Nestler, 2008).

1.10.1 Animal Models of Depression

As depression is such a prevalent problem in the human population, many animal models have been developed to attempt to elucidate the underlying neural mechanisms of depression. Depression is a multivariate illness with a number of symptoms, including depressed mood and irritability, cognitive symptoms such as feelings of guilt and suicidality, anhedonia or inability to experience pleasure, homeostatic symptoms such as weight loss or gain, insomnia or hypersomnia, appetite and energy abnormalities and alterations in psychomotor behavior (Nestler and Hyman, 2010). As such, animal models

of depression can only objectively mimic some but not all symptoms of human depression. As depression in humans is often triggered by stressful life events, current animal models rely on exposing animals to stressful stimuli to induce depression-like behavior. Several chronic stress rodent models of depression have been developed in attempt to mimic the effects of chronic life stress on development of depression in humans. Chronic mild stress or chronic unpredictable stress models of depression rely on exposing animals to daily stressors (i.e. foot shock, hot or cold temperature, swim stress, restraint) for periods of weeks to months or more (Henn and Vollmayr, 2005, Willner, 2005). Following chronic stress, animals develop anhedonia that can be blocked by administration of antidepressants (Willner, 2005), thus providing pharmacological validation of this model. The social defeat model of depression attempts to model depression following chronic abuse in humans. Rodents are subjected to daily encounters with an aggressor and social subordination. After a period of weeks to months, these animals develop depression-like symptoms including anhedonia and social withdrawal that can be blocked by chronic administration of antidepressants (Krishnan et al., 2007). The social defeat model also mimics some of the homeostatic symptoms of depression such as weight gain and insulin resistance (Chuang et al., 2010). The maternal separation model of depression mimics childhood abuse and neglect, and relies on separating pups from their mothers for brief period of time during the first few weeks of life. These animals develop life-long behavioral and neuroendocrine abnormalitites, some of which can be blocked by antidepressants (Meaney, 2001). All of these animal models reliably induce depression-like behavior in rodents, and provide valuable insight into development of depression in humans following stressful life events. However, as

mentioned above, depression in humans can also be triggered by loss of socially rewarding stimuli, such as loss of partner, relative or job (Newport et al., 2002, Henn and Vollmayr, 2005, Krishnan and Nestler, 2008, Nestler and Hyman, 2010, Krishnan and Nestler, 2011). Currently there are no animal models to study effects of loss of social reward in humans. Recently, it has been demonstrated that socially monogamous prairie voles that establish pair bonds develop depression-like behavior when they are separated from their partner (Bosch et al., 2009), however, few mammals establish pair bonds (Kleiman, 1977). Thus a reliable animal model for studying effects loss of social reward on development of depression is needed.

1.11 Neural Circuitry of Depression

An initial trigger for development of depression is activation of the stress system (Holsboer, 2000). The stress system is controlled by the hypothalamic-pituitary-adrenal (HPA) axis. In response to a stressor, corticotropin releasing factor (CRF) is released from the paraventricular nucleus of the hypothalamus (PVN) and acts on the pituitary to cause release of adrenocorticotropic horme (ACTH) (Koob and Heinrichs, 1999). ACTH then acts on the adrenal glands to cause release of cortisol (humans) or corticosterone (rodents) (Koob and Heinrichs, 1999). CRF is the main neurotransmitter in the HPA axis and is also synthesized in the PVN as well as in extrahypothalamic brain regions including the central amygdala (CeA) and bed nucleus of the stria terminalis (BnST) (Swanson et al., 1983). Hyperactivity of the HPA axis is a prominent endophenotype of human depression (Holsboer, 2000). Depressed humans show increased plasma and salivary cortisol, as well as increased size and activity of the adrenal glands (Nemeroff

and Vale, 2005). Depressed humans also show increased CRF mRNA in the cerebrospinal fluid and hypothalamus (Kling et al., 1991). Rodent models of depression including social defeat, maternal separation and chronic stress all lead to hyperactivity of the HPA axis seen as corticosterone hypersecretion (Ayensu et al., 1995, Francis et al., 1999, Francis et al., 2002, Kieran et al., 2010). Increased levels of CRF in the brain are also seen in following chronic stress (Stout et al., 2000), social defeat (Keeney et al., 2006, Marini et al., 2006) and maternal separation (Francis et al., 1999). Thus, it is clear that stress models of depression mimic some of the neural alterations that are present in human depression. Moreover, administration of CRF antagonists has been shown to block development of depression-like behavior in the chronic stress model of depression (Ducottet et al., 2003).

1.11.1 Drug Addiction, Withdrawal and HPA Axis Alterations

The stress system is connected to the limbic system (Herman et al., 2005). As such, exposure to stressful stimuli has been shown to lead to activation of the mesolimbic system, and this activation has been shown to lead to relapse in drug taking in humans and animal models (Sinha, 2008, 2009). Stress induced activation of the mesolimbic system has also been shown to cause sensitization of the locomotor response to psychostimulants (Kalivas and Stewart, 1991, Yap et al., 2005, Mathews et al., 2008), synaptic adaptations of VTA dopamine neurons (Daftary et al., 2009, Hahn et al., 2009) as well as morphological changes in the mPFC (Shansky and Morrison, 2009). Moreover, within the HPA axis CRF signaling appears to play a role in the rewarding aspects of stress-induced relapse associated with drug withdrawal (Hyman et al., 2006) and CRF

antagonists have been shown to reduce effects of stress during drug withdrawal and periods of abstinence (George et al., 2007a, George et al., 2007b, Koob, 2009). Exposure to drugs of abuse increases HPA axis activity (Sarnyai, 1998, Sarnyai et al., 2001, Goeders, 2002, Goeders and Clampitt, 2002) seen as increased corticosterone and ACTH release, as well as activation of CRF neurons in rodents (Sarnyai et al., 2001). Furthermore, during drug withdrawal, rodents demonstrate enhanced ACTH and corticosterone release, and increased levels of CRF (Koob and Kreek, 2007, Koob, 2008).Withdrawal and subsequent abstinence from drugs of abuse in human addicts leads to feelings of depression and anxiety (Koob, 2009), and rodents display anxiety and depression-like behavior following withdrawal from drugs of abuse (Cryan et al., 2003, Perrine et al., 2008). Studies in rodents have also shown that administration of CRF antagonists prevents development of drug withdrawal induced anxiety and depression-like behavior (Sarnyai et al., 1995, Rodriguez de Fonseca et al., 1997, Basso et al., 1999, Sarnyai et al., 2001). Thus, there is evidence that the HPA axis is activated by drugs of abuse and as well as in drug withdrawal. Moreover, CRF signaling appears to be a critical mediator of drug withdrawal induced anxiety and depression.

1.11.2 Alterations in Stress Responses Following Sexual Behavior

Studies have shown that natural reward behaviors such as sexual activity cause activation of the HPA axis. Specifically, following mating to ejaculation both sexually naïve and experienced male rats demonstrate increased plasma corticosterone release (Bonilla-Jaime et al., 2006, Waldherr et al., 2010, Buwalda et al., 2012) and plasma ACTH release (Waldherr et al., 2010). Sexual behavior also causes increased cFos

immunoreactivity in the PVN (Buwalda et al., 2012). Thus, results of these studies indicate that the HPA axis is acutely activated by sexual behavior. When examining longer term effects of sexual behavior in male rats, it has been shown that copulation to ejaculation reduces anxiety-like behavior (Fernandez-Guasti et al., 1989b, Saldivar et al., 1991) and this anxiolytic effect persists for up to four hours following mating (Waldherr and Neumann, 2007). In addition, male rats housed with a sexually receptive female and allowed to mate following contextual fear conditioning, demonstrate decreased fear responses when exposed to fear associated cues (Bai et al., 2009a). Moreover, brief exposure to estrous female odors leads to increased risk taking behaviors towards a predator in male mice (Kavaliers et al., 2001, Kavaliers et al., 2008). In humans sexual behavior has also been shown to lead to decreased stress responses which may persist for hours (Brody, 2006). Thus, results of these studies suggest that sexual behavior leads to decreased stress and anxiety related behaviors in humans and rodents following sexual activity. In rats, sexual behavior has also been shown to lead to dampened stress responses one day following mating seen as decreased corticosterone release in response to acute restraint stress (Ulrich-Lai et al., 2010). Brief exposure to estrous female odors also blocks the increase in corticosterone seen following exposure to a predator (Kavaliers et al., 2001). Furthermore, CRF and cFos mRNA are decreased in the PVN following exposure to forced swim stress in sexually experienced males both 45 minutes and 4 hours following mating (Waldherr et al., 2010). Thus it is clear that mating causes alterations in HPA axis activity as well as alterations in responses to stressors, however it is currently unknown whether there are alterations in stress responses and HPA axis reactivity following a prolonged abstinence period from sexual behavior. Abstinence

from sexual behavior leads to increased vulnerability for drugs of abuse and enhances drug craving, as well as neuroplasticity in the mesolimbic system similar to that seen following repeated exposure to drugs of abuse (Pitchers et al., 2010). A depression and substance abuse are often comorbid, (Kessler et al., 2003, Volkow, 2004, DuPont, 1995) it is hypothesized that abstinence from sexual reward will also lead to depression-like behavior, and underlying neuropathology including increased stress responses.

1.12 Rationale and Objectives: Part 2

Repeated exposure to sexual behavior causes neuroplastic changes in the mesolimbic system, some of which are dependent on a period of abstinence from mating, that may lead to increased vulnerability for drug addiction (Pitchers et al., 2010, Pitchers et al., 2012),. However, it is currently unknown if abstinence from sexual reward leads to increased vulnerability for other disorders related to reward processing, such as depression-like behavior, or alters other systems that are activated by sexual behavior, such as the HPA axis (Buwalda et al., 2012).

1.12.1 Hypothesis

I hypothesize that an extended abstinence period from sexual reward will lead to depression-like behavior, including passive stress coping and anhedonia, as well as underlying neural pathology, including hyperactivity of the HPA axis and alterations in CRF mRNA expression.

1.12.2 Objectives

1. Test the hypothesis that a prolonged period of abstinence from sexual behavior leads to depression-like behavior in male rats using standard tests for depression in rodents, including passive stress coping and anhedonia.

2. Test the hypothesis that abstinence from sexual reward leads to HPA axis hyperactivity, including increased corticosterone and ACTH release following exposure to an acute stressor, and increased CRF mRNA expression in the paraventricular nucleus of the hypothalamus.

3. Determine that CRF is a critical mediator of sexual reward abstinence induced depression-like behavior using pharmacological manipulations of CRF receptors in the nucleus accumbens and medial prefrontal cortex.

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CHAPTER 2

Orexin mediates initiation of sexual behavior in sexually naive male rats, but is not critical for sexual performance.

Sections 2.1-2.5 were published in Hormones and Behavior 58 (3): 397-404, 2010

2.1 INTRODUCTION

Orexin, also known as hypocretin, is a hypothalamic neuropeptide critical for feeding behavior (de Lecea et al., 1998, Sakurai et al., 1998, Sakurai, 2006, Benoit et al., 2008) arousal and sleep (Chemelli et al., 1999, Lin et al., 1999, Furlong and Carrive, 2007, Sakurai, 2007b, Carter et al., 2009, Furlong et al., 2009). Orexin neurons are localized to the lateral hypothalamic area (LHA) and perifornical dorsomedial hypothalamus (PFA-DMH) and produce two neuropeptides, orexin-A and B (de Lecea et al., 1998, Sakurai et al., 1998). Orexin neurons have been shown to project to brain structures involved in mediation of arousal including the locus coeruleus, tuberomammillary nucleus and peduculopontine tegmental nucleus (Peyron et al., 1998, Hagan et al., 1999, Horvath et al., 1999, Baldo et al., 2003). Orexin has also been implicated in reward and motivation, specifically related to food and drugs of abuse (Aston-Jones et al., 2009a, Aston-Jones et al., 2009b) and orexin neurons have been shown to project to reward-related brain structures in the mesolimbic system including the ventral tegmental area (VTA) and nucleus accumbens (NAc) (Peyron et al., 1998, Fadel and Deutch, 2002, Martin et al., 2002, Baldo et al., 2003). Orexin neurons are activated by conditioned contextual cues associated with food and drug reward (Harris et al., 2005, de Lecea L, 2006, Choi et al., 2010) and have been shown to play a role in reward-based feeding behavior (Choi et al., 2010). Moreover, intracerebroventricular (ICV) or intraperitoneal administration of an orexin receptor 1 (ORX1) antagonist results in reduced motivation for palatable food (Thorpe et al., 2005, Nair et al., 2008), whereas ICV orexin-A administration can reinstate this motivation (Boutrel et al., 2005).

The role of orexin in other rewarding behaviors is currently unclear, although several studies have implicated a role for orexin in control of sexual behavior in male rats. It has previously been shown that orexin neurons are activated by copulation in male rats (Muschamp et al., 2007). In addition, administration of orexin-A into the medial preoptic area (mPOA) resulted in enhanced sexual performance evidenced by reduced latencies to mount and intromit, and increased frequencies of mounts and intromission (Gulia et al., 2003). In contrast, ICV administration of orexin-A attenuated sexual motivation by reducing female preference, although only in highly sexually motivated males (Bai et al., 2009). Studies using ORX1 antagonists have also demonstrated contradictory data, as systemic administration of ORX1 antagonist slightly impaired sexual performance by increasing latency to intromit without affecting other parameters of sexual behavior (Muschamp et al., 2007), while ICV administration of ORX1 antagonist had no effect on sexual motivation (Bai et al., 2009). Together these studies suggest that administration of exogenous orexin-A affects sexual performance and motivation; however, endogenous orexin may not play an important role in mediating sexual behavior (Bai et al., 2009). Therefore, the goal of the present study was to determine if endogenous orexin is essential for male rat sexual motivation and performance.

First, it was determined when during sexual behavior orexin neurons are activated, testing the hypothesis that orexin neurons are activated upon introduction of the rewarding stimulus. Moreover, as it has been shown that sexual experience influences sexual performance (Dewsbury, 1969) and the rewarding properties of sexual behavior (Tenk et al., 2009), it was determined whether sexual experience influences orexin

neuron activation during mating. Finally, it was tested whether orexin plays a critical role in sexual motivation and performance using cell body specific lesions of orexin neurons.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Adult male Sprague–Dawley rats (200–250 g) were obtained from Harlan (Indianapolis, IN) or Charles River Laboratories (Sherbrooke, Quebec, Canada) and housed individually or in pairs depending on the individual experiment (see below) in Plexiglas cages. The colony room was maintained on a 12/12 reversed light–dark cycle (lights off at 10 am) and food and water were available *ad libitum* except during behavioral testing. Female Sprague–Dawley rats were obtained from Harlan (Indianapolis, IN) or Charles River Laboratories (Sherbrooke, Quebec, Canada) were bilaterally ovariectomized and implanted subcutaneously with 5% 17-β-estradiol benzoate silastic capsules. Sexual receptivity was induced by subcutaneous injections of progesterone (500 μg in 0.1 mL of sesame oil) approximately 4 h prior to mating sessions. All procedures were approved by the Animal Care Committees at the University of Cincinnati and the University of Western Ontario and conformed to the guidelines outlined by the National Institute of Health and the Canadian Council on Animal Care. All behavioral testing was conducted during the first half of the dark phase under dim red illumination, except when noted otherwise.

2.2.2 Experimental design

2.2.2A cFos expression studies

Male rats (n = 48) were housed individually and half of the animals gained sexual experience in the home cage during 5 twice weekly mating sessions. Mating tests were performed in the home cage to eliminate arousal and cFos expression induced by exposure to a different mating arena and exposure to conditioned cues associated with prior mating (Balfour et al., 2004). A receptive female was introduced into the home cage and males were allowed to mate until one ejaculation or for 60 min. During each test sexual behavior was observed. The total number of mounts and intromissions, as well as the latencies to first mount and intromission (the time from presentation of the receptive female to the first mount or intromission), and ejaculation (the time from the first intromission to ejaculation), was recorded (Agmo, 1997). The remaining half of the animals remained sexually naive. These animals were housed in the same room as the sexually experienced males, were handled and exposed to odors and sounds associated with mating, however did not mate. Naive and experienced animals were each further subdivided into 6 experimental groups (n = 4 per group). The 6 naive and experienced groups included: control males with no exposure to sexual behavior (home cage); males exposed to a non-receptive female in the home cage for 15 min (anestrous female). Males could investigate and interact, however did not mate due to lack of female receptivity; males exposed to the smells of a receptive female placed in a wire mesh box on top of the home cage for 15 min (estrous female); males that displayed mounts, but not intromissions or ejaculation with vaginally masked females (mount); males that displayed mounts and intromissions only (intromission); and males that mated to one ejaculation (ejaculation). One hour after the end of the test, males were sacrificed to analyze cFos expression. Sexually experienced groups were matched on parameters of sexual behavior

and there were no significant differences between groups prior to the final test. Moreover, there were no significant differences between naive and experienced groups in numbers of mounts plus intromissions during the final test.

2.2.2A Perfusions: cFos expression

All males were deeply anaesthetised with sodium pentobarbitol (270 mg/mL) and were transcardially perfused with 4% paraformaldehyde (500 mL; PFA). Following perfusion brains were removed immediately and post-fixed for 1 h in the same fixative then transferred to 20% sucrose solution for cryoprotection. Brains were sectioned on a freezing microtome (Microm, Walldorf, Germany) in coronal sections of 35 µm and collected in 4 parallel series in cryoprotectant solution (30% sucrose in 0.1 M PB containing 30% ethylene glycol and 0.01% sodium azide) and stored at −20 °C until further processing.

2.2.2B Immunohistochemistry

All incubations were performed at room temperature with gentle agitation. Free floating sections were washed extensively with 0.1 M saline buffered sodium phosphate (PBS). Sections were blocked with 1% H_2O_2 (30% stock solution) in PBS for 10 min, then extensively rinsed again with PBS. Sections were incubated with an incubation solution (PBS containing 0.1% bovine serum albumin and 0.4% Triton X-100) for 1 h. Primary antibody incubations were performed in the incubation solution overnight at room temperature. Following staining sections were rinsed in PBS, mounted onto plus charged glass slides and coverslipped with dibutyl phthalate xylene (DPX).

2.2.2C cFos/orexin

One series of sections was immunoprocessed for cFos and orexin. Sections were incubated overnight with a rabbit-raised antibody recognizing cFos (rabbit anti-cFos, sc-52; 1:10 000, Santa Cruz Biotechnology, Santa Cruz, CA) followed by 1 hour incubations with biotinylated goat anti-rabbit (1:500, Vector Laboratories, Burlingame, CA) and an avidin horseradish peroxidase complex (1:1000, ABC kit, Vector Laboratories, Burlingame, CA). Sections were incubated for 10 min in 0.02% diaminobenzidine (DAB) (Sigma, St. Louis, MO) in 0.1 M phosphate buffer (PB) containing 0.012% hydrogen peroxide and 0.08% nickel sulfate, resulting in a blue-black reaction product. Sections were then incubated overnight with a rabbit-raised antibody recognizing orexin-A (rabbit anti-orexin-A, H-003-30; 1:20 000, Phoenix Pharmaceuticals, Burlingame, CA) followed by a 1 hour incubation with biotinylated goat anti-rabbit and ABC, as described above. Finally, the sections were incubated for 10 min with 0.02% DAB in 0.1 M PB containing 0.012% hydrogen peroxide, resulting in a reddish brown reaction product.

All antibodies have been characterized previously (Chen et al., 1999, Satoh et al., 2004, Solomon et al., 2007) Immunohistochemical controls included omission of primary antibodies, western blot analysis demonstrating single bands at appropriate weight (cFos), and loss of immunohistochemical orexin signal with orexin-B-saporin lesions (orexin).

2.2.2D Data analysis

cFos/orexin: Neurons labelled for orexin or orexin and cFos were counted bilaterally in three representative sections per animal known to contain maximal numbers of the orexin neuronal population (Sakurai et al., 1998) spanning −2.3 mm to −3.6 mm from bregma (Paxinos and Watson, 1998) (Figure 2.1), using a drawing tube attached to a

Leica microscope (Leica Microsystems; Wetzlar Germany), by an observer blinded to experimental groups. The PFA-DMH and LHA were delineated based on the location of the fornix (Figure 2.1a). Percentages of orexin neurons expressing cFos were calculated and averaged per hemisphere for each animal, and group means were calculated. Statistical significance between groups was determined using a two-way ANOVA with sexual experience and sexual behavior during the final test as factors followed by Fisher's LSD tests with a 95% confidence level.

2.2.3 Orexin lesion studies

2.2.3A Surgery

Males were housed individually and given one pre-test mating session with a receptive female prior to lesion and sham surgery. Sexual behavior was recorded as described above and groups were matched based on parameters of mating behavior. Male rats were anaesthetised with isoflurane (Abbot Laboratories, St. Laurent, Quebec, Canada) using a Surgivet Isotec4 gas apparatus (Smiths Medical Vet Division, Markham, Ontario, Canada) and placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) with a gas mask covering the nose and mouth to maintain anaesthesia. An incision was made to expose the skull and lambda and bregma were found and determined to be level. A hole was drilled in the skull using a dremel drill (Dremel, Racine, WI) and glass micropipettes (40 µm diameter, World Precision Instruments Inc, Sarasota, FL) filled with a targeted toxin orexin-B-saporin (IT-20, Advanced Targeting Systems, San Diego, CA; 200 ng/µL in PBS); or unconjugated toxin BLANK-saporin (IT-21, Advanced

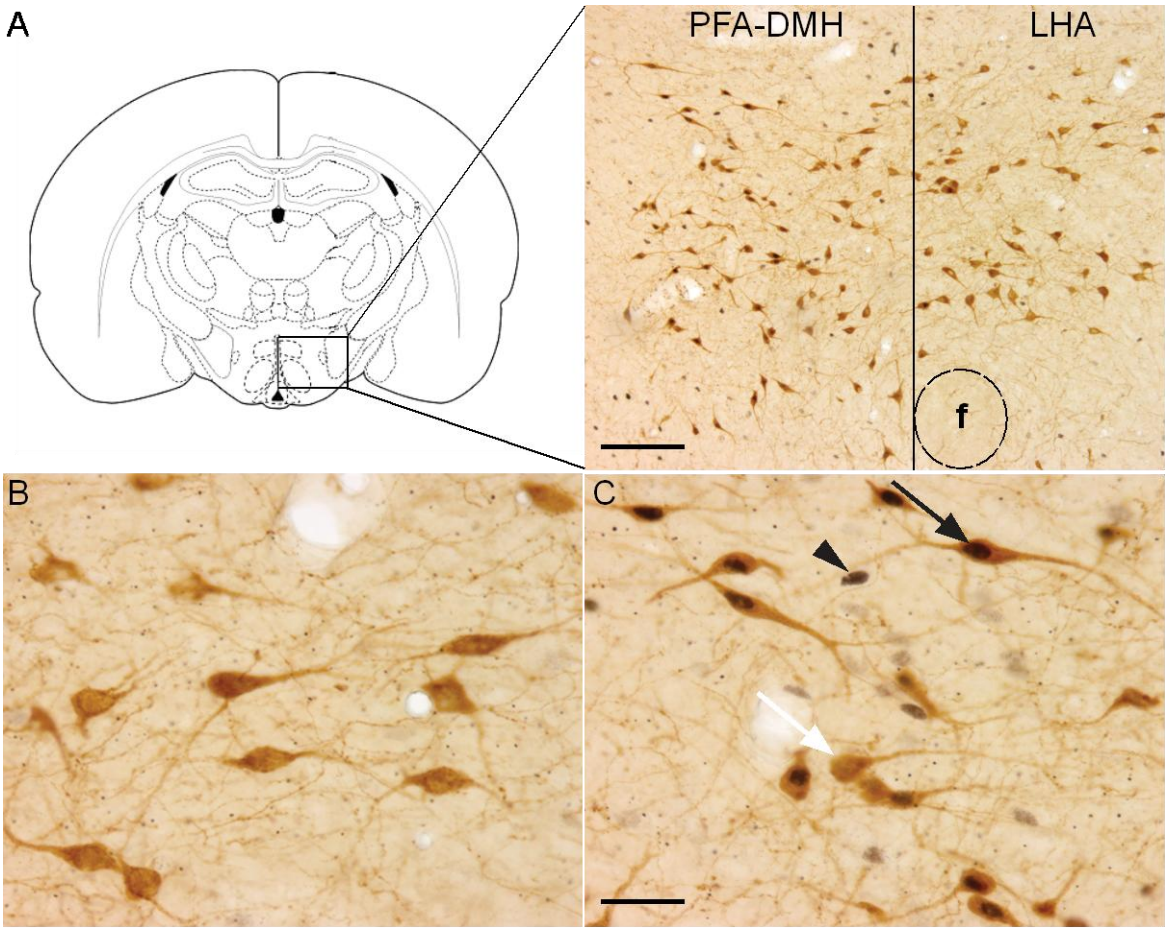

Figure 2.1. Location of orexin neurons in the hypothalamus. (A) Anatomical location of orexin neurons in the hypothalamus (Paxinos and Watson, 1998). Scale bar: 200 µm. (B) Single labelled orexin neurons in the PFA-DMH in an unmated control animal. (C) Orexin neurons in the PFA-DMH expressing cFos following mating. Scale bar: 50 µm. White arrow indicates a single labelled orexin neuron; arrowhead indicates a cFos positive neuron; black arrow indicates an orexin neuron expressing cFos. Abbreviations: PFA-DMH, perifornical dorsomedial hypothalamus; LHA, lateral hypothalamic area; f, fornix.

Targeting Systems, San Diego, CA; 200 ng/μL in PBS; sham controls) were lowered into the hypothalamus. This targeted toxin has been shown to bind with a high affinity to cells expressing orexin receptor 2 (ORX2) and with a significantly lower affinity for cells expressing ORX1 (Gerashchenko et al., 2001), and has been shown to specifically ablate orexin neurons in the hypothalamus (Frederick-Duus et al., 2007). Bilateral infusions of 1 μL (2 per hemisphere) were injected at the following coordinates: AP = −2.8 and −3.2; ML = 0.7 and 0.8; DV = −9.0 (Paxinos and Watson, 1998). After each infusion the needle was left in place for 3 min to allow diffusion. Needles were slowly removed and wounds were closed with wound clips. Two weeks following lesion surgery, all males were tested for sexual experience during four mating trials and were then subjected to the runway and/or elevated plus maze test (see below). Surgeries were performed in three different cohorts, separated by several weeks, to reach sufficient numbers of animals per group.

2.2.3B Sexual behavior

All males were tested for sexual behavior during 4 mating sessions conducted every second day in the home cage. During each session, males mated with a receptive female to one ejaculation or for 60 min, whichever came first. Mating behavior was recorded as described above and copulation efficiency was also calculated [numbers of intromissions / (numbers of mounts + numbers of intromissions)]. Statistical differences in parameters of sexual performance were compared between lesion and sham groups for each trial using a one-way ANOVA with lesion surgery as a factor and Fisher's LSD test with a 95% confidence level, or when appropriate, non-parametric tests were run using a Kruskal–Wallis one-way ANOVA with lesion surgery as a factor and Dunn's test with a

95% confidence level. In addition, data for each group were compared to the pre-surgery data using paired t-tests.

2.2.3C Sexual motivation: runway test

Following testing of sexual behavior, a subgroup of the now sexually experienced males were tested for sexual motivation using a straight runway apparatus (MED Associates Inc., St. Albans, VT) (120 cm long;(Lopez et al., 1999)). Males habituated to the runway apparatus over two subsequent 10 minute trials conducted on the same day. Next, two test trials were conducted. During the first trial, a stimulus animal (estrous female, anestrous female or male) was placed in a goal box with perforated dividers at the end of the runway. A fan was used to blow the scents of the stimulus animals towards the male. Experimental males were placed in the start box, the door was opened to allow access to the runway, and time to reach the goal box was recorded. Once reaching the goal box, males were given 30 s to interact with the stimulus animal behind the screen. An identical second trial followed 1 h later. Statistical significance between times to reach the goal box between trial 1 and trial 2 was analyzed using paired t-tests with a 95% confidence level. Statistical significance between groups was determined using a one-way ANOVA with lesion surgery as a factor followed by Fisher's LSD tests with a 95% confidence level.

2.2.3D Anxiety-like behavior: elevated plus maze

A subgroup of the now sexually experienced males was tested for anxiety-like behavior to determine if effects of orexin lesions on sexual performance or motivation

were due to changes in anxiety or arousal. Males were exposed to the elevated plus maze apparatus (EPM; MED Associates Inc., St. Albans, VT) in a brightly lit room during the end of the light phase. The EPM consisted of 4 arms each 50 cm in length extending from a central junction and was elevated 75 cm. Two arms of the maze were open to the outside environment and the other two were enclosed with dark siding 40 cm high. Animals were placed on the EPM and monitored for 5 min. Time spent in open and closed arms, and total numbers of entries into each arm were recorded using photobeam arrays. Statistical significance between groups was determined using a one-way ANOVA with lesion as a factor followed by Fisher's LSD tests with a 95% confidence level.

2.2.3E Perfusions and mating-induced cFos

Following all behavioral testing, all males were deeply anaesthetised with sodium pentobarbitol (270 mg/mL) and were transcardially perfused with 500 mL of 4% PFA for lesion verification as described previously. In addition, to test the effects of orexin lesions on mating-induced cFos expression, groups of sham and lesion males mated until one ejaculation. One hour following ejaculation, males were transcardially perfused with 4% PFA as described above. Half of the males in this group were not introduced to a female and were perfused from the home cage to serve as unmated controls.

2.2.3F Immunohistochemistry

Brains were sectioned using a freezing microtome in 4 parallel series of 35 μm coronal sections and stored as described above. For lesion verification, one series of sections containing the hypothalamus from all lesion experiments was single labelled for

orexin using the same rabbit anti-orexin-A and DAB protocol as described above. One series of sections from the animals that mated was stained for cFos and orexin as described above.

2.2.3G Lesion verification

In each animal, numbers of orexin neurons immunoreactive for orexin were counted bilaterally in the PFA-DMH and LHA in 3 sections expressing maximal numbers of orexin cells in non-surgery controls, −2.3 mm to −3.6 mm from bregma as described above. Cells per hemisphere were averaged for each animal, and group means were calculated. Non-surgery control animals (from cFos experiments) were used to determine intact/baseline numbers of orexin neurons and data are expressed as percentages compared to the non-surgery control males (Figure 2.2). Males that had fewer than 20% orexin cells compared to non-surgery control animals were included in the lesion group. Animals with greater than 20%, but fewer than 80% of orexin neurons remaining were included in a partial lesion group. Sham controls did not have significant changes in numbers of orexin cells. Statistical significance between sham, partial and complete lesion animals was calculated using a one-way ANOVA and Fisher's LSD test with a 95% confidence level.

2.2.3H Lesion specificity

To verify that lesions were restricted to orexin neurons, one series of sections containing the hypothalamus from a subset of sham and lesion animals (n = 20) was

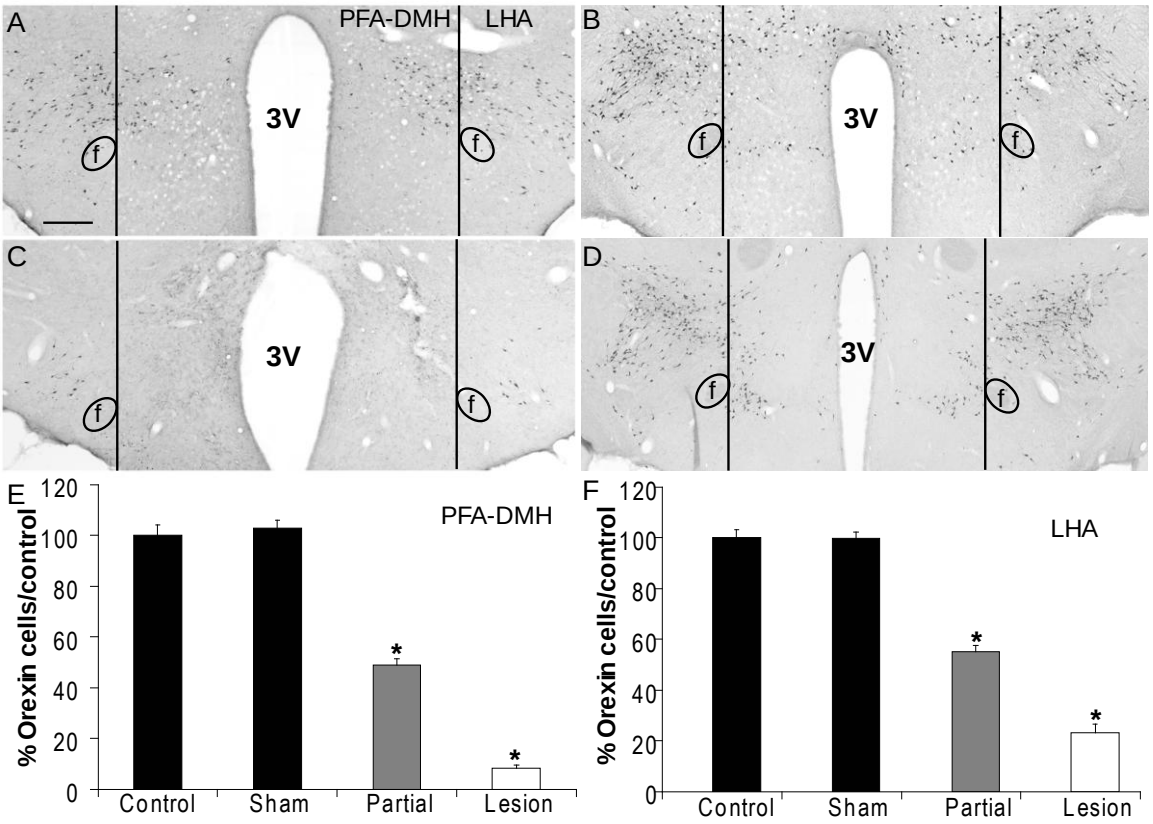

Figure 2.2. Lesion verification. Representative images showing orexin (A) and MCH (B) cells in a sham lesion animal injected with BLANK-saporin. Representative images showing loss of orexin cells (C), but intact MCH cells (D) in a lesion animal injected with orexin-B-saporin. Scale bar: 400 µm; (E) quantification of orexin neurons in the PFA-DMH (E) and LHA (F) of sham, partial and lesion males, expressed as percentages of non-surgery controls.*Indicates significant difference from all other groups ($p < 0.001$). Sham: n = 35; partial lesion: n = 45; lesion: n = 19. Abbreviations: PFA-DMH: perifornical dorsomedial hypothalamus; LHA: lateral hypothalamic area; f: fornix.

immunoprocessed for melanocyte concentrating hormone (MCH), a hypothalamic peptide that has overlapping location (but no colocalization) with orexin neurons (Broberger et al., 1998), using a rabbit-raised antibody recognizing MCH (rabbit anti-MCH, H-070-47; 1:150 000, Phoenix Pharmaceuticals, Burlingame, CA) and DAB as described previously. MCH neurons express ORX1 (Backberg et al., 2002) but not ORX2, and are not significantly reduced following orexin-B-saporin treatment (Frederick-Duus et al., 2007). MCH immunoreactive cells were counted bilaterally in two sections per animal (sham: n = 7; lesion n = 5), using alternate sections to those analyzed for orexin neurons. Lesions did not significantly reduce numbers of MCH neurons in either PFA-DMH or LHA (Table 2.1, Figure 2.2B and D; PFA-DMH: $p = 0.47$; LHA: $p = 0.33$). Furthermore, mating-induced cFos expression was counted bilaterally in one representative section per animal (sham: n = 4; lesion n = 3), using alternate sections to those analyzed for orexin neurons. Lesions did not affect mating-induced cFos expression in the PFA-DMH or LHA (Table 2.1; PFA-DMH: $p = 0.53$; LHA: $p = 0.82$). Finally, representative sections used for orexin cell counts (animals: sham: n = 6; lesion: n = 6) were Nissl counterstained using cresyl violet (5 g cresyl violet acetate (C-5042, Sigma, St. Louis, MO), 0.5 g of sodium acetate trihydrate (S209, Thermo Fisher Scientific, Ottawa, Ontario, Canada), and 1 L double distilled water with glacial acetic acid (AX0073-6, EMD Chemicals, Mississauga, Ontario, Canada) at pH: 3.14). Counts of Nissl-stained neurons were performed in standard areas of analysis (250 µm × 200 µm) in the general location of orexin neurons. Numbers of Nissl-stained neurons did not differ between sham and lesion groups (Table 2.1, PFA-DMH: $p = 0.23$; LHA: $p = 0.33$). Since a deficiency in orexin has been shown to contribute to narcolepsy in mice

	Sham		Lesion	
	PFA-DMH	**LHA**	**PFA-DMH**	**LHA**
Nissl	93.3 ± 7.2	84.1 ± 7.4	82.8 ± 3.9	75.3 ± 4.6
MCH	101.3 ± 4.1	136.2 ± 8.1	88.2 ± 16.6	119.1 ± 13.5
Mating-induced cFos	45.5 ± 6.5	30.1 ± 2.7	40.0 ± 3.8	32.0 ± 5.0

Table 2. 1. Verification of lesion specificity: analysis of the numbers of neurons stained for Nissl, MCH or mating-induced cFos demonstrated that there was no significant loss of neurons in general, MCH cells, or mating-induced neural activation in the PFA-DMH or LHA following infusions of orexin-B-saporin. Abbreviations: PFA-DMH, perifornical dorsomedial hypothalamus; LHA, lateral hypothalamic area; MCH, melanocyte concentrating hormone.

dogs (Lin et al., 1999) and humans (Siegel, 1999, Nishino et al., 2000, Peyron et al., 2000, Thannickal et al., 2000) animals were observed to ensure the absence of a narcoleptic phenotype. Animals were observed for the duration of all behavioral tests reported in this study and did not display characteristics of narcolepsy.

2.2.3I Mating-induced cFos expression in lesion animals

Numbers of cFos-immunoreactive cells were counted bilaterally in 3 sections per animals in standard areas of analysis in the ventral tegmental area (VTA; 900 × 900 μm), mPOA (400 × 600 μm); nucleus accumbens (NAc) core and shell (400 × 600 μm) and the prelimbic, infralimbic and anterior cingulate subregions of the medial prefrontal cortex (mPFC) (600 × 800 μm per subregion) by an observer blinded to experimental groups. Counts were averaged for each animal, and group means were calculated. Statistical significance was calculated using a two-way ANOVA with sexual experience and lesion as factors followed by Fisher's LSD test with a 95% confidence level.

2.3 RESULTS

2.3.1 Orexin neuron activation during sexual behavior

A significant increase in cFos expression in orexin neurons was observed following sexual behavior in both the PFA-DMH ($F_{(5,31)}$ 63.4; $p < 0.001$; Figure 2.3a) and LHA ($F_{(5,31)}$ 10.4; $p < 0.001$; Figure 2.3b), with no effect of sexual experience. Specifically, in both sexually naive and experienced animals, all experimental groups of males displaying different parameters of sexual behavior (investigation of anestrous female, exposure to estrous female odors, display of mounting, intromissions, or

ejaculation) showed equal induction of cFos compared to home cage controls with a higher percentage of orexin cells activated in the PFA-DMH (60–80%) versus the LHA (14–33%), without differences between the experimental groups. These results suggest that orexin neurons are activated following exposure to the stimulus female without further activation during sexual performance. Moreover, the activation is not dependent on incentive salience of the female stimulus as both non-receptive and receptive females induced activation in sexually experienced males.

2.3.2 Effects of orexin lesions

2.3.2A Sexual behavior

Orexin lesions resulted in facilitation of sexual behavior (mount latency: $F_{(2,47)}$ 3.962; $p = 0.034$; intromission latency: $H = 9.104$; $p = 0.011$). During the first mating trial, lesion males showed shorter latencies to mount and intromission compared to sham animals (mount latency: $p = 0.03$; intromission latency: $p = 0.01$; Figures 2.4a and b) and compared to latencies during the pre-surgery mating trial (mount latency: $p = 0.02$; intromission latency: $p = 0.03$; data not shown). Partial lesion males did not differ significantly from sham males, and neither group differed from the pre-surgery mating trial. Effects of lesions on mount and intromission latencies were attenuated with sexual experience, as there were no differences between groups, during any of the subsequent trials (trial 4 shown in Figures 2.4a and b). Ejaculation latencies (Figure 2.4c), numbers of mounts (Figure 2.4d) and intromissions (Figure 2.4e) as well as copulation efficiency (Figure 2.4f) did not significantly differ between groups during any of the trials or within each group between the first mating trial and the pre-surgery test.

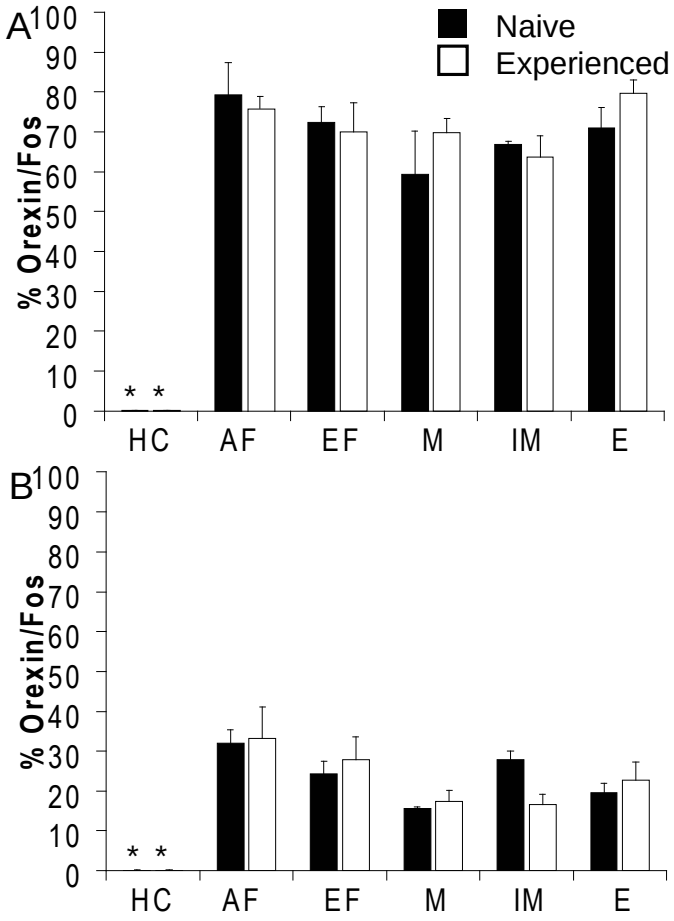

Figure 2.3. Orexin neurons in PFA-DMH (A) and LHA (B) expressed cFos following all parameters of mating behavior in naive and experienced animals. Abbreviations: HC, home cage; AF: anestrous female; EF, estrous female; M, mount; IM, intromission; E, ejaculation. *Indicates significant difference from all other groups ($p < 0.001$). n = 4 per group.

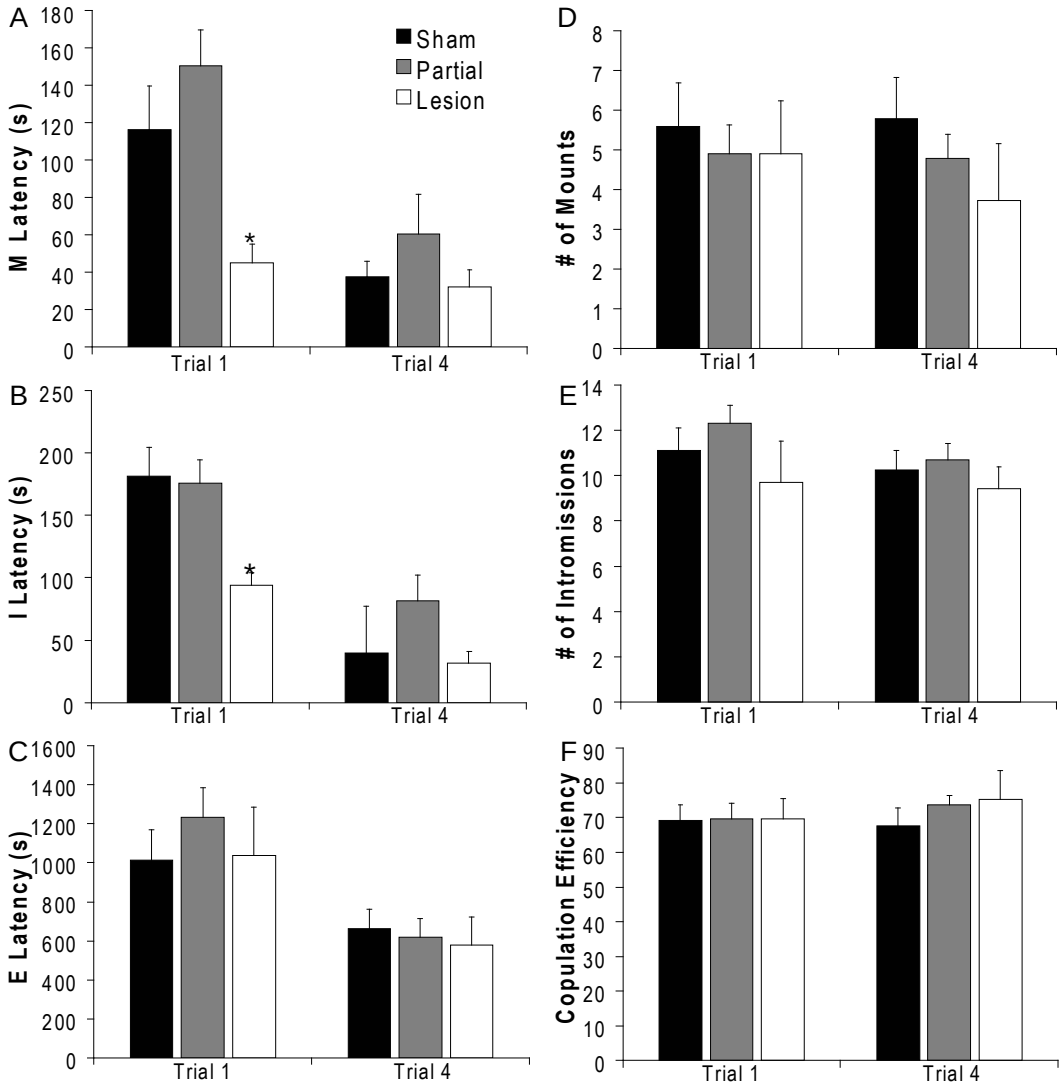

Figure 2.4. Orexin lesions shortened latencies to mount and intromission in sexually naive males during trial 1. Orexin lesions did not affect mating during trial 4, after males gained sexual experience. (A) Mount latency. (B) Intromission latency. (C) Ejaculation latency. (D) Number of Mounts. (E) Number of intromissions. (F) Copulation efficiency. *Indicates significant difference from sham. Sham: n = 19; partial lesion: n = 23; lesion: n = 7.

2.3.2B Runway test

Orexin lesions did not affect sexual motivation assessed in a straight runway test in sexually experienced males. Over the course of two test trials, lesion males ran significantly faster towards an estrous female in the second trial compared to the first trial ($p = 0.03$; Figure 2.5). Such increased run time is indicative of sexual motivation (Lopez et al., 1999). Partial lesion and sham males also ran faster towards an estrous female during trial 2 ($p = 0.03$), although this failed to reach significance in sham males ($p = 0.052$). None of the groups showed increased speed to run towards an anestrous female or a male during trial 2. Moreover, no significant differences were observed between sham, partial and lesion males on speed to run towards any stimulus animal on neither trial 1 nor trial 2, demonstrating lack of differences in general activity on the runway.

2.3.2C Anxiety-like behavior

Results thus far suggest that lesions may facilitate initiation of sexual behavior in naive animals via a potential effect on responses to novelty and/or anxiety-like behaviors when the males encounter a novel female. In support, lesion males showed decreased anxiety-like behavior on the EPM, seen as a decreased percentage of time spent in the closed arms, ($p = 0.012$; Figure 2.6) and an increased percentage of time on the open arms ($p = 0.023$; Figure 2.6) compared to sham males. Partial lesions had no significant effect. These data further support that lesion decreased anxiety-like behavior.

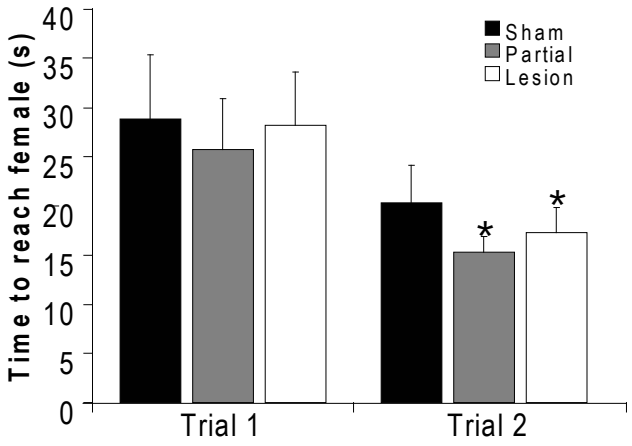

Figure 2.5. Orexin lesions did not affect sexual motivation in sexually experienced males. Shown are times to reach an estrous female in the runway test during both trials 1 and 2. *Indicates significant reduction in time to reach the female in trial 2 compared to trial 1. Sham: n = 24; partial lesion: n = 26; lesion: n = 12.

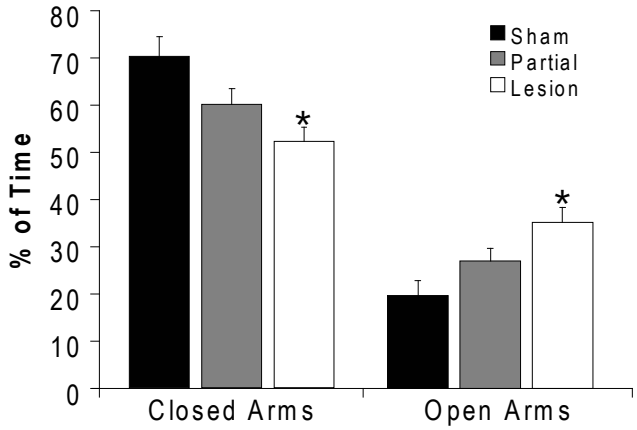

Figure 2.6. Orexin lesions decreased anxiety-like behavior on the elevated plus maze. Percentage of time spent in the closed arms (left) were decreased and percentage of time spent in open arms (right) were increased in lesioned males. *Indicates significant difference from sham. Sham: n = 27; partial lesion: n = 36; lesion: n = 12.

2.2.3D cFos expression

To assess whether endogenous orexin contributes to mating-induced neuronal activation in orexin-innervated brain regions, analysis of mating-induced cFos expression in the VTA, NAc core and shell, mPOA and the mPFC was conducted. In both lesion and sham males, mating significantly increased cFos in all of the analyzed brain areas compared to unmated controls (Table 2.2) Lesions did not affect neural activation, as sham and lesion animals did not differ in baseline or mating-induced cFos expression.

2.4 DISCUSSION

These studies investigated the role of endogenous orexin in sexual performance and motivation in the male rat. It was found that orexin is not essential for sexual motivation or performance. Instead, orexin neurons are activated by the female stimulus, independent of the hormonal status of the female or sexual experience of the male. Moreover, removal of endogenous orexin by orexin cell-specific lesions decreased anxiety-like behaviors and facilitated initiation of sexual behavior in sexually naive males. Thus, the results of this study support a role for orexin in arousal (de Lecea et al., 2006, Harris and Aston-Jones, 2006, Furlong and Carrive, 2007, Sakurai, 2007a, Boutrel et al., 2009, Furlong et al., 2009) and anxiety (Suzuki et al., 2005, Davis et al., 2009, Li et al., 2010), but do not support a critical role for orexin in sexual motivation or performance.

The results of these studies further clarify the role of endogenous orexin and the apparent contrasting findings of the previous studies examining the role of orexin in male sexual behavior using pharmacological tools. Intra-mPOA infusions of exogenous orexin-

Brain Area	Non Mating			Mating		
	Sham	Partial	Lesion	Sham	Partial	Lesion
VTA	13.2 ± 9	5.8 ± 3.3	3.2 ± 2.2	83.3 ± 3.3 *	79.4 ± 4.4 *	63.3 ± 20 *
NAc Core	7.9 ± 1.3	7.0 ± 3.0	2.7 ± 1.2	54.8 ± 3.4 *	61.4 ± 5.3 *	53.8 ± 24.2 *
NAc Shell	5.3 ± 3.2	5.0 ± 3.9	2.1 ± 1.2	57.4 ± 2.7 *	60.3 ± 5.2 *	38.6 ± 13.7 *
mPOA	5.9 ± 3.3	7.9 ± 3.3	8.3 ± 5.7	197.8 ± 21.4 *	184.3 ± 11.7 *	224.1 ± 22.4 *
mPFC	28.0 ± 13.2	24.0 ± 11.3	8.6 ± 6.2	296.3 ± 79.9 *	309.8 ± 44.3 *	263.4 ± 98.2 *

Table 2.2. Mating-induced cFos in sham, partial and lesion groups compared to non-mating controls of the same lesion status. *Indicates significant difference from non-mating control ($p < 0.001$ for all groups). No differences between groups were detected in either baseline or mating-induced cFos expression. Abbreviations: VTA: ventral tegmental area; NAc: nucleus accumbens, mPOA: medial preoptic area; mPFC: medial prefrontal cortex. There were no significant differences between cFos counts in any subregion of the mPFC, thus the combined expression of the three subregions is shown. Sham: n = 8; partial lesion: n = 9; lesion: n = 6.

A led to increased sexual arousal and improved sexual performance, suggesting that orexin may act in the mPOA to increase motivation and performance of sexual behavior (Gulia et al., 2003). However, in contrast, ICV infusions of orexin-A attenuated sexual motivation and arousal (Bai et al., 2009), while an orexin receptor antagonist had no effect on sexual arousal (Bai et al., 2009), indicating that endogenous orexin may not play a role in sexual motivation. Finally, ORX1 blockade by systemic injections was shown to only slightly impair copulatory performance (Muschamp et al., 2007). From these conflicting studies, a few conclusions can be drawn. First, application of exogenous orexin-A may affect behavior, but ORX1 blockade is without major effects, suggesting a minor role for endogenous orexin in regulation of male sexual behavior (Bai et al., 2009). The current results support this possibility. The current studies using removal of orexin, by orexin cell-specific lesions indicate that endogenous orexin is not essential for sexual motivation or performance, in line with observations by Bai et al., (2009). It is important, however to note that lack of effects of orexin lesions on sexual motivation in the runway may be due to the fact that animals had gained sexual experience prior to sexual motivation testing, therefore lack of effect in the runway test may have been due to the sexual experience of the males. Future experiments may address this caveat by testing the effects of orexin lesions on sexual motivation in naive males.

It is also possible that the two orexin ligands and the two subtypes of orexin receptors (ORX1 and ORX2; Sakurai et al., 1998) may regulate sexual behavior in opposite directions. By utilizing orexin cell lesion techniques, the ligands for both subtypes of orexin receptors (orexin-A and B) were eliminated in the current study. The two receptor subtypes are expressed in different brain areas (Trivedi et al., 1998, Marcus

et al., 2001) and have been shown to differentially regulate memory for cue induced cocaine-seeking (Smith et al., 2009). Previous studies on sexual behavior have primarily focused on the role of orexin-A and ORX1 (see discussion above). The orexin receptor antagonist SB334867 used in the studies thus far specifically targets ORX1 which has a high affinity for orexin-A and significantly lower affinity for orexin-B (Sakurai et al., 1998). Likewise, orexin-A has been used as the exogenous orexin in previous studies (Gulia et al., 2003, Bai et al., 2009). Future studies are needed to investigate the role of orexin-B and ORX2 in regulation of male sexual behavior.

The current study tested the effects of long term loss of orexin. Muschamp et al., (2007) suggested that a long term reduction of orexin following castration may account for the loss of sexual motivation and performance. This hypothesis was contradicted by the current findings as orexin cell lesions did not reduce sexual motivation or performance. It is possible that the long term orexin loss in the current study may have resulted in compensatory mechanisms, although no changes in mating-induced neural activation within the circuit mediating sexual behavior were detected. Nonetheless, it is clear that reduced or lack of orexin does not prevent sexual behavior. Moreover, the results of the current study do not support a major role for orexin in induction of cFos expression by sexual behavior. It has been clearly established that orexin contributes to activation of neurons in the VTA (Korotkova et al., 2003, Borgland et al., 2006, Narita et al., 2006, Vittoz et al., 2008). However, orexin cell lesions did not block mating-induced neural activation in the VTA, or in any other reward-related brain regions analyzed, despite the presence of orexin-immunoreactive fibers in close proximity to the activated

neurons in sham males. Thus, mating-induced neural activation in these brain regions does not appear to be dependent on orexin action.

A somewhat unexpected finding of the current study was the effect of orexin lesions on facilitation of the initiation of sexual behavior in sexual naive, but not experienced animals. This was shown to be correlated with a reduction in anxiety-like behaviors. Therefore, the effects of orexin lesions on sexual motivation and performance may be secondary to its effects on anxiety and arousal. Indeed previous studies have suggested a role for orexin in anxiety as ICV infusion of orexin-A decreased time on the open arms of the EPM in mice (Suzuki et al., 2005). Infusion of orexin-A into the paraventricular nucleus of the thalamus of male rats decreased time spent in the center area of an open field chamber and decreased novel object exploration, indicating that orexin may be involved in the generation of anxiety-like behavior (Li et al., 2010). In addition, dominant male rats that show increased risk taking on the EPM have increased levels of ORX1 mRNA in the mPFC (Davis et al., 2009). Orexin has also been shown to alter responses to stress (Ida et al., 1999, Ida et al., 2000), and stimulation of orexin receptors increases release of corticotrophin releasing factor (Al-Barazanji et al., 2001, Singareddy et al., 2006), corticosterone (Ida et al., 2000, Kuru et al., 2000) and adrenocorticotropic hormone (Kuru et al., 2000). Orexin antagonists are currently in clinical trials for treatment of insomnia, a disorder which is often comorbid with anxiety disorders (Sullivan and Neria, 2009), and it is hypothesized that orexin antagonists could potentially be used to treat anxiety disorders (Mathew et al., 2008). Given the growing body of evidence of a role for orexin in anxiety and arousal it appears that orexin lesions

may facilitate the initiation of sexual behavior in naive males by reducing anxiety-like responses associated with the introduction of a novel stimulus, i.e. the female.

Significant activation of orexin neurons was seen following sexual arousal and sexual behavior in both sexually naive and experienced animals in both the PFA-DMH and LHA, with 60–80% and 14–33% of orexin cells expressing cFos, respectively. There is a body of evidence supporting a dichotomy in orexin neuronal function within the orexin cell population, with the PFA-DMH being critically involved in arousal and the LHA being critical for reward-related behaviors (Harris et al., 2005, Harris and Aston-Jones, 2006, Aston-Jones et al., 2009a). Hence, activation of the PFA-DMH orexin cells by the female stimulus supports the hypothesis that orexin is activated by and is critical for arousal, including sexual arousal in naive and experienced males, and anxiety associated with the novel female stimulus in naive males. However, PFA-DMH cells were activated to similar levels independent of the experience of the males and the hormonal status of the female, suggesting that the PFA-DMH cells were activated during general arousal and not specifically by sexual arousal. Moreover, our studies do not fully support the existence of a completely dichotomous orexin cell population as there was a significant activation of the LHA following exposure to all parameters of sexual arousal and performance, regardless of whether the behaviors were associated with reward. Thus, experienced males exposed to an anestrous female showed equal levels of orexin cell activation in the LHA compared to experienced males that copulated to ejaculation. However, only the latter group will form a conditioned place preference for mating (Tenk et al., 2009); suggesting that copulation to ejaculation is more rewarding than other

elements of mating. The current study did not specifically test the role of orexin in sexual reward; hence future studies are needed to address that question.

In summary, the results of these studies demonstrate that orexin is not critical for sexual performance or motivation. Instead, orexin cell lesions were demonstrated to reduce anxiety, suggesting that endogenous orexin is involved in increasing anxiety. Moreover, removal of orexin resulted in facilitation of initiation of sexual behavior in sexually naive males, suggesting that endogenous orexin may inhibit initiation of mating, possibly by increasing anxiety in response to the novel stimulus, i.e. the female. These findings further elucidate the neural circuitry involved in sexual performance and anxiety, and add to a growing body of literature on the role of orexin in mediation of arousal and anxiety.

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CHAPTER 3

Lesions of orexin neurons block conditioned place preference for sexual behavior in male rats.

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3.1 INTRODUCTION

The hypothalamic neuropeptide orexin (hypocretin) is found exclusively in the lateral hypothalamic area (LHA) and the perifornical-dorsomedial hypothalamus (PFA-DMH) and consists of two active peptides, orexin-A and orexin-B (de Lecea et al., 1998, Sakurai et al., 1998). Orexin is critical for food intake (de Lecea et al., 1998, Sakurai et al., 1998, Sakurai, 2006, Benoit et al., 2008), arousal and sleep (Chemelli et al., 1999, Lin et al., 1999, Sakurai, 2007a, b, Carter et al., 2009). Recent studies have shown that orexin also plays a critical role in mediation of reward (DiLeone et al., 2003, Aston-Jones et al., 2009, Aston-Jones et al., 2010) and orexin cells have extensive projections throughout the brain, including to reward associated brain areas such as the nucleus accumbens (NAc) and ventral tegmental area (VTA) (Peyron et al., 1998, Fadel and Deutch, 2002, Martin et al., 2002).

Orexin neurons are activated by conditioned contextual cues associated with food and drug reward in a conditioned place preference (CPP) paradigm (de Lecea et al., 2006), a standard paradigm used to determine reward seeking behavior (Tzschentke, 2007). Moreover, excitotoxic lesions of orexin neurons in the LHA or orexin receptor-1 antagonists in the VTA significantly reduce morphine preference in a CPP paradigm (Harris et al., 2007). In addition, LHA orexin neuronal stimulation, and intra-VTA orexin-A administration reinstate morphine CPP following extinction (Harris et al., 2005). Orexin-A administration into the LHA increases self administration of palatable food (Thorpe et al., 2005) while orexin receptor-1 antagonists block self administration of food (Nair et al., 2008), ethanol (Lawrence et al., 2006) and nicotine (Hollander et al.,

2008). Thus, there is ample evidence that orexin plays a role in reward processing related to food intake and drugs of abuse.

Orexin neurons are activated by sexual behavior in male rats (Muschamp et al., 2007, Di Sebastiano et al., 2010). In addition, exogenous orexin-A administration into the medial preoptic area enhances copulatory performance in male rates, evidenced by shortened latencies to mount and intromission, and increased frequency of mounts and intromissions (Gulia et al., 2003). However, a critical role for endogenous orexin in sexual behavior is not supported by findings that orexin cell-specific lesions do not disrupt sexual motivation or performance (Di Sebastiano et al., 2010), that intracerebroventricular (ICV) administration of an orexin receptor-1 antagonist does not disrupt sexual motivation (Bai et al., 2009), and that systemic administration of antagonist only slightly inhibits sexual performance (Muschamp et al., 2007). However, a role for endogenous orexin in mediation of sexual reward has yet to be elucidated. Therefore, the goal of the current study was to test the hypothesis that orexin plays a critical role in processing of sexual reward. First, it was determined whether orexin neurons are activated by conditioned cues predicting sexual reward by exposing male rats to an environment associated with prior sexual behavior. Next, orexin cell-specific lesions were utilized to determine a specific role for orexin in sexual reward processing using a CPP paradigm (Agmo and Gomez, 1993, Tenk et al., 2009).

3.2 MATERIALS AND METHODS

3.2.1 Animals

Adult male Sprague Dawley rats (200-250g) were obtained from Harlan (Indianapolis, IN) or Charles River Laboratories (Sherbrooke, Quebec, Canada) and were pair housed for the duration of experiments in Plexiglas cages. The colony room was maintained on a 12/12 reversed light-dark cycle (lights off at 10 am) with food and water available at all times except during behavioral testing. Female Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN) or Charles River Laboratories (Sherbrooke, Quebec, Canada), were bilaterally ovariectomized and received subcutaneous implants of 5% 17-β-estradiol benzoate in silastic capsules. Sexual receptivity was induced by subcutaneous progesterone injections (500 μg in 0.1 ml of sesame oil) approximately 4 h prior to mating sessions. All procedures were approved by the Animal Care Committees at the University of Cincinnati and the University of Western Ontario and conform to guidelines outlined by the National Institute of Health and the Canadian Council on Animal Care.

3.2.2 Experiment 1: cFos Expression Studies

3.2.2A Apparatus

The CPP apparatus (MED Associates, St. Albans, VT) consisted of three chambers with different visual and tactile cues. The two test chambers (28 x 22 x 21 cm) had black walls and parallel bar flooring or white walls and metal grid flooring and were separated by a central compartment (13 x 22 x 21) with grey walls and a smooth, grey Plexiglas floor. The three chambers were connected by guillotine doors in colors

matching the chamber with which they were attached and males were confined to one chamber or allowed to move freely between chambers.

3.2.2B Experimental Design

On the day 1 (pre-test) males (n=5; paired males) were allowed free access to the entire apparatus for 15 min and the initial preference for each animal was determined. As a group, animals did not display preference for one chamber, but each animal had a slight (less than 60 second) preference (Pitchers et al., 2009, Tenk et al., 2009). On days 2 and 3 (conditioning trials), males mated to one ejaculation in the home cage and mating behavior was observed and recorded (Agmo, 1997). A receptive female was placed in the home cage and total numbers of mounts and intromissions as well as latencies to first mount and intromission (time from presentation of a receptive female to first mount or intromission) as well as latency to ejaculation (time from the first intromission to ejaculation) were recorded during each trial. Immediately following ejaculation males were placed into the paired chamber (initially non-preferred chamber for 30 minutes. For control pairings, males were placed into the unpaired (initially preferred) chamber without mating for 30 minutes. Half of the animals received sex pairing on day 2 and control pairing on day 3. The remaining animals received sex pairing on day 3 and control pairing on day 2. On day 4, a post-test procedurally identical to the pre-test was conducted and conditioned preference was determined. Another group of males (n=5; unpaired males) served as a control group and were placed in the chambers without mating on both conditioning days. A preference score (percentage of time spent in the sex-paired chamber) and difference score (time spent in the sex-paired chamber minus

the time spent in the control chamber) were calculated for each animal and compared using a paired t-test, with a 95% confidence level. Indeed, males formed a significant CPP for the sex paired chamber seen as an increased preference score ($p=0.038$) and difference score ($p=0.04$) in the post-test compared to the pre-test following one pairing with ejaculation, confirming previous reports (Straiko et al., 2007, Tenk et al., 2009, Webb et al., 2009a, Webb et al., 2009b), while control males did not form a preference for either chamber.

3.2.2C Tissue Processing

One hour following the end of the post-test, males were anesthetised with sodium pentobarbitol (270mg/mL) and were transcardially perfused with a 0.9% saline solution followed by 500 mL of 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB). Brains were quickly removed and post-fixed for one hour in the same fixative and transferred to a 20% sucrose solution for cryoprotection. Brains were sectioned into 35 µm coronal sections on a freezing microtome (Microm, Walldorf, Germany) and collected in 4 parallel series in cryoprotectant solution (30% sucrose in 0.1 M PB containing 30% ethylene glycol and 0.01% sodium azide). Brains were stored at -20 C until further processing.

3.2.2C Immunohistochemistry: cFos/Orexin

Incubations were performed with gentle agitation at room temperature. Free floating sections were extensively washed in 0.1M saline buffered sodium phosphate (PBS). Sections were blocked for 10 minutes in 1% H_2O_2 (30% stock solution) in PBS,

then again rinsed extensively with PBS. Sections were then incubated for 1 hour in an incubation solution (PBS containing 0.1% bovine serum albumin and 0.4% Triton X-100). Primary antibody incubations were performed overnight at room temperature in the same incubation solution. Following staining sections were rinsed extensively with PBS, mounted onto plus charged glass slides and coverslipped with dibutyl phthalate xylene (DPX).

One series of sections was immunoprocessed for cFos and orexin. Sections underwent an overnight incubation with a rabbit raised antibody recognizing cFos (rabbit anti-cFos, SC52; 1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA) which was followed by incubation with biotinylated goat anti-rabbit (1:500, Vector Laboratories, Burlingame, CA) for 1 hour and a 1 hour incubation with avidin horseradish peroxidase complex (1:1000, ABC kit – Vector Laboratories, Burlingame, CA). Finally, sections underwent incubation for 10 minutes in 0.02% diaminobenzidine (DAB) (Sigma, St. Louis, MO) in 0.1M PB containing 0.012% hydrogen peroxide and 0.08% nickel sulfate, which resulted in a blue-black reaction product. Next, sections were incubated overnight with a rabbit raised antibody recognizing orexin-A (rabbit anti-orexin-A, H-003-30; 1:20,000, Phoenix Pharmaceuticals, Burlingame, CA) followed by 1 hour incubation with biotinylated goat anti-rabbit and ABC, as described above. Immunoreactivity was visualized by a 10 minute incubation with 0.02% DAB in 0.1M PB containing 0.012% hydrogen peroxide, resulting in a reddish brown reaction product.

All antibodies were previously characterized (Chen et al., 1999, Satoh et al., 2004, Solomon et al., 2007). Controls for immunohistochemistry included: primary antibody omission, western blot analysis demonstrating bands of appropriate weight (cFos) and

loss of immunohistochemical signal following lesions of orexin neurons with orexin-saporin (orexin).

3.2.2E Data Analysis

Neurons labelled for orexin or orexin and cFos (Figure 3.1) were bilaterally counted in the PFA-DMH and LHA in 3 sections per animal known to contain maximal numbers of orexin neurons, spanning a distance from -2.3 mm to -3.6mm (Paxinos and Watson, 1998), using a Leica microscope (Leica Microsystems; Wetzlar Germany). Anatomical location of the PFA-DMH and LHA was determined based on location of the fornix. Data were expressed as percentages of orexin cells that expressed cFos and averages were calculated per hemisphere, per section, for each animal, and group means were calculated. Statistical significance between paired and unpaired groups was determined for PFA-DMH and LHA using student's t-test with a 95% confidence level.

3.2.3 Experiment 2: Orexin Cell-Specific Lesion Studies

3.2.3A Lesion Surgery

Males underwent one pre-test mating session in a clean Plexiglas mating cage ($60\times45\times50$ cm^3) prior to undergoing lesion or sham surgery. A receptive female was placed into the cage and males were allowed to mate to one ejaculation or for 60 minutes. Sexual behavior was recorded as described above and groups were matched on parameters of mating behavior. Male rats were anesthetized with isoflurane (Abbot Laboratories, St. Laurent, Quebec, Canada) in a Surgivet Isotec4 gas apparatus (Smiths Medical Vet Division,

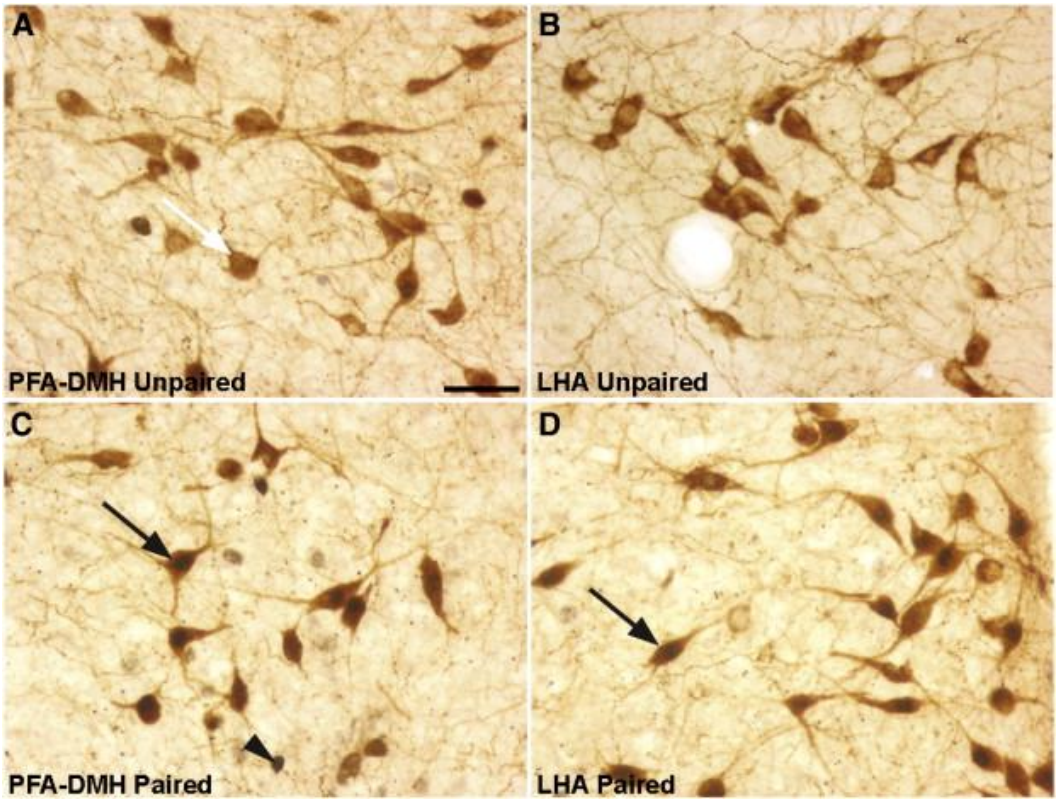

Figure 3.1. Orexin and cFos immunoreactivity in PFA-DMH (A,C) and LHA (B,D) in unpaired control (A,B) and paired (C,D) males. White arrow indicates an example single labelled orexin neuron; arrowhead indicates an example single labelled cFos neuron; black arrow indicates an example orexin neuron expressing cFos; Scale bar: 50 µm.

Markham, Ontario, Canada). A gas mask was placed over the nose and mouth to maintain anaesthesia and males were secured in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). An incision was made, the skull was exposed, and lambda and bregma were found and determined to be level horizontally. A hole was drilled into the skull using a dremel drill (Dremel, Racine, WI). Glass micropipettes (40µm diameter, World Precision Instruments Inc, Sarasota, FL) were filled with the targeted toxin orexin B-saporin (Advanced Targeting Systems, San Diego, CA; 200ng/µL in PBS) or unconjugated toxin BLANK-saporin (Advanced Targeting Systems, San Diego, CA; 200ng/µL in PBS; sham controls) and were lowered into the hypothalamus. This toxin has a high affinity for orexin receptor 2 expressing cells, and a significantly lower affinity for orexin receptor 1 expressing cells (Gerashchenko et al., 2001), and has been shown to specifically lesion orexin neurons in the hypothalamus (Frederick-Duus et al., 2007, Di Sebastiano et al., 2010). To ensure the toxin spread would encompass the majority of the orexin neuronal population, two injections were made per hemisphere at different rostro-caudal coordinates. 1 µL infusions (2 per hemisphere) were infused bilaterally at the following coordinates: AP = -2.8 and -3.2, ML = 0.7 and 0.8, DV = -9.0. Following each infusion the needles were left in place for 3 minutes to allow diffusion. Micropipettes were removed slowly and incisions were closed with wound clips.

Following a two week recovery from surgery, males received four mating sessions to gain sexual experience and were subsequently subjected to either a conditioned place preference (CPP) or conditioned place aversion (CPA) paradigm. All tests were performed during the second half of the dark phase under dim red illumination.

3.2.3B Conditioned Place Preference (CPP)

The CPP test was conducted as described above, with conditioning trials repeated twice over the course of 4 consecutive days. A separate group of sham control males (n=15) was included that did not receive mating paired with either chamber and served as a negative control group to demonstrate lack of non-specific change in preference. The post-test was conducted on the sixth day as described previously and preference score (percentage of time spent in paired chamber) and difference score (time spent in paired chamber minus the time spent in the control chamber) were calculated. Time in the paired chamber in the post-test compared to the pre-test was also calculated. Statistical differences in the pre-test and post-test data were compared using a paired t-test, with a 95% confidence level. Formation of CPP was defined as a significant increase in both preference score and difference score in the post-test compared to the pre-test.

3.2.3C Conditioned Place Aversion (CPA)

The CPA experiment was conducted in the same apparatus used in the CPP paradigm using lithium chloride (LiCl) induced visceral illness as the aversive stimulus. Males underwent a pre-test as described above. Over the course of 4 consecutive conditioning days immediately following the pre-test, males were injected with a 20 mL/kg dose of 0.15M LiCl and placed into the initially preferred chamber. On alternate days males were injected with a 20 mL/kg dose of 0.9% saline and placed into the initially non-preferred chamber. The post-test was conducted on the sixth day as described above, and preference score and difference score were calculated in the same manner. Statistical differences in the pre-test and post-test data were compared using a

paired t-test, with a 95% confidence level. Formation of CPA was defined as a significant decrease in both preference score and difference score in the post-test compared to the pre-test.

3.2.3D Tissue Processing

Following completion of all experimental testing, males were anesthetized with sodium pentobarbital (270mg/mL) and perfused transcardially with 4% PFA as described above. Brains were removed and stored in cryoprotectant for further processing and lesion analysis.

3.2.3E Immunohistochemistry

*Orexin***:** One series of sections from the CPP and CPA experiments was immunoprocessed for orexin using the same rabbit raised antibody recognizing orexin-A (rabbit anti-orexin-A, H-003-30; 1:20,000, Phoenix Pharmaceuticals, Burlingame, CA) and DAB for lesion verification.

3.2.3F Lesion Verification

Numbers of orexin neurons in the PFA-DMH and LHA were bilaterally counted in 3 sections per animal, containing the maximum number of orexin neurons, spanning a distance from -2.3 mm to -3.6mm relative to bregma (Paxinos and Watson, 1998) using a Leica microscope (Leica Microsystems; Wetzlar Germany). Cells per hemisphere, per section were averaged for each animal, and group means were calculated. Numbers of orexin cells were also counted in a separate group of males that had not undergone

surgery (n=20) and counts in lesion males were expressed as percentages of these non-surgery control values. Lesions were classified as follows: males that had fewer than 20% of the total number of orexin cells compared to non-surgery control animals were included in the lesion group. Animals with greater than 20%, but fewer than 80% of orexin cells were included in a separate partial lesion group (Figure 3.2). Sham control animals did not have significant changes in numbers of orexin cells when compared to non-surgery controls (Table 1). Statistical significance between sham, partial and lesion animals was calculated using a one-way ANOVA and Fisher's LSD test with a 95% confidence level. Experiments were performed on multiple separate surgical cohorts of animals due to the low portion of orexin-B saporin treated animals that were verified to have complete lesions. For CPP two separate experiments were performed to include intact sham controls (exp.1: n=8 and exp. 2: n=10; combined n=18), males with partial lesions (exp. 1: n=8 and exp. 2: n=17; combined n=25) and males with complete lesions (combined n=8). The CPA experiment was performed using a third surgical cohort of animals. In this experiment 10 males had complete lesions, 19 were partial lesions and 17 were intact sham controls.

3.2.3G Lesion Specificity

In order to determine if lesions specifically targeted orexin neurons, analysis of melanocyte concentrating hormone (MCH) and Nissl stained neurons in the PFA-DMH and LHA were conducted, as these markers have been shown previously to be unaltered

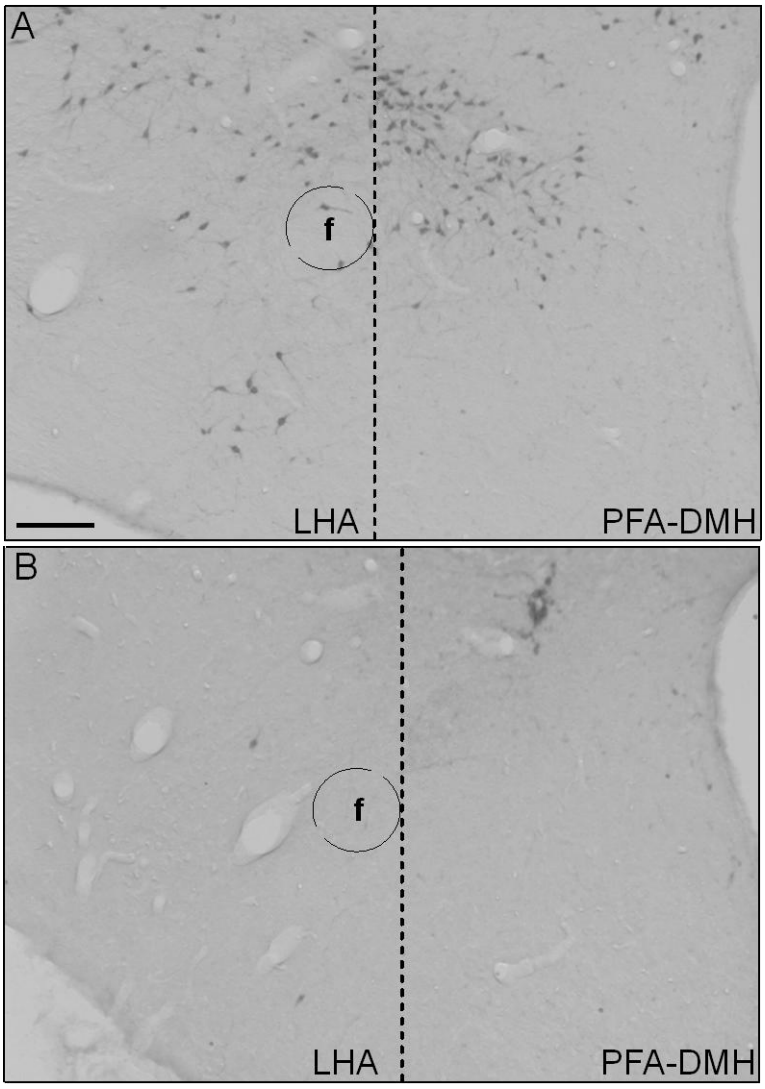

Figure 3.2. Representative images showing orexin neurons in the hypothalamus of a sham animal injected with BLANK-saporin (A) and loss of orexin neurons in the hypothalamus of a lesion animal injected with orexin-B-saporin (B). Abbreviations: PFA-DMH: perifornical-dorsomedial hypothalamus; LHA: lateral hypothalamic area; f: fornix. Scale bar: 200 µm.

following administration of orexin-B saporin (Frederick-Duus et al., 2007, Di Sebastiano et al., 2010). One series of sections from a subset of animals (n=20) was immunoprocessed for MCH, another hypothalamic peptide with overlapping location, but no colocalization with orexin neurons (Broberger et al., 1998) using a rabbit-raised antibody recognizing MCH (rabbit anti-MCH, H-070-47; 1:150,000, Phoenix Pharmaceuticals, Burlingame, CA). Analysis of MCH immunoreactivity was quantified in two bilateral sections per animal, using alternate sections to those quantified for orexin neurons, and no significant damage to the MCH population was observed, indicating lesions were specific to orexin neurons. To further verify lesion specificity representative sections from animals used for orexin cell counts (sham: n=6; lesion: n=6) were Nissl counterstained using cresyl violet. Nissl stained neurons were counted in standard areas of analysis (250 μm x 200 μm) in the general location of the orexin neuronal population and there were no significant differences in numbers of Nissl stained neurons in lesion animals compared to sham.

3.4 RESULTS

3.4.1 Orexin neuron activation by sexual reward

To determine if orexin neurons are activated during seeking of sexual reward, cFos expression was analyzed in animals that formed a mating-induced CPP and compared to unpaired control males (Figure 3.3). Exposure to the CPP apparatus resulted in a significantly greater percentage of orexin neurons that expressed cFos in paired compared to unpaired males in the PFA-DMH ($p=0.0019$) and LHA ($p=0.026$). In both

	Sham		Partial		Lesion	
	PFA-DMH	LHA	PFA-DMH	LHA	PFA-DMH	LHA
CPP	97.5 ± 2.6%	97.6 ± 3.7%	49.1 ± 3.2%	58.0 ± 2.9%	7.1 ± 2.1%	24.0 ± 2.9%
CPA	108.8 ± 3.6%	103.8 ± 3.4%	48.9 ± 3.6%	51.4 ± 3.9%	9.3 ± 2.1%	22.7 ± 4.6%

Table 3.1. Quantitative data for orexin lesion verification. The percentages of orexin neurons relative to non-surgery control males are listed for the CPP and CPA experiments. In each experiment, percentages of orexin neurons are significantly lower in the partial and lesion groups compared to sham controls in the PFA-DMH and LHA ($p<0.001$). All data represent mean percentages ± standard error of the mean (SEM). * indicates significant difference from sham control PFA-DMH; # indicates significant difference from sham control LHA. Abbreviations: PFA-DMH: perifornical-dorsomedial hypothalamus; LHA: lateral hypothalamic area; CPP: conditioned place preference; CPA: conditioned place aversion. Numbers of animals per group: CPP: Sham n=18; Partial n=25; Lesion n=8. CPA: Sham: n=17; Partial: n=19; Lesion: n=10.

unpaired and paired males, exposure to the CPP chamber resulted in higher percentages of cFos-positive orexin cells in the PFA-DMH compared to the LHA orexin population (unpaired: $p=0.003$; paired: $p<0.0001$). Although the increase in percentages of activated orexin cells in paired compared to unpaired males appeared greater in the PFA-DMH (28%) than LHA (7.6%), the fold change in orexin activation was similar in both regions (1.7 fold increase). These results indicate that orexin neurons in PFA-DMH and LHA are activated by contextual cues associated with sexual reward.

3.4.2 Mating-induced Conditioned Place Preference (CPP)

To determine if orexin is critically involved in sexual reward processing, effects of orexin lesions on mating-induced CPP were determined. Lesions did not affect any parameter of sexual behavior during the CPP pairing test (Table 2), confirming our previous report that orexin lesions do not alter sexual behavior in sexually experienced male rats (Di Sebastiano et al., 2010) However, lesion males failed to develop a conditioned preference for a mating-paired chamber, while sham and partial lesion males did form a mating-induced CPP. In particular, sham and partial lesion males, but not lesion males, showed a significant increase in preference score (percentage of time spent in the sex-paired chamber; sham: $p=0.003$; partial: $p=0.04$; Figure 3.4a) and a significant increase in difference score (time spent in the sex-paired chamber minus time spent in the control chamber; sham $p=0.005$; partial: $p=0.04$; Figure 3.4b) during the post-test. Control unpaired males that did not associate mating with either chamber of the CPP apparatus did not form a preference for either chamber, confirming that repeated exposure to the CPP apparatus did not result in preference (Figure 3.4a/b). Sham males

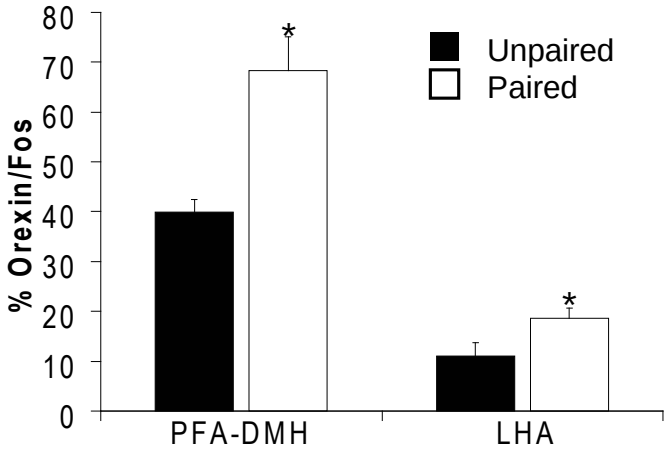

Figure 3.3. Percentages of orexin neurons expressing cFos in the PFA-DMH and LHA following exposure to the CPP apparatus in unpaired control males (black bars) and mating-paired males (white bars). All data are mean ± SEM ; n= 5 animals per group. * indicates significant difference from unpaired group.

	Sham	Partial	Lesion
Mount Latency (s)	13.0 ± 2.1	13.3 ± 2.1	12.3 ± 3.0
Intromission Latency (s)	18.5 ± 4.1	21.3 ± 5.3	24.2 ± 7.9
Ejaculation Latency (s)	449.0 ± 53.1	541.5 ± 58.4	412.1 ± 70.5
# of Mounts	6.1 ± 1.7	5.3 ± 1.0	4.3 ± 1.3
# of Intromissions	11.8 ± 1.0	11.7 ± 0.9	9.1 ± 1.8

Table 3.2. Sexual behavior during the CPP conditioning trial. All data are mean ± SEM. No significant differences between groups were detected in any parameter of sexual behavior. Numbers of animals per group: CPP: Sham n=18; Partial n=25; Lesion n=8.

spent significantly more time in the paired chamber during the post-test than pre-test, compared to control unpaired males (95.4 ± 29.7s versus 30.6 ± 13.7s; p=0.017). Lesion males did not spend more time in the paired chamber during the post-test than pre-test (-10.9 ± 56.2s). Although partial lesion males spent 67.7 ± 32.5s in the paired chamber during the post-test than pre-test, this failed to reach statistical significance.

Finally, as described in the methods, the CPP experiment was conducted in two separate surgical cohorts and in both experiments sham (n=8 and n=10 resp.) as well as partial lesioned (n=8 and n=17 resp.) males showed a significant preference (p=0.03-0.004) and difference score (p=0.04-0.005). Hence, the failure to detect significant mating induced CPP in lesion animals is not a result of a smaller number of animals in that group (n=8), but rather a result of the orexin lesions.

3.4.3 Conditioned Place Aversion (CPA)

To determine if orexin lesions blocked CPP by affecting associative learning for a particular context, males underwent a CPA paradigm. Lesion males, as well as sham and partial lesion males, formed a significant aversion for the LiCl paired chamber. Specifically all groups showed a significant decrease in preference score ($p<0.001$; Figure 3.5a) and a significant decrease in difference score ($p<0.001$; Figure 3.5b). Therefore, orexin lesions do not disrupt associative learning and memory in general.

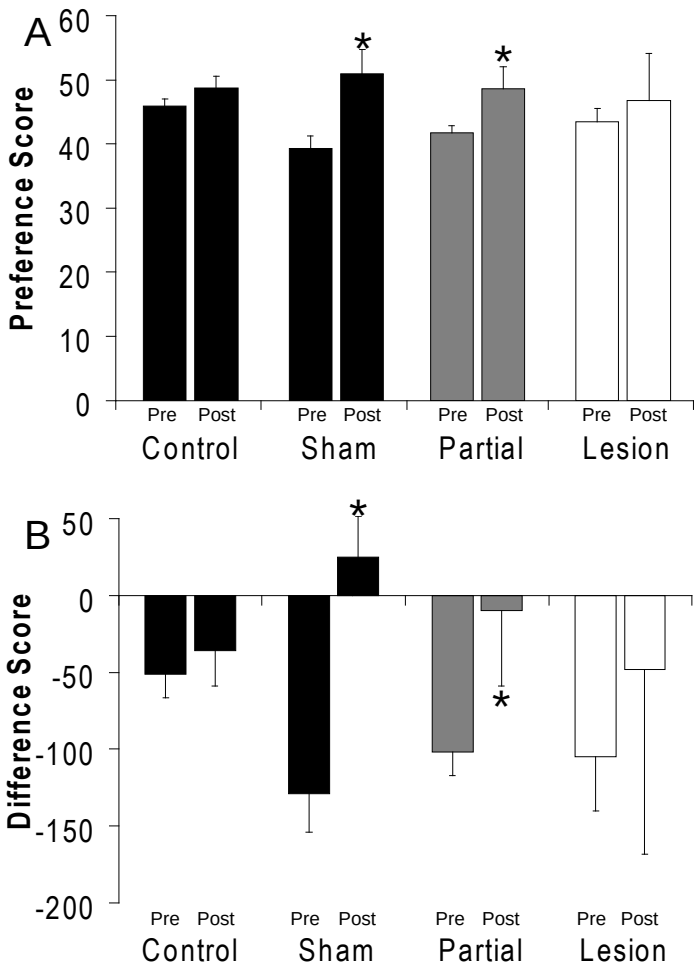

Figure 3.4. Orexin lesions prevent mating-induced CPP. (A) Percentages of time spent in the sex-paired chamber (preference score) during pre- and post-test. (B) Time (s) spent in the sex-paired chamber minus the time spent in the control chamber (difference score) during pre- and post-test. All data are mean ± SEM. * indicates significant difference from pre-test. Numbers of animals per group: Control: n=16; Sham: n=18; Partial: n=25; Lesion: n=8.

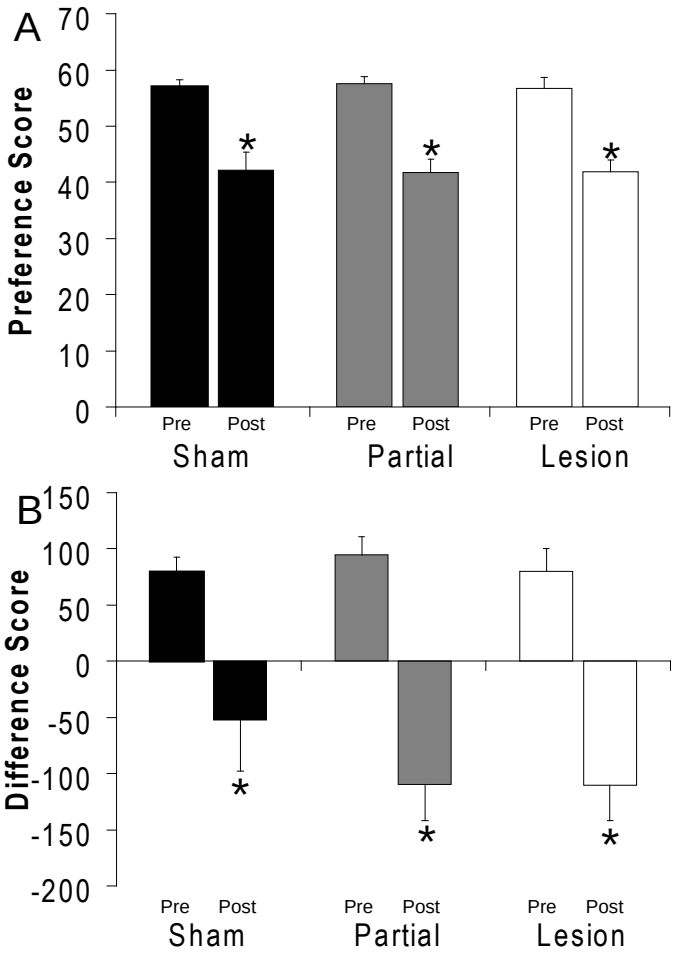

Figure 3.5. Orexin lesions do not prevent CPA for an aversive stimulus. (A) Percentages of time spent in the sex-paired chamber (preference score) during pre- and post-test. (B) Time (s) spent in the sex-paired chamber minus the time spent in the control chamber (difference score) during pre- and post-test. All data are mean ± SEM * indicates significant difference from pre-test. Numbers of animals per group: Sham: n=17; Partial: n=19; Lesion: n=10.

3.4 DISCUSSION

Results of this study showed that orexin neurons are activated during exposure to sexual reward-associated contextual signals and are critical for cue-induced seeking of sexual reward. Thus, the present results expand on a growing body of literature demonstrating a critical role for orexin in reward processing (Harris and Aston-Jones, 2006, Aston-Jones et al., 2009, Aston-Jones et al., 2010) . These results also add to findings on the role of orexin in sexual behavior and sexual reward processing. It has been shown previously that orexin neurons are activated by sexual behavior in male rats (Muschamp et al., 2007). However, endogenous orexin does not appear to be essential for sexual performance. In a previous study, it was demonstrated that orexin lesions facilitate initiation of sexual behavior in sexually naive males (Di Sebastiano et al., 2010). Orexin lesions also reduced anxiety-like behavior, hence the effect of orexin lesions on initiation of mating may be due to reduced anxiety associated with the introduction of a novel female stimulus . The facilitative effects of orexin lesions were attenuated with sexual experience and orexin lesions did not affect any parameter of sexual performance in sexually experienced male rats (Di Sebastiano et al., 2010). In addition, orexin lesions did not alter sexual motivation as determined using a straight runway test (Lopez et al., 1999, Di Sebastiano et al., 2010). In agreement, Bai and coworkers showed that ICV administration of orexin receptor antagonists did not disrupt sexual motivation (Bai et al., 2009). In the current study sexually experienced males with orexin lesions did not show impaired sexual behavior, therefore endogenous orexin does not appear to be essential for sexual performance or motivation, but may be critical for processing of sexual reward and conditioned cue-induced sexual reward seeking behavior.

The current findings that orexin is essential for sexual reward processing and specifically for mating-induced CPP are consistent with previous findings that orexin is critical for the acquisition and expression of food and drug induced CPP (Harris et al., 2005, Harris et al., 2007). In addition, orexin is essential for conditioned responses, but not for the reinforcement of cocaine (Aston-Jones et al., 2010). Specifically, the orexin receptor antagonist SB-334867 attenuated reinstatement of extinguished cocaine (Smith et al., 2009) and ethanol (Lawrence et al., 2006) seeking induced by conditioned cues, but not induced by cocaine priming (Smith et al., 2009). Perhaps along the same lines, the current study shows that orexin lesions prevented conditioned seeking of sexual reward, but did not affect initiation (seeking) or expression of sexual behavior in the presence of the receptive female. A potential alternate explanation for these findings is that orexin is involved in the acquisition or expression of associative memory in general (Jaeger et al., 2002, Aou et al., 2003, Smith and Pang, 2005, Akbari et al., 2008) and thus effects of manipulations could be caused by disruption of associative memory in the CPP paradigm, rather than disruption of conditioned reward seeking. Therefore, the current study set out to demonstrate that males with orexin lesions maintained the ability to form a conditioned aversion to lithium chloride induced visceral illness in the same contextual environment as was used for CPP. Hence, orexin lesions in the current experiment did not disrupt the ability to form associative contextual memories.

There is ample evidence supporting a dichotomy in orexin neuronal function, with PFA-DMH neurons critically involved in arousal and waking and LHA neurons involved in reward (Harris and Aston-Jones, 2006, Aston-Jones et al., 2009, Aston-Jones et al., 2010). LHA orexin neurons, but not PFA-DMH orexin neurons, are activated by

conditioned cues associated with cocaine, morphine, or food reward (Harris et al., 2007) and stimulation of LHA orexin neurons induces or reinstates conditioned preference for drug reward (Harris et al., 2005, Aston-Jones et al., 2010). The findings in the current study do not conform with this dichotomy, as neurons in both LHA and PFA-DMH were activated by cues associated with sexual reward. Other recent evidence also suggests a role for the PFA-DMH in reward processing, as this population of neurons becomes activated following exposure to contextual cues associated with cocaine reward and is involved in cocaine seeking behavior (Hamlin et al., 2008). In addition, intra-NAc injections of the μ-opioid receptor agonist DAMGO leading to high fat palatable food intake caused activation of the PFA-DMH and not LHA, further implicating the PFA-DMH may have some role in reward processing (Zheng et al., 2007). In further support, PFA-DMH orexin neurons have projections to the VTA (Fadel and Deutch, 2002, Geisler and Zahm, 2005). Future studies are needed to determine the specific role of each of the two orexin neuronal subpopulations in sexual reward processing using pharmacological manipulations rather than cell-specific lesions, as the latter resulted in lesions of both LHA and PFA-DMH.

The results of this study implicate a role for orexin in conditioned cue-induced seeking of sexual reward. However, the mechanism by which orexin contributes to this aspect of sexual function is currently unclear. Orexin activates VTA dopamine neurons (Korotkova et al., 2003, Borgland et al., 2006, Narita et al., 2006, Borgland et al., 2008) and thus it is possible that orexin-dopamine interactions may be a critical mediator of sexual reward induced CPP. However, dopamine neurotransmission does not appear to be critical for mating-induced CPP (Agmo and Berenfeld, 1990, Garcia Horsman and

Paredes, 2004, Paredes and Agmo, 2004, Ismail et al., 2009). Instead, endogenous opioids appear involved in development and expression of mating-induced CPP (Agmo and Berenfeld, 1990, Mehrara and Baum, 1990, Agmo and Gomez, 1993, Paredes and Martinez, 2001, Ismail et al., 2009). During sexual behavior, endogenous opioids are acting on μ-opioid receptors in the medial preoptic area (Coolen et al., 2004), a brain region critical for mating induced CPP (Agmo and Gomez, 1993). Moreover, in the VTA, μ-opioid receptors are activated by exposure to conditioned contextual cues associated with sexual reward (Balfour et al., 2004). Hence, orexin may be interacting with the endogenous opioid system to mediate conditioned-cue induced seeking of sexual reward. Indeed, orexin-A induced feeding behavior is blocked by opioid receptor antagonists naloxone (Clegg et al., 2002) and naltrexone (Sweet et al., 2004). In addition, ICV administration of the orexin receptor-1 antagonist SB 334867 reduced the effects of the μ-opioid receptor agonist DAMGO on high fat diet intake (Zheng et al., 2007). Orexin neurons that respond to the exogenous opioid morphine have been shown to express μ-opioid receptors (Georgescu et al., 2003) and orexin-1 antagonists block morphine induced CPP (Harris et al., 2007). Furthermore, injection of orexin-A into the hypothalamus leads to increased enkephalin release in the VTA, the paraventricular nucleus of the hypothalamus and the central amygdala (Karatayev et al., 2009) indicating a role for orexin in mediation of opioid release in the brain. Therefore with ample evidence for the role of orexin-opioid interactions in mediation of natural and drug reward, future studies may address a role for these interactions in development of mating-induced CPP.

In conclusion, the current study demonstrates a role for orexin in conditioned cue-induced seeking of sexual reward and contributes novel information to our knowledge of the role of orexin in reward processing. These findings provide further elucidation of the neural circuitry involved in natural reward and sexual reward in particular.

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CHAPTER 4

Loss of Sexual Reward Causes Depression-like Behavior in Male Rats

4.1 INTRODUCTION

Depression is mood disorder in which feelings of sadness, loss, anger, or frustration interfere with everyday life for a prolonged period of time (Krishnan and Nestler, 2008). It is estimated that 16.2% of adults in the United States will experience a major depressive episode at some point in their lifetime (Kessler et al., 2003). Depression is often triggered by stressful life events such as childhood abuse or neglect, chronic stress or chronic abuse. In addition, depression can also be caused by loss of rewarding social interactions such as death of partner, friend or relative, or loss of job (Newport et al., 2002, Henn and Vollmayr, 2005, Krishnan and Nestler, 2008, Nestler and Hyman, 2010, Krishnan and Nestler, 2011).

Animal models used to study depression frequently rely on exposing animals to stressful stimuli. Rodents subjected to early life stress such as maternal separation (Newport et al., 2002, Millstein and Holmes, 2007), chronic stress via exposure to a daily unpredictable stressor (Henn and Vollmayr, 2005, Krishnan and Nestler, 2008) or chronic social defeat by daily encounters with an aggressor (Nestler and Hyman, 2010, Krishnan and Nestler, 2011) all develop depression-like behaviors, including passive stress coping behavior, anhedonia, and increased anxiety. In contrast, there is currently no established animal model to study development of depression following loss of social reward. Monogamous pair bonded male prairie voles displayed depression-like behavior following loss of partner (Bosch et al., 2009), but few other mammals establish pair bonds (Kleiman, 1977). Therefore, the goal of the current study was to determine if loss of social reward in male rats can cause depression-like behavior. In male rats the most rewarding social interaction is sexual behavior. Males will perform operant tasks (Everitt

et al., 1987, Everitt and Stacey, 1987) climb barriers (Sheffield et al., 1951) or cross electrified grids (Moss, 1924) to gain access to a sexually receptive female, and form a conditioned place preference for sexual reward (Agmo and Berenfeld, 1990, Tenk et al., 2009).

Sexual behavior affects stress and anxiety-related behaviors, including decreased anxiety-like behavior (Fernandez-Guasti et al., 1989, Saldivar et al., 1991, Waldherr and Neumann, 2007) and decreased fear responses (Bai et al., 2009) shortly following mating. However, the effects of a prolonged period of abstinence from sexual reward on anxiety and depression-like behaviors have not been tested. Recently, it has been shown that an abstinence period from sexual reward increases vulnerability for drugs of abuse in male rats (Frohmader et al., 2010, Pitchers et al., 2010) while drug craving was not altered shortly after mating. Therefore, we hypothesize that long term loss of sexual reward will also cause vulnerability to other disorders related to reward processing, in particular depression-like behavior. The current studies demonstrate that a prolonged loss of sexual reward in male rats causes depression-like behavior, including increased passive stress coping and decreased social interaction, and support the potential development of using deprivation of this natural reward behavior in male rats as a paradigm to study depression following loss of social reward.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Adult male Sprague Dawley rats (200-250g) were obtained from Charles River Laboratories (Sherbrooke, Quebec, Canada or Wilmington, MA, USA) and were housed

in same-sex pairs for the duration of experimental testing in Plexiglas cages. To identify animals, tails were labeled with permanent marker. In all experiments, cages mates underwent the same experimental treatment. The colony room was maintained on a 12/12 reversed light-dark cycle (lights off at 10 am) with food and water available ad libitum except during behavioral testing. Female Sprague-Dawley rats were obtained from Charles River Laboratories (Sherbrooke, Quebec, Canada or Wilmington, MA, USA), were bilaterally ovariectomized and received subcutaneous implants of 5% 17-β-estradiol benzoate in silastic capsules. Females were administered a subcutaneous progesterone injection (500 μg in 0.1 ml of sesame oil) to induce sexual receptivity 4 hours prior to mating sessions. All procedures were approved by the Animal Care Committees at the University of Western Ontario and University of Michigan and conform to guidelines outlined by the Canadian Council on Animal Care and the National Institute of Health.

4.2.2 Sexual Behavior

All sexual behavior testing took place during the first half of the dark phase, 3-6 hours after lights off. Adult male Sprague-Dawley rats were randomly divided into two groups that either mated (experienced group) or were handled and remained sexually naïve (naïve group) over the course of five consecutive days. On each day, males in the experienced group were placed in a clean Plexiglas mating cage (60×45×50 cm3) for 10 minutes to acclimate. Subsequently, a sexually receptive female was placed into the cage and males were allowed to mate to one ejaculation (females were removed after completion of the post ejaculatory interval) or for 60 minutes, which ever occurred first.

Durations of the mating sessions averaged 16.7 minutes on the first trial and reduced to 8.7 minutes average on the fifth trial, reflecting the effects of sexual experience on faster initiation and completion of mating (Pitchers et al., 2012). Mating behavior was observed and analyzed as described previously (Di Sebastiano et al., 2010, Di Sebastiano et al., 2011). During each trial latencies to first mount and intromission as well as latency to ejaculation (time from the first intromission to ejaculation) were recorded. For experiments that included multiple sexually experienced groups, all groups were matched on parameters of sexual behavior, including latencies to mount, intromission and ejaculation during the final mating session as well as numbers of ejaculations during the 5 sessions. Numbers of mounts and intromissions were also recorded. Males in the naïve group were handled and placed in a clean Plexiglas cage (60×45×50 cm3) for 60 minutes in the same rooms as sexually experienced males and thus were exposed to the same levels of disturbance and to distant female odors and sounds of mating.

4.2.3 Forced Swim Test

Sexually experienced or sexually naïve males were tested for depression-like behavior following a one day, one week or one month period of abstinence from sexual behavior (n=16 per time point) or handling (n=12 per time point), using the forced swim test. The forced swim test was conducted in a large cylindrical container, 25.5 cm in diameter; 45 cm in height, filled with cold tap water (20 ± 1˚C) to a depth of 30 cm. Testing was conducted during the light phase, 2-6 hours after lights on. 1,7 or 28 days following last sexual experience or handling males underwent a pre-exposure session to the forced swim apparatus in which they were placed in the water for 15 minutes. 24

hours following this pre-test (hence 2, 8 and 29 days following last mating or handling) males underwent a test session in which they were placed in the water again for 5 minutes (Porsolt et al., 1977a, Porsolt et al., 1977b, Detke et al., 1995, Porsolt et al., 2001). Following each swimming session rats were removed from the water, towel dried and placed under a heat lamp until fur was dry. Forced swimming sessions were recorded from above with video cameras. Behavior videos were scored by an observer blind to animal's treatment using automatic timer software (Stopwatch+; Center for Behavioral Neuroscience, Atlanta, GA: (http://www.cbnatl.org/research/behavioralcore.shtml (Bosch et al., 2009). Behavior was scored as follows: Struggling (Climbing): movements when forelimbs break the surface of the water; Swimming: coordinated movements of the forelimbs and hindlimbs propelling the animal forward, without breaking the water's surface; Floating (Immobility): very slight movements of the limbs sufficient to keep the trunk at equilibrium. The percentage of time animals spent floating (immobile) during the second test is considered indicative of a passive stress coping and depression-like behavior (Porsolt et al., 1977b). Statistical significance in percentage of time animals displayed immobility was compared between naïve and experienced males using Student's t-test with a 95% confidence level.

4.2.4 Tail suspension test

The same groups of sexually experienced or sexually naïve males (1, 7 or 28 days following last sexual experience (n=16 per time point) or handling (n=12 per time point)) were then tested for depression-like behavior using the tail suspension test, 24 hours after forced swim test, during the light phase 2-6 hours after lights on. Male rats

were suspended by their tail from a hook 90 cm off the ground for one minute (Steru et al., 1985) (Chermat et al., 1986). Test sessions were recorded using a video camera. The duration of immobility, defined as passive hanging was scored using Stopwatch+ software (Bosch et al., 2009) by an observer blind to the animal's treatment as a measure of passive stress coping. Statistical significance in the percentage of time animals were immobile was compared between naïve and experienced males using Student's t-test with a 95% confidence level.

4.2.5 Social Interaction Test

Another group of sexually experienced (n=14) and sexually naïve (n=10) animals were tested for anhedonia using the social interaction test 28 days following last mating or handling, during the dark phase, 3-6 hours after lights off. The social interaction apparatus was a large plastic open field arena (84 x 42 x 30 cm) with a wire mesh box (18 x 11.5 x 8 cm) along the short wall. Males were placed into the apparatus for a 5 minute habituation period with an empty wire mesh goal box. Following habituation, males were removed from the apparatus and placed back in the home cage for 1 hour. Next, a social target animal (unfamiliar adult male Sprague Dawley rat) was placed in the wire mesh goal box and experimental males were placed back in the arena for 5 minutes to measure social interaction. Approximately 1 hour later, a sexually receptive female (ovariectomized, estradiol and progesterone-primed, adult Sprague Dawley rat) was placed in the wire mesh box and experimental males were again placed in the box for 5 minutes to measure social interaction. The social interaction arena was cleaned with 70% ethanol between trials. All social interaction sessions were recorded from above using a

video camera. Social interaction was measured using video tracking software (Stoelting Any-Maze, Wood Dale, IL) to measure time spent in the interaction zone (28 x 42 cm area around the wire mesh cage), the avoidance zone (28 x 42 cm) and the neutral zone (remaining center area of the chamber, 28 x 42 cm). Time spent in each zone was calculated and statistical differences between naïve and experienced animals were calculated using a two-way ANOVA and Tukey's test with a 95% confidence interval.

4.2.6 Sucrose Preference Test

Another group of sexually experienced (n=20) or naïve (n=20) or male rats underwent a sucrose preference test for anhedonia using methods described by (Willner et al., 1987) 28 days following last mating or handling. Males were individually housed on Day 1 of the test and were given two bottles of tap water in the home cage for 48 hours (Days 1 & 2) to allow for habituation. On Days 3 & 4 one of the water bottles were substituted for a bottle containing a 1% (w/v) sucrose solution, and animals were once again given 48 hours to habituate to drinking the sucrose solution. On Day 5, animals were food and water deprived for 6 hours during the second half of the dark phase prior to testing to maximize sucrose consumption. Bottles containing 1% sucrose or tap water were weighed and placed into the home cage for 1 hour to measure sucrose preference, during the first hour of the light phase. Following the 1 hour test bottles were removed, weighed again and sucrose preference was calculated as follows: (sucrose consumed (g)/(sucrose consumed (g) + water consumed (g) x 100). Statistical difference in sucrose consumption between naïve and experienced animals was calculated using Student's t-test with a 95% confidence level.

4.2.7 Conditioned Place Preference

Adult male Sprague Dawley rats mated (n=12) or were handled and remained sexually naïve (n=12). 28 days following last sexual experience or handing males were tested for CPP for sexual reward, using an unbiased apparatus (MED Associates, St. Albans, VT) consisting of three distinct chambers. On Day 1 (pre-test) males were given free access to the entire CPP apparatus for 15 minutes to determine initial preference for either chamber (Di Sebastiano et al., 2011). On Days 2 and 3 (conditioning trials), males were placed into a larger mating cage and mated until 1 ejaculation and were then placed into the initially non-preferred chamber (paired chamber) for 30 minutes, or were taken from the home cage and placed into the initially preferred chamber (unpaired chamber). Pairings were conducted in a counter-balanced design. A post-test procedurally identical to the pre-test was conducted on the fourth day to determine CPP. A preference score (percent of time spent in the sex-paired chamber) was calculated for each animal. Statistical significance between pre-test and post-test were compared using a two-way repeated measures ANOVA and Holm-Sidak test with a 95% confidence level.

4.2.8 Antidepressant Administration

Next, it was tested whether passive stress coping can be blocked by antidepressant administration, and hence can be considered depression-like behavior. A group of adult male Sprague-Dawley rats mated (experienced) or were handled and remained sexually naïve (naïve) over the course of five consecutive conditioning days. Mating behavior was recorded as described above and males were matched on parameters of mating behavior

and divided into 4 groups: naïve males treated with saline (n=10), experienced males treated with saline (n=12), naïve males treated with antidepressant (n=12), experienced males treated with antidepressant (n=12). 28 days following last sexual experience or handling males underwent the forced swim test. Sexually naïve or experienced males underwent a 15 minute pre-exposure session to the forced swim test and were then administered the antidepressant imipramine (Sigma-Aldrich, St. Louis MO, Cat #I7379; 10 mg/kg in 0.9% saline, intraperitoneal) or saline vehicle (10 mg/kg i.p.) 24, 5 and 1 hours before testing in the 5 minute forced swim test (29 days following last mating or handling, n=12 per group). Behavior was recorded using video cameras and analyzed as described above by an observer blind to animal's treatment. Statistical significance between naïve and experienced saline or imipramine treated males was determined using a one-way ANOVA and Fishers LSD test with a 95% confidence level.

4.2.9 Rescue of depression-like behavior by sexual experience

To determine if abstinence from sexual behavior during the 28 day period is essential for development of depression-like behavior, adult male Sprague-Dawley rats mated or were handled and remained sexually naïve over the course of 5 consecutive trials as described above. One group of experienced males (n=11) mated twice weekly during the 28 day period and were then tested for depression in the forced swim test to determine if development of depression-like behavior is dependent on an abstinence period from mating and not caused by other explanations, i.e. length of time from onset of mating behavior. These males had their final mating session on day 27, underwent the forced swim pre-test on day 28 and were tested for depression-like behavior in the forced

swim test 24 hours following the pre-test (day 29). A second group of experienced males (n=11) underwent a 27 day period of abstinence and mated to ejaculation on day 27, underwent the forced swim pre-test on day 28 and the forced swim post-test 24 hours following the pre-test, to determine if development of depression-like behavior can be rescued by a single re-exposure to sexual behavior. Finally, animals in the experienced control (n=9) and naïve control (n=8) groups were tested on the forced swim test 28-29 days following last handling or sexual experience, with no mating during the entire 28 day abstinence period. Behavior in the forced swim test was recorded and analyzed as described above. Statistical significance between groups was determined using a one way ANOVA and Fishers LSD test with a 95% confidence level.

4.2.10 Anxiety-like behavior: Elevated plus maze

As depression is often comorbid with anxiety in both humans (Kessler et al., 1994) and animal models (Berton et al., 2006) the same groups of sexually experienced (n=16 per time point) or sexually naïve (n=12 per time point) males used for the FST and tail suspension tests in 4.2.3 and 4.2.4 were tested for anxiety-like behaviors 1, 7 or 28 days following last sexual experience or handling. Anxiety-like behavior was tested using the elevated plus maze apparatus (EPM; MED Associates Inc., St. Albans, VT) during the end of the light phase in a brightly lit room, prior to the forced swim pre-test. The EPM was elevated 75 cm off the ground and consisted of 4 arms of equal length (50 cm) extending from a central junction. Two of the arms were enclosed with dark siding 40 cm high and the other two arms were open to the external environment. Males were placed on the central junction and were allowed to freely explore the EPM for five minutes and

time spent in closed and open arms was monitored using photobeam arrays. Statistical significance in time spent in open or closed arms between naïve and experienced males was determined using Student's t-test with a 95% confidence level.

4.3 RESULTS

4.3.1 Forced Swim Test and Tail Suspension Test

A 28 period of abstinence from sexual behavior caused significantly higher display of passive stress coping behavior. Sexually experienced males demonstrated a significantly higher percentage of immobility behavior in the forced swim test and tail suspension test compared to sexual naïve males 28 days following last sexual experience (Figure 4.1a; $p=0.012$ and $p=0.036$ respectively), but not 1 (Figure 4.1c) or 7 days (Figure 4.1b) following last sexual experience.

4.3.2 Antidepressant Treatment

Antidepressant administration prevented development of passive stress coping behavior following a 28 day abstinence period from sexual behavior (Figure 4.2a; $F_{(3,40)}$ 5.551; $p<0.003$). Sexually experienced males pre-treated with imipramine spent significantly less time immobile compared to experienced saline treated males (Figure 4.2a; $p<0.001$). Moreover, sexually experienced males pre-treated with saline showed increased time immobile during the post-test compared to naïve saline treated controls (Figure 4.2a; $p=0.003$), confirming previous results. These results confirm that passive stress coping behavior seen following 28 days of abstinence from mating is indeed depression-like behavior, as this behavior can be rescued by antidepressant treatment.

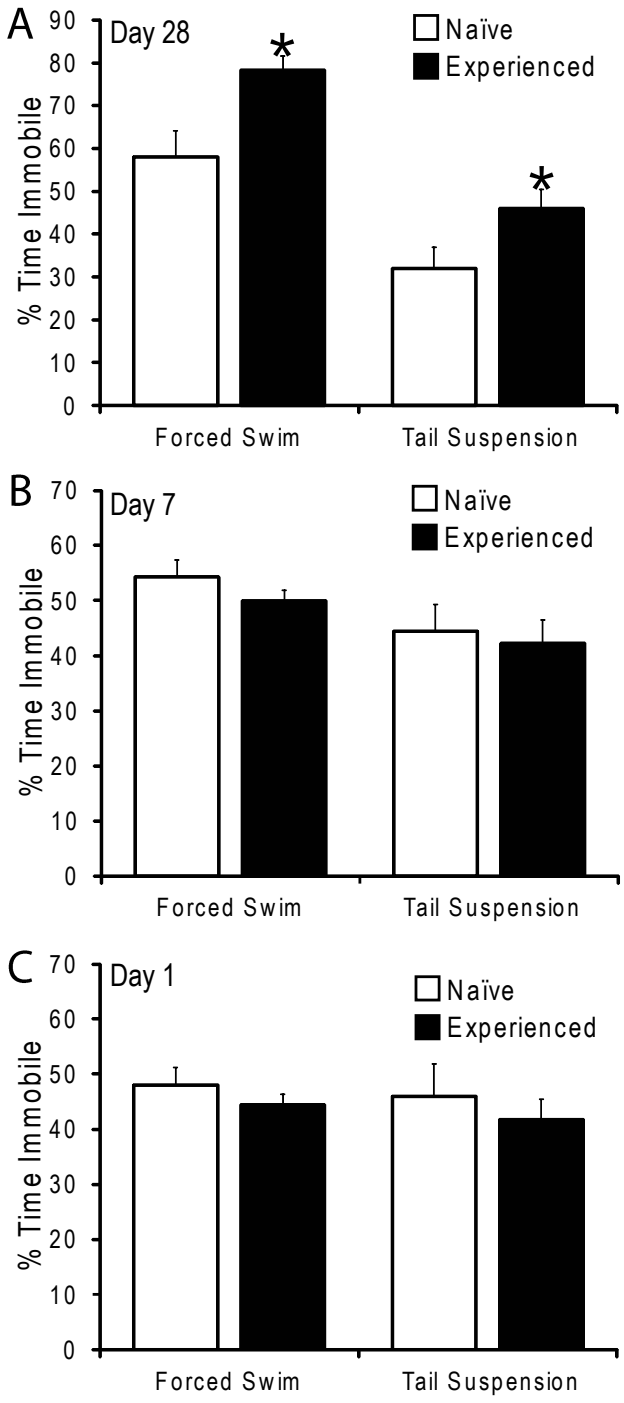

Figure 4.1. Percentage of time spent immobile in forced swim test and tail suspension test of sexually naïve (white bars) and experienced (black bars) males at 28 (A), 7 (B), or 1 (C) days following last handling or mating session. Data are presented mean ± SEM.* Indicates significant difference from naïve males in the same test and same time point.

4.3.3 Rescue of depression-like behavior by sexual experience

Development of depression-like behavior is dependent on a period of abstinence from mating, as males that mated twice weekly during the 28 day period did not develop increased passive stress coping behavior in the forced swim test (Figure 4.2b; $F_{(3,30)}$ 4.643; p=0.01). First, sexually experienced males that underwent a 28 day abstinence period from mating displayed longer duration of floating compared to sexually naïve males, confirming our results from the previous experiments (Figure 4.2b; p=0.005). In contrast, males that mated twice weekly during the 28 days following the initial 5 daily mating sessions, spent significantly less time immobile in the forced swim test compared to experienced males that underwent a 28 day period of sex abstinence (Figure 4.2b; p=0.007) and did not differ significantly in time immobile compared to naïve males. Males that underwent a 27 day period of abstinence and mated one day prior to first exposure to the forced swim test still demonstrated higher immobility compared to naïve males (Figure 4.2b; p=0.032) and experienced males that mating during the 28 day period (Figure 4.2b; p=0.041) but were not significantly different from sexually experienced males with prolonged abstinence (p=0.214), indicating that a single experience with sexual behavior following a 27 day abstinence period from mating did not rescue development of depression-like behavior. This was not due to a deficit in mating behavior, as parameters of initiation and performance did not differ after 27 days of abstinence compared to the same animals' final mating session of the five consecutive daily mating sessions (Mount Latency: Day 5, 53.6 ±14.9 s vs. Day 27 46.5± 15.7 s; p=0.67; Intromission Latency: Day 5, 54.4 ±15 s vs. Day 27, 64.2 ± 18.4 s; p=0.8; Ejaculation Latency: Day 5, 656.8 ± 137.5s vs. Day 27, 893 ±156.4; p=0.35). These results confirm facilitation of sexual behavior is maintained following a one month period

of abstinence from mating (Pitchers et al., 2012), and indicate that lack of a rescue effect of mating one day prior to testing on the forced swim test on development of depression-like behavior in sexually experienced animals is not due to impairments in copulatory performance or sexual motivation.

4.3.4 Anhedonia: Social Interaction

28 days of abstinence from mating caused anhedonia seen as decreased social interaction (Figure 4.3a; $F_{1,62}$ 9.368; p=0.003). Sexually experienced males spent significantly less time interacting with a novel male (Figure 4.3a; p=0.038) and receptive female (Figure 4.3a; p=0.03) compared to sexually naïve males. Naïve and experienced males did not differ in time spent interacting with an empty goal box during habituation (p=0.357).

4.3.5 Anhedonia: Sucrose Preference

Abstinence from sexual reward did not cause anhedonia in the sucrose preference test, as sexually experienced males did not differ significantly in volume of sucrose consumed compared to sexually naïve males 28 days following last mating or handling (Figure 4.3b).

4.3.6 Mating-induced Conditioned Place Preference

A 28 day period of abstinence from sexual reward did not affect mating-induced CPP, as sexually naïve and experienced males both formed a significant preference for a sexual reward-paired chamber (Figure 4.3c $F_{1,47}$ 8.45; p=0.008). Both sexually naïve and

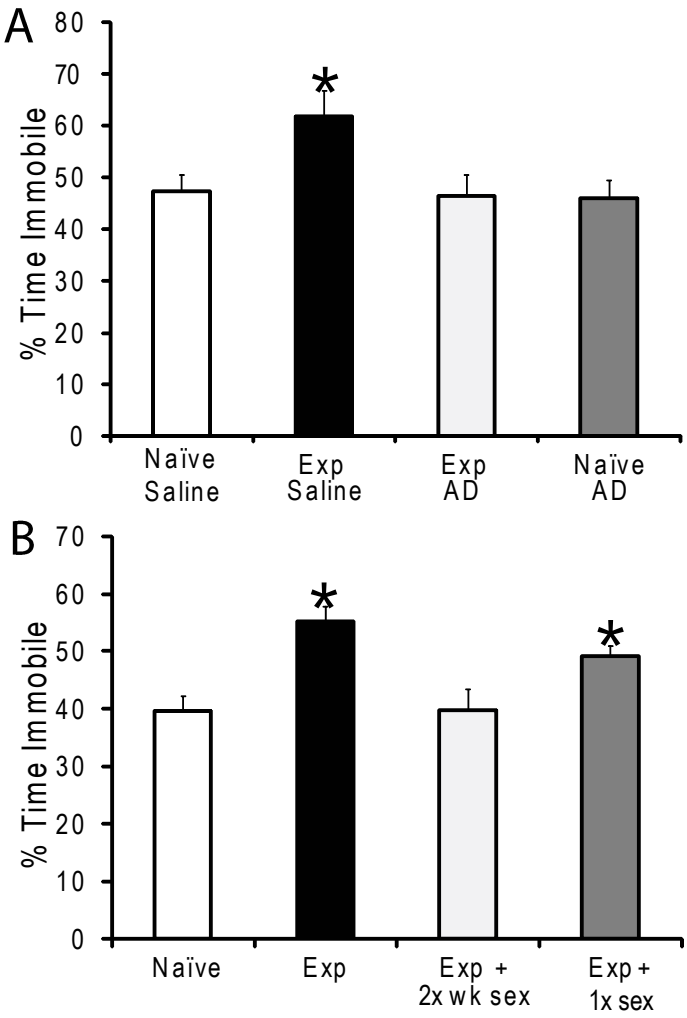

Figure 4.2. (A) Percentage of time spent immobile in saline-treated sexually naïve males (Naïve Saline) and sexually experienced males (Exp Saline); and in imipramine-treated experienced (Exp AD) and sexually naïve (Naïve AD) males. **(B)** Percentage of time spent immobile in sexually naïve males (Naïve) and sexually experienced males with a 28 day period of sex abstinence (Exp), experienced males that mated twice weekly during the 28 day period (Exp + 2x wk sex), or sexually experienced males that mated once 1 day prior to testing on the forced swim test (Exp + 1x sex). Data are presented as mean ±SEM * indicates significant difference from naïve saline (A) or naïve (B) controls.

experienced males showed a significant increase in preference score (percentage of time spent in the sex-paired chamber) during the post-test compared to the pre-test: naive: (Figure 4.3c; 0.037); experienced: (Figure 4.3c; p=0.037). Moreover, initiation or performance of sexual behavior were not affected by the 28 day abstinence period as sexually experienced males did not differ in any parameters from their final day of mating during the 5 consecutive days and were significantly facilitated compared to sexually naïve males (Mount Latency: Naïve, 559.5 ± 193.8 s vs. Experienced, 104.4 ± 18 s; p=0.02; Intromission Latency: Naïve, 813.5±228.3 vs. Experienced;161.8 ± 36.4 p=0.01; Ejaculation Latency: Naïve, 1934.3 ± 322.4 vs. Experienced, 678.75 ± 46.4; $p<0.001$).

4.3.7 Anxiety-like Behavior: Elevated Plus Maze

Neither sexual behavior nor a period of abstinence caused alterations in anxiety-like behaviors. There were no significant differences in time spent in the open arms (Figure 4.4a) and closed arms (Figure 4.4b) on the EPM between sexually naïve and experienced males 1, 7 or 28 days following last sexual experience.

4.4 Discussion

The results of these studies demonstrate that an extended abstinence period from sexual behavior; a powerful social reward behavior, causes a depression-like phenotype in male rats. Depression-like behavior was evident as enhanced passive stress-coping behavior in the forced swim and tail suspension tests. Immobility in these tests is indicative of depression-like behavior and was blocked by treatment with the tricyclic

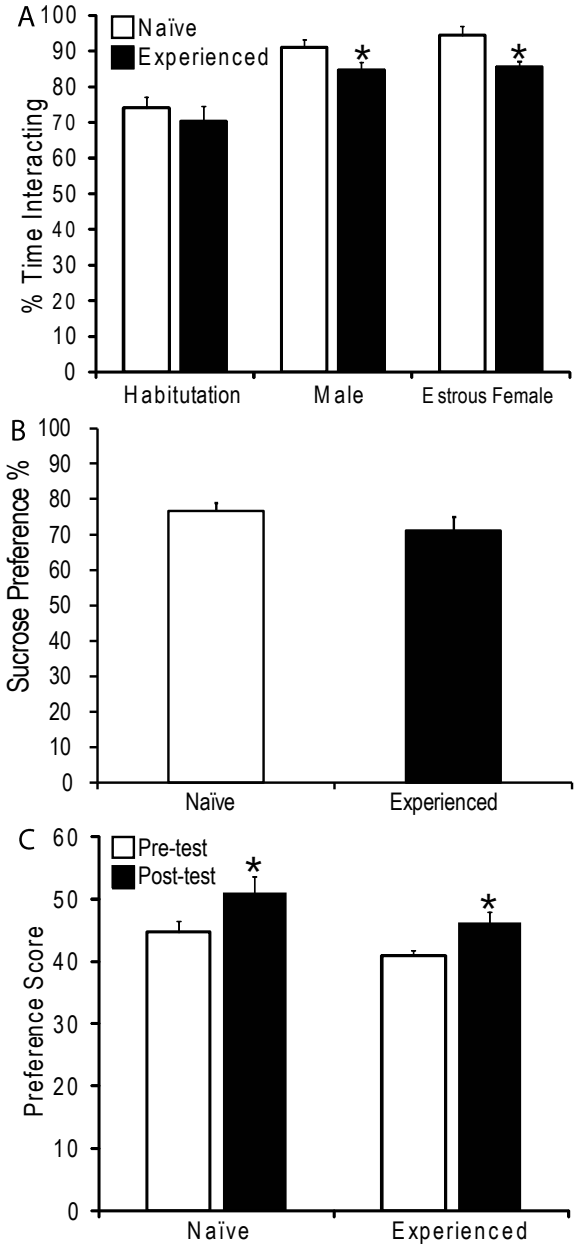

Figure 4.3 (A) Percentage of time spent interacting with an empty goal box (habituation) novel male and estrous female in the social interaction test in sexually naïve (white bars) and sexually experienced (black bars) males, 28 days following last handling or mating. **(B)** Percentage of sucrose consumed in the sucrose preference test in sexually naïve (white bars) and sexually experienced males (black bars) 28 days following last handling or mating. **(B)** Preference score: % time spent in sex reward paired chamber in the pre- and post-test during the CPP test 28 days following last handling or mating. Data are presented mean ± SEM.* Indicates significant difference from naïve males in the same test and same time point.

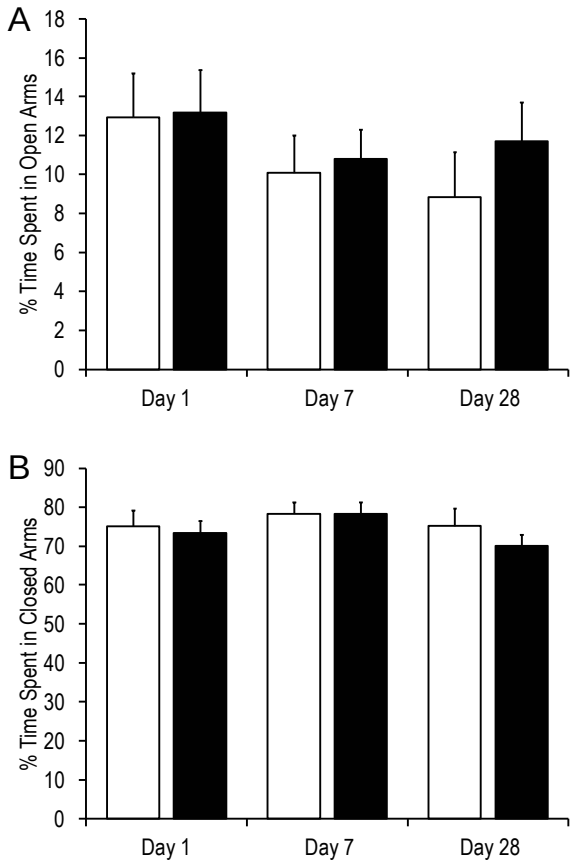

Figure 4.4. Time spent in **(A)** open arms and **(B)** closed arms on the elevated plus maze in sexually naïve (white bars) and experienced males (black bars) 1, 7, or 28 days following last handling or mating session. Data are presented as mean ± SEM.

antidepressant imipramine (Porsolt et al., 1977a, Porsolt et al., 1977b). Development of depression-like behavior was dependent on a period of abstinence from mating, as mating twice weekly during the 28 day period prevented enhanced passive-stress coping behavior in the forced swim test. Furthermore, engaging in sexual activity one time prior to testing in the forced swim test was not sufficient to rescue depression-like behavior, confirming that development of depression-like behavior requires loss of social reward, and is not caused by other explanations such as length of time following first mating session. Thus, theses studies suggest that development of depression-like behavior following loss of sexual reward is dependent on a prolonged period of abstinence from mating, and may provide a new model to study human depression following loss of social reward.

In the current studies we observed depression-like behavior, including increased passive stress coping following a prolonged abstinence period from sexual reward, comparable to depression-like phenotypes observed in stress models of depression. In the social defeat model of depression, defeated animals also demonstrate increased immobility in the forced swim test to a similar magnitude as that observed in the current study in both rats (Hollis et al., 2010) and mice (Keeney et al., 2006). Maternal separation in rats has also been shown to lead to increases in passive stress coping behavior, again to a similar magnitude as that seen in the current studies (Aisa et al., 2007, Lee et al., 2007). (Hulshof et al., 2011). Finally, in the chronic mild stress model of depression, mice and rats also demonstrate increased passive stress coping (Bielajew et al., 2003, Mineur et al., 2006). Thus, loss of sexual reward appears to induce depression-like behavior to a similar magnitude of that seen in stress models of depression, such as social defeat, maternal

separation and chronic mild stress. Alterations in passive stress coping behaviors provide an effective method for screening depression-like behaviors in rodents, due to the responsivity of the forced swim and tail suspension tests to antidepressant activity (Porsolt et al., 1977a, Porsolt et al., 1977b, Chermat et al., 1986). However, these stress models of depression are also associated with alterations in stress axis activity (Ayensu et al., 1995, Francis et al., 1999, Francis et al., 2002, Kieran et al., 2010), as well neuropathology similar to that observed in human depression (Francis et al., 1999, Stout et al., 2000, Keeney et al., 2006, Marini et al., 2006). Thus, loss of sexual reward may also lead to alterations in stress responsivity and underlying neuropathology of depression.

Loss of sexual reward also caused anhedonia, another hallmark of depression (Nestler and Hyman, 2010) seen as decreased social interaction with a novel male and an estrous female 28 days following last mating. However, social defeat appears to induce a higher magnitude of social avoidance than the current study, in both rats (Hollis et al., 2010) and mice (Berton et al., 2006). This difference in magnitude is likely due to the fact that in the social defeat paradigm, animals are chronically being attacked by another conspecific, and may thus develop a higher level of avoidance, whereas in the current study decreased social interaction more likely reflects a loss of motivation for social interaction. The effects of maternal separation in the social interaction test are unclear, as maternally separated animals have been shown to have decreased social interaction (Maciag et al., 2002), while other studies have shown that maternal separation does not alter social interaction (Hulshof et al., 2011). Finally, it has been shown that animals

exposed to the chronic mild stress paradigm do not show alterations in the social interaction test (D'Aquila et al., 1994).

Loss of sexual reward did not cause reduced sucrose preference. The chronic mild stress model of depression has been shown to reliably induce anhedonia in the sucrose preference test following long-term exposure to stressors (Willner et al., 1987, Pucilowski et al., 1993, Willner, 2005). Likewise, chronic social defeat in rats (5 consecutive weeks of defeat) (Rygula et al., 2005) or mice (10 consecutive days of defeat) (Krishnan et al., 2007) causes decreased sucrose preference. In contrast, rats exposed to shorter term social defeat (4 consecutive days) did not demonstrate anhedonia in the sucrose preference test (Hollis et al., 2010), even though they did develop increased passive stress coping in the forced swim and tail suspension tests, as well as decreased social interaction. Moreover, male rats exposed to maternal separations do not demonstrate decreased sucrose preference (Matthews et al., 1996, Shalev and Kafkafi, 2002). These results suggest that development of anhedonia in the sucrose preference test requires long term exposure to a stressor and provide a potential explanation for the lack of decreased sucrose preference in the current study. Moreover, we hypothesize that lack of anhedonia for sucrose in the current study may be due to the need in this paradigm to expose the animals to the rewarding sucrose stimulus for 2 days prior to testing, potentially preventing development of anhedonia.

Another measure of anhedonia is animal's interest in pleasurable activities such as sexual behavior (Nestler and Hyman, 2010). We have demonstrated that following a 28 day period of abstinence from mating males do not display decreased sexual motivation or performance (Pitchers et al., 2012), and have unaltered sexual reward. Thus, these

results suggest loss of sexual reward induces anhedonia in the form of reduced social interaction, but there is no loss of reward associated with the consumption of a rewarding substance or consummatory behavior with a rewarding stimulus. Hence, abstinence from sexual reward induced decreased "wanting" or incentive motivation for a salient stimulus, but did not alter the "liking" or reward for the consumption of the salient stimulus (Robinson and Berridge, 1993). In contrast, males that have been socially defeated demonstrate decreased attempts to copulate with a receptive female (Rygula et al., 2005). In the chronic mild stress paradigm, sexually experienced males show impairments in copulatory performance seen as increased latencies to mount and intromission, and decreased frequency of mounts and ejaculations (Gronli et al., 2005). Maternally separated males are also impaired in their copulatory performance during their first mating trial in adulthood, as latencies to mount and intromission are lengthened, and ejaculation frequency is decreased (Rhees et al., 2001). Likewise, human depressed patients often lose interest in sex and demonstrate sexual dysfunction (Mathew and Weinman, 1982, Dell'Osso et al., 2009, Galecki et al., 2011). However, depressed humans have also been reported to engage in sexual risk behaviors, including multiple partners and decreased condom use (Shrier et al., 2001, Khan et al., 2009). In our lab, we have shown that 28 days of abstinence from mating not only causes depression-like behavior, but also causes increased vulnerability for drugs of abuse (Pitchers et al., 2010). As sexual risk behavior is also associated with drug taking (Peugh and Belenko, 2001, Rawson et al., 2002, Raj et al., 2007, Fisher et al., 2011), and depressed patients have a high incidence of substance abuse (Volkow, 2004), loss of sexual reward may be a valuable model to study comorbid depression and substance abuse.

Abstinence from mating did not result in anxiety-like behavior. Thus, in contrast to the anxiolytic effects of sexual behavior shortly following mating, there do not appear to be long term effects of sexual behavior on anxiety. Moreover, this finding is in contrast to the induction of anxiety in other depression models, in which animals are exposed to stressors. Stress models of depression such as social defeat, chronic stress or maternal separation all lead to alterations in anxiety-like behavior as well as in depression-like behavior (Daniels et al., 2004, Vyas and Chattarji, 2004, Krishnan et al., 2007). As an abstinence period from sexual behavior is not a stressor such as attack, restraint, cold, or physical separation from the mother, it does not cause anxiety-like behavior. Moreover, in the current studies males were housed with a same sex cage mate and some environmental enrichment during the entire abstinence period, to avoid social isolation stress, which has also been shown to cause anxiety and depression-like behavior (Karim and Arslan, 2000, Dandekar et al., 2009, Djordjevic et al., 2012). Thus, loss of sexual reward appears to be an animal model of depression without anxiety that is not caused by stress or agonistic attacks. Even though in humans anxiety can be comorbid with depression, this is not the case for all individuals suffering depression (Prigerson et al., 1995, Kessler et al., 1996)

Recently it was shown that monogamous, pair bonded male prairie voles that are separated from their partner for 3-5 days develop depression-like behavior, seen as increased immobility in the forced swim and tail suspension tests (Bosch et al., 2009). These males also demonstrated increased anxiety-like behavior, seen as decreased numbers of entries into the open arms of the EPM (Bosch et al., 2009). However males that were separated from a same sex sibling partner also demonstrated decreased entries

into the open arms (Bosch et al., 2009), a suggesting that effects on anxiety may be due to the fact that these males are socially isolated during partner separation. In order to form a pair bond males prairie voles were housed for five consecutive days with a female, during which mating behavior occurred (Bosch et al., 2009). In the current studies males were only housed with females during a once daily mating session for course of 5 consecutive days. Mating sessions lasted on average 16.7 minutes on the first trial, and were reduced to 8.7 minutes by the fifth trial, suggesting that development of depression-like behavior following loss of sexual reward requires only brief daily exposure to sexual behavior, as opposed to long-term cohabitation with a female. Moreover, in the current studies, development of depression-like behavior was observed following a prolonged (28-29 day) abstinence period from sexual behavior, and not at earlier timepoints, (1 or 7 days), whereas prairie voles separated from their partner developed depression depression-like behavior following 3-5 days of partner separation (Bosch et al., 2009). It is currently unknown whether depression-like behavior following partner separation is maintained at longer intervals of partner separation.

In conclusion, the current study demonstrates that loss of sexual reward causes depression-like behavior in the absence of chronic stressors or social isolation. The primary difference of the current findings compared to established rodent models for depression is that development of depression in the latter are dependent on chronic stress or long-term exposure to agonistic attacks. Instead, the findings presented here may resemble depression induced by loss of socially rewarding events and we propose this to be a useful model to study depression-like behavior following loss of social reward in rodents. This model meets criteria to validate animal models of depression, as loss of

social reward causes anhedonia and passive stress coping, giving this model homology to symptomology seen in human depression or *face validity* (Krishnan and Nestler, 2010). Furthermore, this model has *pharmacological validity* (Krishnan and Nestler, 2010) as passive stress coping following loss of social reward can be blocked by antidepressants. This model also displays *construct validity* (Krishnan and Nestler, 2010), as development of depression-like behavior is dependent on a period of abstinence from mating, and as such requires loss of social reward, similar to development of depression-like behavior following loss of social rewards in humans. Moreover, abstinence from sexual reward has also been shown to cause increased vulnerability for psychostimulant abuse in male rats (Pitchers et al., 2010) and increased ethanol consumption in drosophila (Shohat-Ophir et al., 2012). Given the comorbidity of depression and substance abuse, it will be important to determine which neural alterations are caused by loss of sexual/social reward to cause such behavioral pathology.

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CHAPTER 5

Loss of Sexual Reward Causes Enhanced Stress Reactivity in Male Rats

5.1 INTRODUCTION

In humans, mood disorders such as depression can be triggered by stressful life events (Kessler, 1997, Brown, 1998, Kendler et al., 1999, Pine et al., 2002). Stress activates the hypothalamic-pituitary-adrenal (HPA) axis, causing release of corticotropin releasing factor (CRF) from the paraventricular nucleus of the hypothalamus (PVN), which stimulates the anterior pituitary to secrete adrenocorticotropic hormone (ACTH), leading to release of cortisol (humans) or corticosterone (cort; rodents) from the adrenal glands. These adrenal steroids then regulate the stress response via negative feedback and activation of glucocorticoid receptors in hypothalamus (Jones et al., 1977, Keller-Wood and Dallman, 1984, Evanson et al., 2010), pituitary (Cole et al., 2000, Russell et al., 2010), hippocampus (Sapolsky et al., 1984, Jacobson and Sapolsky, 1991, Furay et al., 2008), and prefrontal cortex (PFC) (Hill et al., 2011, Radley and Sawchenko, 2011). CRF is also produced and released from neurons in extra-hypothalamic brain regions, namely the bed nucleus of the stria terminalis (BnST) and central amygdala (CeA) (Koob and Heinrichs, 1999, Bale and Vale, 2004). CRF acts on two receptors, CRF receptor 1 (CRFR1) and CRF receptor 2 (CRFR2) (De Souza, 1987, Bale and Vale, 2004) expressed in areas such as the nucleus accumbens (NAc) and prefrontal cortex (PFC) (Van Pett et al., 2000), brain areas that are critical for mediation of the emotional aspects of the stress response (Menzaghi et al., 1993, Morgane et al., 2005, Jankord and Herman, 2008). CRFR1 and CRFR2 have been shown to play opposite roles in mediation of the stress response, with CRFR1 inducing stress responses and CRFR2 attenuating stress responses (Bale and Vale, 2004, Contarino and Papaleo, 2005).

Human depression is associated with hyperactivity of the HPA axis (Holsboer, 2000). Depressed patients show increased CRF levels in the cerebrospinal fluid (Nemeroff et al., 1984) as well as increased CRF mRNA in the hypothalamus (Kling et al., 1991). In addition, depressed suicide patients show atrophy of the PFC and NAc (Manji et al., 2001) as well as decreased CRF receptor binding sites in the PFC (Nemeroff et al., 1988). In animal models of depression that are dependent on exposure to stressful stimuli, such as social defeat, chronic stress or maternal separation these animals show increased CRF mRNA in the PVN (Francis et al., 1999, Stout et al., 2000, Keeney et al., 2006, Marini et al., 2006). Furthermore, development of depression-like behavior in the chronic stress model of depression can be blocked by systemic administration of CRF receptor antagonists (Ducottet et al., 2003), while intra-NAc infusion of CRF induces depression-like behavior (Chen et al., 2012). Both CRFR1 and CRFR2 have been shown to play a role in development of depression, again with some evidence for opposing roles, with a pro-depressant role for CRFR1 activation and anti-depressant effects of CRFR2 activation. (Shaham et al., 1998, Hammack et al., 2003, Wang et al., 2005, Henry et al., 2006, Wang et al., 2006, Wang et al., 2007).

In the previous chapter (Chapter 4), it was demonstrated that an extended (28 day) period of abstinence from sexual behavior caused depression-like behavior, including enhanced passive stress-coping behaviors and decreased social interaction. However, it is unknown if the CRF system plays a regulatory role in these behavioral alterations. Therefore, the goal of the current studies was to test the hypothesis that loss of sexual reward leads to increased HPA axis activity, including increased CRF levels in the PVN as well as enhanced cort and ACTH release in response to an acute stressor. In addition,

we tested the hypothesis that the CRF system is critical for the development of depression-like behavior following loss of sexual reward by chronically infusing CRF receptor antagonists either into the cerebroventricular system or locally into the NAc or PFC, mesolimbic brain regions that are important for development of depression (Krishnan and Nestler, 2008), during the final week of mating abstinence.

5.2 MATERIALS MATERIALS

5.2.1 Animals

Adult male Sprague Dawley rats (200-250g) were obtained from Charles River Laboratories (Sherbrooke, Quebec, Canada or Wilmington, MA, USA). For the duration of experimental testing males were housed in same-sex pairs in Plexiglas cages. To identify animals, tails were labeled with permanent marker. In all experiments, cage mates underwent the same experimental treatment. The colony room was maintained on a 12/12 reversed light-dark cycle (lights off at 10 am). Food and water were available *ad libitum* except during behavioral testing. Female Sprague-Dawley rats were obtained from Charles River Laboratories (Sherbrooke, Quebec, Canada or Wilmington MA, USA). Females were ovariectomized bilaterally and were implanted subcutaneously with 5% 17-β-estradiol benzoate in silastic capsules. 4 hours prior to mating sessions females were administered a subcutaneous progesterone injection (500 μg in 0.1 ml of sesame oil) to induce sexual receptivity. All procedures were approved by the Animal Care Committees at the University of Western Ontario and University of Michigan and conform to guidelines outlined by the Canadian Council on Animal Care and the USA National Institute of Health.

6.2.2 Sexual Behavior

Testing for sexual behavior took place 3-6 hours after lights off, during the first half of the dark phase. Over the course of five consecutive days, adult male Sprague-Dawley rats were either mated (experienced group) or were handled and remained sexually naïve (naïve group). Males in the experienced group were placed in a clean Plexiglas mating cage (60×45×50 cm) on each of the five conditioning days for 10 minutes. Following the 10 minute habituation, a sexually receptive female was placed into the cage and males were allowed to copulate ejaculation or for 60 minutes. Mating behavior was recorded and analyzed as previously described (Di Sebastiano et al., 2010, Di Sebastiano et al., 2011). Latencies to first mount and intromission and latency to ejaculation were recorded during each trial as well as numbers of mounts and intromissions. Males in the sexually naïve group were placed into clean Plexiglas cages (60×45×50 cm3) for 60 minutes, in the same rooms and at the same time as the experienced males were allowed to mate and were thus exposed to the same handling and environment, but without mating.

5.2.3 Activation of CRF neurons by sexual behavior

5.2.3A Tissue Collection

To determine if CRF neurons are activated by sexual behavior, cFos expression was determined after mating in sexually naïve or experienced males. Adult male Sprague-Dawley rats were divided into experimental groups as follows: Naïve no sex (NNS): handled for five consecutive days. Experienced no sex (ENS): mated to one ejaculation on five consecutive days. Both of these groups were perfused 24 hours after

last handling or mating from the home cage and served as controls. Naïve sex (NS): handled for four consecutive days, and mated to one ejaculation on fifth day. Experienced sex (ES): mated to one ejaculation on five consecutive days (n=5 per group). Both of these groups were perfused one hour following the end of mating. Males were perfused transcardially with 0.9% saline followed by 500 mL of 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB). Brains were quickly removed and post-fixed for one hour in the same fixative, then transferred to 20% sucrose for cryoprotection. Brains were sectioned into 35 µm coronal sections on a freezing microtome (Microm, Walldorf, Germany) and collected in 4 parallel series in a cryoprotectant solution (30% sucrose in 0.1M PB containing 30% ethylene glycol and 0.01% sodium azide) and were stored at -20ºC until further processing.

5.2.3B Immunohistochemistry

One series of sections for each animal was immunoprocessed for CRF and cFos as a marker for neural activation using immunohistochemistry. Free floating sections were washed extensively with 0.1M saline buffered sodium phosphate (PBS) between incubation steps and all incubations were performed at room temperature using gentle agitation. Sections were pre-incubated for 10 minutes using 1% H_2O_2 (30% stock solution) in PBS and for 1 hour in the incubation solution (PBS with 0.1% bovine serum albumin and 0.4% Triton X-100). Sections were incubated overnight with a rabbit raised antibody recognizing cFos (rabbit anti-cFos, SC52; 1:10,000 in incubation solution, Santa Cruz Biotechnology, Santa Cruz, CA), followed by biotinylated goat anti-rabbit (1 hour; 1:500 in incubation solution, Vector Laboratories, Burlingame, CA) and avidin

horseradish peroxidase complex (ABC; 1 hour; 1:1000 in PBS; Vector Laboratories, Burlingame, CA). cFos-immunoreactivity was visualized using nickel-enhanced diaminobenzidine (DAB; Sigma, St. Louis, MO; 0.02% in 0.1M PB containing 0.012% hydrogen peroxide (Fisher, Pittsburg, PA) and 0.08% nickel sulfate (Sigma, St. Louis, MO) resulting in a blue-black reaction product. Sections were next incubated overnight with a rabbit raised antibody recognizing CRF (rabbit anti-CRF, T-4036 1:2,000, Peninsula Labs, San Carlos CA) followed biotinylated goat anti-rabbit and ABC, as described above. CRF immunoreactivity was visualized with DAB (10 minutes; 0.02% in 0.1M PB containing 0.012% hydrogen peroxide). Both antibodies have been characterized previously (Bovetto et al., 1996, Di Sebastiano et al., 2010, Di Sebastiano et al., 2011). Following completion of staining sections were rinsed extensively with PB, mounted onto plus charged glass slides and coverslipped with dibutyl phthalate xylene (DPX).

5.2.3C Analysis

Neurons labeled for CRF or CRF and cFos were counted bilaterally in all sections containing CRF positive neurons in the, PVN, CeA and BnST using a Leica microscope (DMR5; Leica, Walldof, Germany). Counts were averaged per hemisphere for each animal, group means were calculated and statistical significance between naïve and experienced groups was determined using a two-way ANOVA with a 95% confidence level.

5.2.4 Changes in CRF mRNA following mating abstinence

5.2.4A Tissue Collection

Adult male Sprague Dawley rats gained sexual experience over the course of five consecutive mating trials (n=10) or were handled and remained sexual naïve (n=10). 1 or 28 days following last sexual experience or handling males were euthanized with sodium pentobarbital (270mg/kg); brains were rapidly removed and flash frozen on dry ice in sterile RNAse free conditions. Microdissections of the BnST, CeA and PVN were performed by isolating tissue with a sterile blunt end needle from frozen coronal sections.

5.2.4B mRNA isolation and real-time polymerase chain reactions

Tissue punches were homogenized and RNA was isolated using TriZol reagent (Invitrogen, Carlsbad CA) for quantitative real time reverse transcription polymerase chain reaction (qRT-PCR). RNA was checked for concentration and quality using spectrophotometer (Nanodrop; Thermo Scientific, Wilmington, DE). Reverse transcription of RNA was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA) according to manufacturers' instructions and a BioRad Thermal Cycler (Hercules, CA), using equal amounts of RNA for each sample. Quantitative analysis of gene expression was determined using real-time qRT-PCR. Each reaction for was performed with TaqMan chemistry using 2µL of cDNA, 10µL of TaqMan Universal PCR Master Mix 2X (Applied Biosystems Inc., Foster City, CA), 0.8µL of 25mM MgCl2 (Invitrogen, Carlsbad CA), 6.2µL DEPC and 1µL of TaqMan gene expression assay solution for the gene of interest. qRT-PCR reactions were performed for CRF (Rn01462137_m1), and glyceraldehydes-3-phosphate dehydrogenase

(GAPDH; Rn99999916_s1) in the PVN, CeA and BnST. Reactions were performed using the Rotorgene (Corbett Life Sciences, Valencia CA) and the following PCR conditions: hold at 50ºC for 2 min, hold at 95ºC for 10 min, 40 cycles of 95ºC for 15 sec and 58ºC for 1 min. Cycle threshold (Ct) levels were determined using a optimal threshold line generated from standard curve 5-log dilutions of cDNA from the brain region of interest for each probe. Standard curves were considered optimal when a line of best fit through the Ct values of the dilutions had an efficiency value between 0.8-1 and an R2 greater than 0.99. Each sample was analyzed in triplicate and Cts for each experimental sample were determined and averaged over the replicates. CRF levels within a sample were normalized to their respective GAPDH levels to generate the ΔCt value. The ΔΔCt value was calculated as 2^(ΔCt (sample) - ΔCt (average of naïve control group)) x100 and, CRF in each animal was expressed as a change over the average of the naïve control group within each time point. Statistical significance between naïve and experienced groups were calculated using Student's t-test with a 95% confidence level.

5.2.5 Acute Restraint Stress and Blood Collection

Sexually experienced (n=12) or naïve (n=10) males underwent acute restraint to measure stress responses according to methods described previously (Vahl et al., 2005). All stress testing was conducted during the light phase, 2-5 hours after lights on. 28 days following last mating or handling males were transported to a testing room and placed in a cylindrical polyethylene tail access restrainer (Model #51335, Stoelting, Wood Dale IL). Immediately, a small laceration was made at the base of the tail and a baseline blood sample was collected in an eppendorf tube filled with 10µL of 100µM EDTA. Males

were then restrained for 29 minutes, after which another blood sample was collected (30 minutes, end of restraint). Males were placed back in the holding cage and blood was again collected 60 and 120 minutes post restraint. All blood samples (200-400µL) were collected within 1-2 minutes and immediately placed on ice. Blood samples were centrifuged; plasma was extracted and stored at -20ºC until further processing. Cort and ACTH were measure using ImmuChem™ 125I radioimmunoassay (RIA) kits (cort, cat #07120102; ACTH, cat # 07106102; MP Biomedicals, Solon, OH) according to manufacturer's instructions. The cort assay required 10µL of plasma and intra- and interassay coefficients of variation were 7.1% and 7.2%. The ACTH assay required 100µL of plasma and had intra- and interassay coefficients of variation of 4.1% and 3.9%. Statistical differences in cort and ACTH levels between naïve and experienced males over time was calculated using a two way repeated ANOVA (factors: experience and time) and Tukey's test, with a 95% confidence level.

5.2.6 Chronic ICV CRF receptor antagonist administration via osmotic minipumps

5.2.6A Surgery

Adult male Sprague-Dawley rats mated (experienced) or were handled and remained sexually naïve (naïve) over the course of five consecutive mating trials. Experienced groups were matched on parameters of sexual behavior. 21 days following last sexual experience males were implanted with an osmotic minipump (Model 2001, Alzet, Cupertino CA). Pumps were filled 12 hours prior to implantation, with CRF receptor 1 antagonist: CP 154-526 (n=8) 1mg/mL, in 0.45% acetic acid vehicle in sterile saline; Cat. # 2779, Tocris Bioscience, Ellisville MO), CRF receptor 2 antagonist:

Antisauvagine-30 (n=7) 2mg/ml in saline; Cat # 2071, Tocris Bioscience, Ellisville MO), or vehicle (acetic acid n=9 or saline n=10). Pumps were connected to the brain infusion cannula with vinyl catheter tubing (Alzet, Brain Infusion Kit 2, Cupertino CA) and stored in 37°C incubator with cannulas in sterile saline. Male rats were anesthetized with isoflurane (MWI, Boise ID) in a Surgivet Isotec4 gas apparatus (Smiths Medical Vet Division, Markham, Ontario, Canada), secured in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) with a gas mask secured over the nose and mouth to maintain anesthesia. An incision was made to expose the skull, lambda and bregma were found determined to be level. A hole was drilled into the skull using a Dremel drill (Dremel, Racine, WI) at coordinates relative to bregma: AP= -0.5mm, ML= 1.5mm, DV= -3.9mm. The unilateral brain infusion cannula (Alzet, Brain Infusion Kit 2, Cupertino CA) was implanted and secured with dental cement (Lang Dental Manufacturing Company, Wheeling IL) and the osmotic minipump was implanted (Alzet, Model 2001, Cupertino CA) under the flank. Surgical incisions were closed with wound clips and males were allowed one week to recover from surgery during which osmotic minipumps chronically infused vehicle, CRFR1 antagonist or CRFR2 antagonist at a rate of 1.0μL/hr for the remaining 6.5 days (infusions thus ended on day 27).

5.2.6B Forced Swim Test

One day after termination of chronic infusion and 28 days following last mating session, males were tested for depression-like behavior in the forced swim test. The forced swim test was conducted during the light phase, 2-6 hours after lights on. The forced swim apparatus was a large cylindrical container, 25.5 cm in diameter, 45 cm in

height, and was filled with cold tap water (20 ± 1˚C) to a depth of 30 cm. 28 days after last mating or handling, rats were pre-exposed to forced swim test and were placed in the water for 15 minutes. 24 hours following pre-exposure (thus, 29 days following last mating or handling) males underwent the test session and were placed in the water for 5 minutes (Porsolt et al., 1977a, Porsolt et al., 1977b, Detke et al., 1995, Porsolt et al., 2001). After swimming sessions were completed, males were removed from the water; towel dried and placed under a heat lamp until fur was dry. All forced swimming sessions were recorded from above. Behavior was scored using automatic timing software (Stopwatch+; Center for Behavioral Neuroscience, Atlanta, GA: (http://www.cbnatl.org/research/behavioralcore.shtml) (Bosch et al., 2009) by an observer blind to the animal's treatment. Behavior was scored as follows: Struggling (Climbing): movements of both the fore- and hindlimbs that break the surface of the water; Swimming: movements that propel the animal forward, requires coordinated movement of both the fore- and handlings. Floating (Immobility): slight movements of the fore- and hindlimbs to keep body at equilibrium, with very little movement. Percentage of spent floating (immobile) during the second test is indicative of a passive stress coping and depression-like behavior (Porsolt et al., 1977b). Experimental groups included: naïve males treated with vehicle (saline; n=4 or 0.45% acetic acid vehicle; n=5; n=9 total), experienced males treated with vehicle (saline; n=5 or 0.45% acetic acid vehicle; n=5; n=10 total), experienced males treated with CRFR1 antagonist (n=9), experienced males treated with CRFR2 antagonist (n=8). Statistical significance in time spent immobile between groups was determined using a one-way ANOVA and Fisher's LSD test with a 95% confidence level.

5.2.6C Tissue collection real time qPCR

To determine if chronic administration of a CRFR1 antagonist during the final week of mating abstinence blocked the increase in CRF mRNA levels in the PVN observed following a 28 day period of abstinence from mating, males were overdosed with sodium pentobarbital (270mg/kg); brains were rapidly removed and flash frozen on dry ice in sterile RNAse free conditions. Microdissections of the PVN were performed, and qRT-PCR for CRF was conducted as described above.

5.2.7 Acute ICV CRF receptor antagonist administration

As males in the osmotic minipump experiment were tested in the forced swim test without antagonist infusions at the time of testing, another group of males was used to test the effects of acute CRFR1 antagonist administration on depression-like behavior. Adult male Sprague-Dawley rats mated or were handled and remained sexually naïve over the course of five consecutive mating trials. Experienced groups were matched on parameters of sexual behavior. All males were implanted with a 21 gauge unilateral guide cannula in the lateral ventricle (Plastics One, Roanoke VA), using the same stereotaxic surgical techniques and coordinates as described above. During the 1 week surgical recovery period, males were handled daily to habituate them to the process of intracranial injections. Following 1 week recovery from surgery males received a single intracranial infusion 15 minutes prior to the testing on the second day of the forced swim test session of vehicle (5 µL; 0.45% acetic acid in sterile saline; n=8) or CRFR1 antagonist, CP 154-526 (n=8) 1mg/mL, in 0.45% acetic acid vehicle in sterile saline; Cat. # 2779, Tocris Bioscience, Ellisville MO). Infusions were conducted using a microinfusion pump

(Harvard Apparatus, Holliston, MA) connected to a 26 gauge microinjector (4.1mm with 0.5 mm projection, Plastics One, Roanoke VA) over a 1 minute period after which microinjectors were left in place for 1 minute to allow diffusion. Behavior was recorded and scored as described above. Statistical significance in percentage of time spent immobile between groups was calculated using a one-way ANOVA and Fishers LSD test with a 95% confidence level.

5.2.8 Local Manipulations CRFR1 in Nucleus Accumbens or Prefrontal Cortex

Local manipulations of CRFR1 were conducted in a manner procedurally identically to described above, but bilateral brain infusion cannulas (Plastics One, Roanoke VA) were directed at the NAc (coordinates relative to AP = 1.7 mm, ML= 1.2 mm, DV= -7.3 mm) or mPFC (coordinates relative to bregma AP = 2.7 mm, ML= 0.3 mm, DV= -3.5mm) and CRFR1 antagonist (CP 154-526; 1mg/mL, in 0.45% acetic acid vehicle in sterile saline) was infused using osmotic minipumps Alzet, Model 1007D (Cupertino CA) at 0.5µL/hr for 6.5 days following surgery. Experimental groups consisted of: naïve males treated with vehicle (0.45% acetic acid in sterile saline, n=9; in the NAc), experienced males treated with vehicle (n=9, in the NAc), naïve males treated with CRFR1 antagonist in the NAC (n=9) or mPFC (n=10), experienced males treated with CRFR1 antagonist in the NAc (n=11) or mPFC (n=10). Statistical significance in time spent immobile between groups was calculated using a Kruskal-Wallis One Way ANOVA Ranks and Dunn's test 95% confidence level.

5.3 Results

5.3.1 Activation of CRF neurons by sexual behavior

Sexual activity did not activate CRF neurons in the PVN, CeA, or BnST. Specifically, cFos was not expressed in any CRF neurons following mating in either sexually naïve or experienced males in the PVN, CeA, or BnST (Figure 5.1). cFos immunoreactive cells were detected in the vicinity of CRF neurons. However, sexual experience (independent of mating on final test day) reduced total numbers of CRF neurons in the CeA (Figure 5.2d; $p=0.049$) but did not affect numbers of CRF neurons in the in PVN (Figure 5.2e) or BnST (Figure 5.2f).

5.3.2 Activation of CRF mRNA by abstinence from sexual behavior

28 days of abstinence from sexual behavior caused significant up regulation of CRF mRNA in the PVN of sexually experienced males compared to sexually naïve males (Figure 5.3a; $p=0.012$). CRF mRNA in the PVN was not significantly different between experienced and naïve males 1 day following last mating or handling (Figure 5.3a). In the CeA, CRF mRNA levels were significantly decreased in sexually experienced animals both 1 and 28 days following last sexual experience compared to sexually naïve males 1 and 28 days following handling respectively (Figure 5.3a; $p=0.034$ and $p=0.0004$, respectively). In the BnST, CRF mRNA did not differ significantly between sexually naïve and experienced males either 1 or 28 days following last handling or mating (Figure 5.3c).

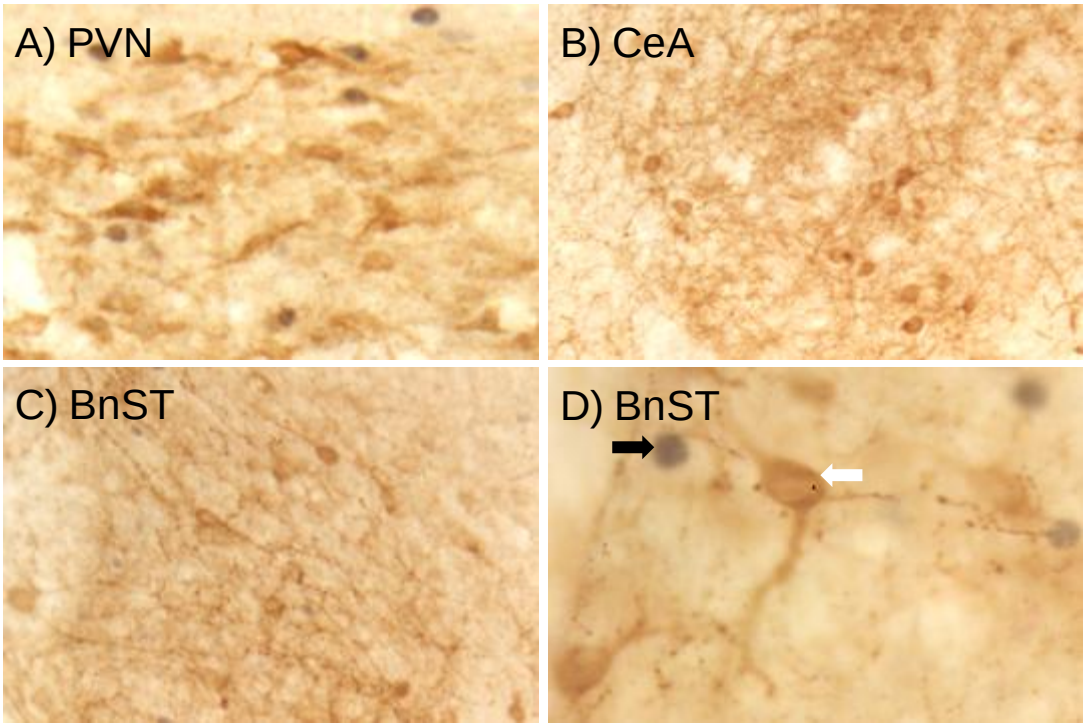


Figure 5.1. Representative images of neurons in the (A) PVN, (B) CeA, (C) BnST expressing CRF (brown, cytosolic) and cFos (black, nuclear) at 10x magnification. (D) Representative image of CRF expressing neuron (white arrow) and cFos expressing neuron (black arrow) that are not colocalized in the BnST (40x magnification). Abbreviations: PVN, paraventricular nucleus of the hypothalamus; CeA, central amydgals; BnST, bed nucleus of the stria terminalis.

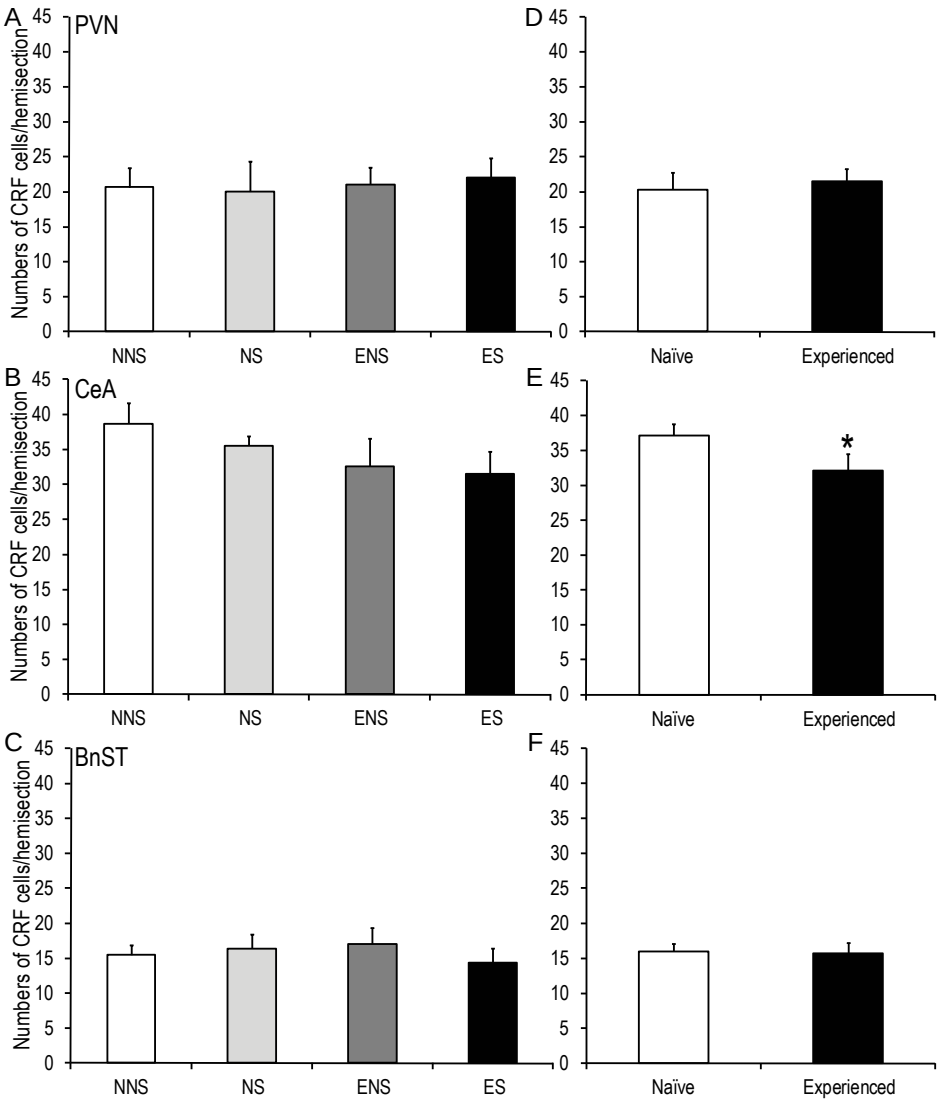


Figure 5.2. Mean numbers of CRF neurons in naïve and experienced males that mated 1 hour before (NS: naïve sex, ES: experienced sex) or 24 hours prior to tissue collection (ENS: experienced no sex, NNS: naïve no sex) in the PVN (A), CeA (B) and BnST (C). Mean numbers of cells based on all animals in naïve or experienced groups, independent of mating behavior immediately prior to tissue collection, in the PVN (D), CeA (E) and BnST (F). Data are presented mean ± SEM. * Indicates significant difference from naïve.

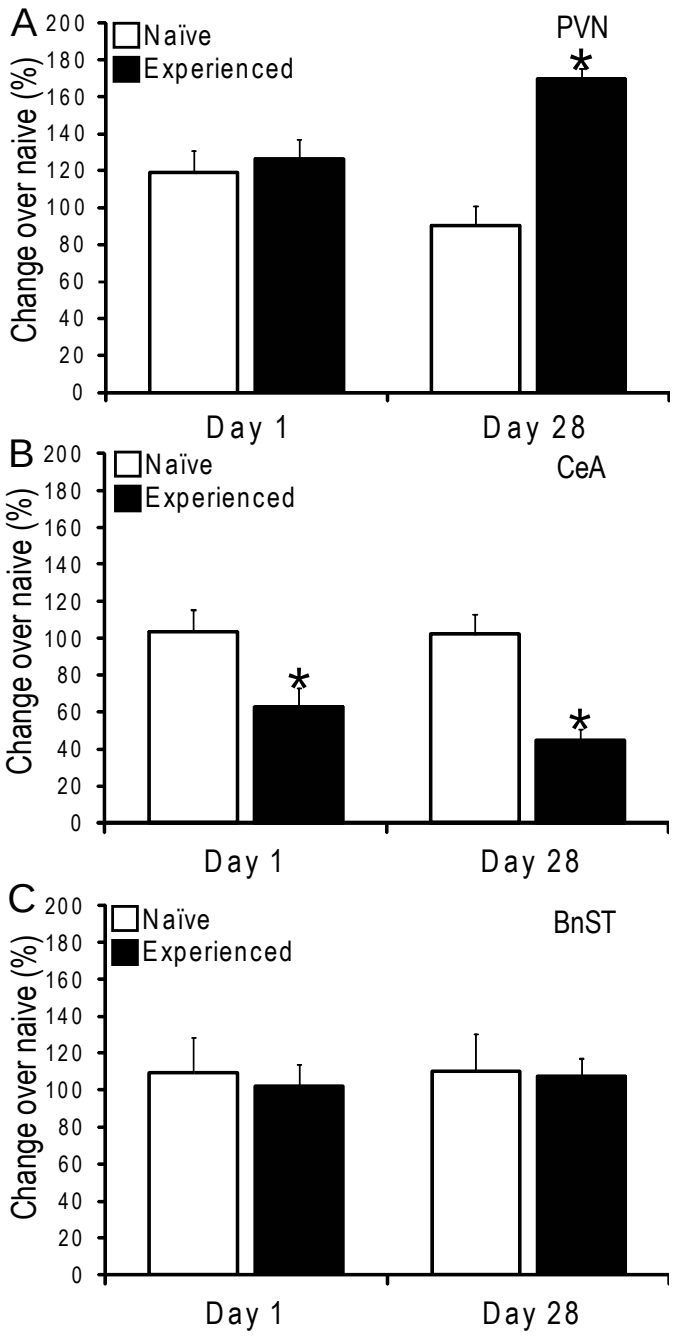


Figure 5.3. CRF mRNA expression in the PVN **(A)** CeA **(B)** and BnST **(C)** of sexually naïve and experienced males 1 and 28 days following last handling or mating. Data are presented mean ± SEM.* Indicates significant difference from naïve males.

5.3.3 Acute Stress Responses

28 days of abstinence from sexual behavior did not alter baseline cort and ACTH release, as sexually naïve and experienced males did not differ in plasma cort or ACTH levels at the 0 minute timepoint. However, 28 days of abstinence from sexual behavior caused increased plasma cort (Figure 5.4a; $F_{1,75}$ 12.283, $p<0.001$) and ACTH (Figure 5.4b; $F_{1,56}$ 20.031, $p<0.001$) release in response to acute restraint stress. Both sexually naïve and experienced males displayed enhanced cort and ACTH release in response to acute restraint stress at 30 minutes post restraint, and enhanced cort release at 60 minutes post restraint, compared to baseline levels ($p<0.001$ for cort and ACTH, both timepoints). However, sexually experienced males displayed significantly greater cort and ACTH release in response to acute restraint stress 28 days following last mating compared to sexually naïve males. Specifically, sexually experienced males showed a higher magnitude of peak cort at 30 (Figure 5.4a; $p=0.042$) and 60 minutes (Figure 5.4a; $p<0.001$) and ACTH at 30 (Figure 5.4b; $p<0.001$) and 60 minutes (Figure 5.4b; $p=0.034$) compared to sexually naïve males. Sexually experienced males also demonstrated prolonged cort and ACTH release as cort remained elevated from the 30 to the 60 minute timepoint in experienced males, but decreased significantly at 60 minute timepoint compared to the 30 minute timepoint in sexually naïve males (Figure 5.4b; $p<0.001$) . ACTH release was also prolonged in experienced males, as ACTH levels returned to baseline levels at the 60 minute timepoint in sexually naive males, but remained elevated compared to baseline in sexually experienced males (Figure 5.4b; $p<0.043$).

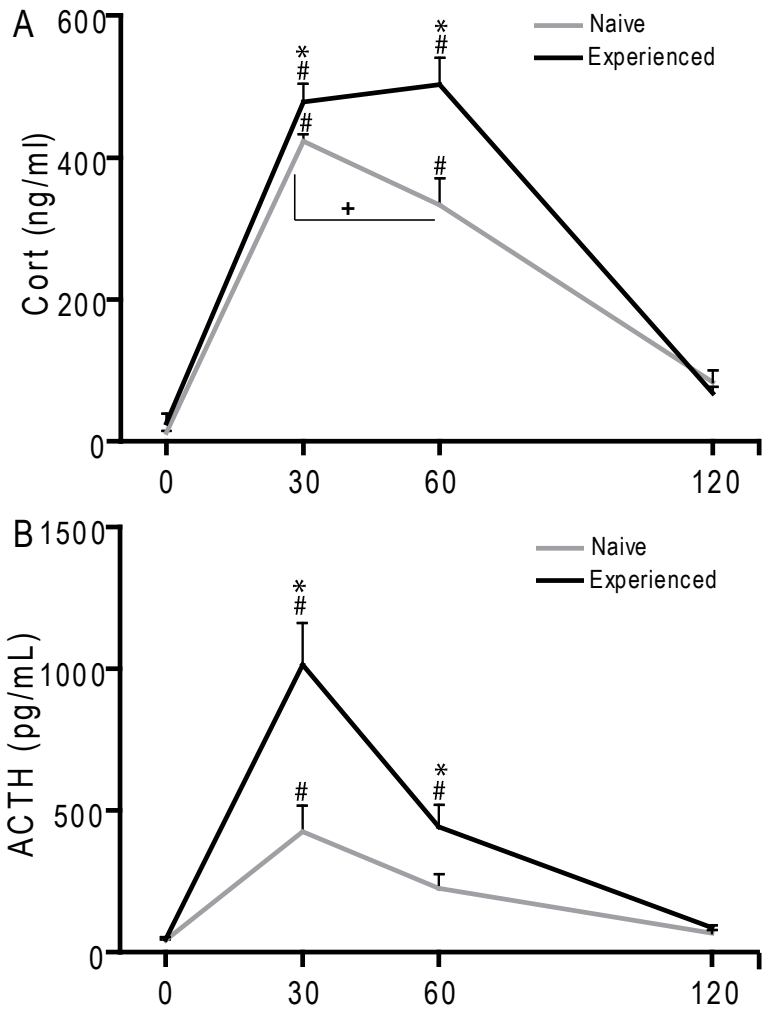


Figure 5.4. Plasma corticosterone (Cort) **(A)** and ACTH **(B)** levels in response to acute restraint stress in sexually naïve and experienced males 28 days following last handling or mating. Data are presented mean ± SEM. * indicates significant difference from naïve males at the same timepoint. # indicates significant difference from 0 minute timepoint. + indicates significant difference between the 30 and 60 minute timepoints.

5.3.4 Effects of chronic ICV treatment with CRF receptor antagonists

Chronic treatment with a CRFR1 antagonist during days 21-27 of mating abstinence prevented development of depression-like behavior seen following a 28 day period of abstinence from mating (Figure 5.5a; $F_{3,38}$ 4.016; p=0.015). Specifically, males treated with CRFR1 antagonist-treated spent significantly less time immobile compared to experienced vehicle treated males (Figure 5.5a; p=0.002), and were not significantly different from naïve vehicle treated males. Males treated with a CRFR2 antagonist did not show less time immobile compared to experienced vehicle-treated males, but also did not show significantly more time immobile compared to naïve-vehicle-treated males (Figure 5.5a), suggesting that CRFR2 antagonist administration had a partial effect on this phenotype. A 28 day period of abstinence from mating induced depression-like behavior as experienced vehicle treated males spent significantly increased time immobile compared to naïve vehicle treated males (Figure 5.5a; p=0.015), confirming results of Chapter 4. Furthermore, chronic administration of CRFR1 receptor antagonist blocked the sex abstinence induced increase in CRF mRNA expression in the PVN. CRF mRNA in sexually experienced males treated with CRFR1 antagonist (109.8 ± 27.5%;) did not differ from sexually naïve males (101.1 ± 8.3%), while vehicle-treated sexually experienced males had a strong trend towards increased CRF mRNA (159.1 ± 30.8%; p=0.059) compared to naïve males

5.3.5 Effects of acute CRFR1 antagonist administration on depression-like behavior

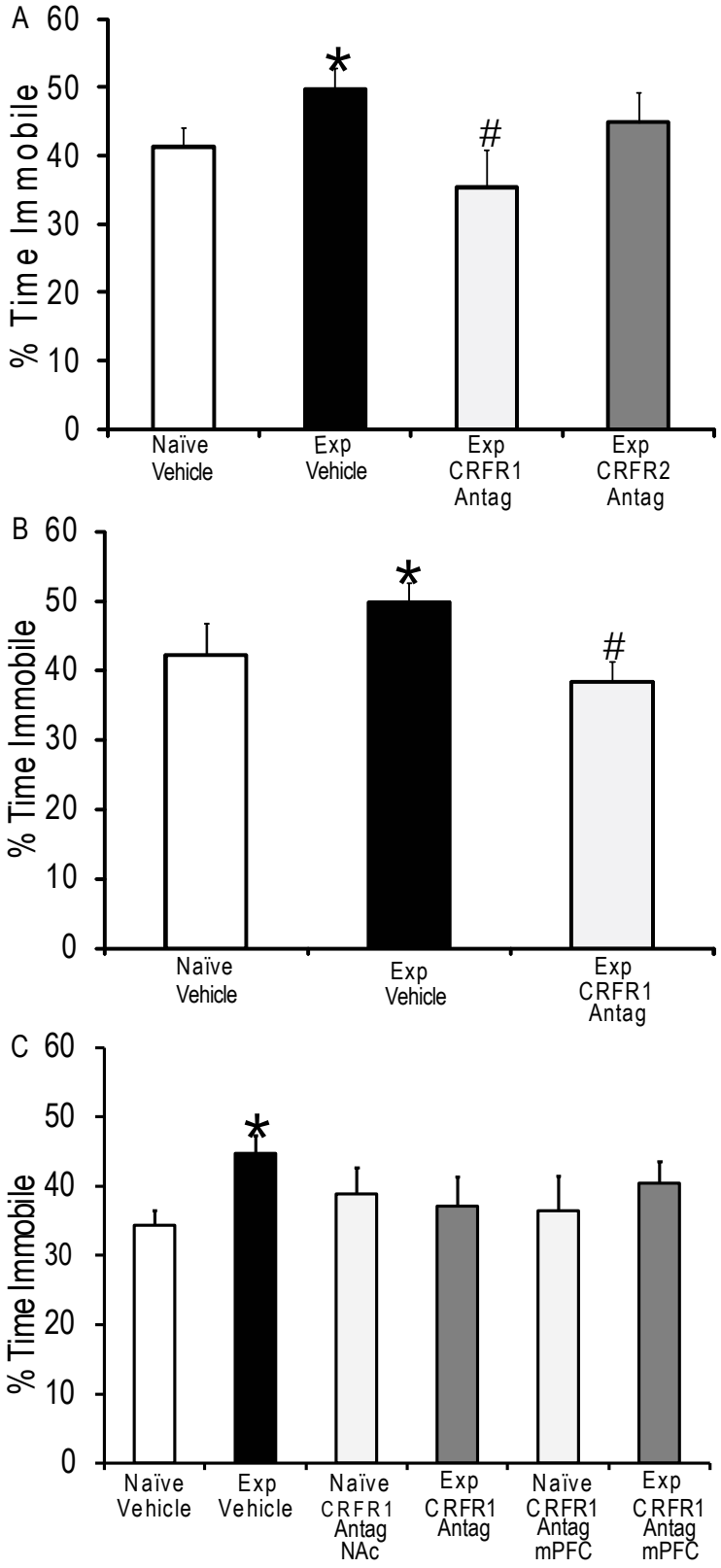
Administration of CRFR1 antagonist immediately prior to the forced swim test prevented expression of depression-like behavior in sexually experienced male rats (Figure 5.5b; $F_{2,37}$ 3.855; p=0.031). Males treated with CRFR1 antagonist showed

decreased time immobile compared to experienced vehicle-treated males (Figure 5.5b; p=0.019) and did not differ from naïve vehicle-treated males. Experienced vehicle-treated males again spent increased time immobile compared to naïve vehicle-treated males (Figure 5.5b; p=0.037).

5.3.6 Local Manipulations of CRFR1 in the Nucleus Accumbens and Medial Prefrontal Cortex

To determine where in the brain CRF is acting on CRF1 receptors to cause depression-like behavior following loss of sexual reward, CRFR1 antagonists were infused into the NAc or mPFC for days 21-27 of the 28 day abstinence period. Indeed, CRFR1 antagonism in either NAc or mPFC prevented development of depression-like behavior (Figure 5.5c; H = 9.157; p=0.027). Specifically, Experienced vehicle treated males spent increased time immobile compared to naïve vehicle treated males (Figure 5.5c; $p<0.05$) confirming the previous findings. Antagonism of CRFR1in either the NAc or mPFC prevented development of passive stress coping, as CRFR1 antagonist-treated males did not differ significantly in time spent immobile compared to naïve vehicle- or antagonist-treated males (Figure 5.5c). However, experienced NAc antagonist-treated or PFC antagonist-treated males also did not differ significantly in time spent immobile from experienced vehicle-treated males (Figure 5.5c), indicating a partial effect of the local CRFR1 antagonism. Therefore, these results suggest that CRFR1 may be acting simultaneously in multiple brain regions to cause development of depression-like behavior.

Fig. 5.5. (A) Percentage of time spent immobile in vehicle-treated sexually naïve males (Naïve Vehicle), sexually experienced males (Exp Vehicle); sexually experienced CRFR1 antagonist treated males (Exp CRFR1 Antag) and sexually experienced CRFR2 antagonist treated males males (Exp CRFR2 Antag) that received vehicle or antagonist infusions during the final 7 days of abstinence **(B)** Percentage of time spent immobile in vehicle-treated sexually naïve males (Naïve Vehicle), sexually experienced males (Exp Vehicle); sexually experienced CRFR1 antagonist treated males (Exp CRFR1 Antag) that were acutely treated with vehicle or CRFR1 antagonist 15 minutes prior to testing on the forced swim test. **(C)** Percentage of time spent immobile in sexually naïve (Naïve) or experienced (Exp) males treated with either vehicle or CRFR1 antagonist in the NAc or mPFC. Data are presented as mean ±SEM * indicates significant difference from naïve vehicle, # indicates significant difference from experienced vehicle.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5.4 DISCUSSION

Previously, we showed that sexual experience followed by a prolonged (28 days), but not short (1-7 days) period of abstinence from sexual reward caused increased depression-like behaviors, consisting of increased passive stress coping and decreased social interaction. In the current study, we demonstrated that these depression-like behaviors are associated with enhanced HPA activity, namely increased stress-induced ACTH and cort release and increased CRF mRNA in the PVN. Thus, these results provide further insights into the mechanisms by which loss of sexual reward causes development of depression and validation of loss of sexual reward in male rats as a model for depression-like behavior, as this model demonstrates homology to pathology seen in human depression, or *pathological validity* (Krishnan and Nestler, 2010). In humans, major depression is associated with hyperactivity of the HPA axis, increased CRF mRNA and protein levels in the PVN and CRF secretion into the cerebrospinal fluid (Kling et al., 1991), as well as increased plasma and salivary cortisol (Nemeroff and Vale, 2005).

A potential mechanism for the observed increases in PVN CRF and enhanced stress-induced release of cort and ACTH may be decreased glucocorticoid negative feedback (Reul and de Kloet, 1985, Nemeroff, 1993, Koob and Heinrichs, 1999). It is hypothesized that the hyperactivity of the HPA axis seen in depressed humans may be due to alterations in glucocorticoid negative feedback, as depressed patients fail to mount an appropriate negative feedback response in the dexamethasone (synthetic glucocorticoid) suppression test (Rush et al., 1996, Holsboer, 2000). Studies in depressed humans have demonstrated decreased glucocorticoid receptor (GR) mRNA expression in the hippocampus and prefrontal cortex (Webster et al., 2002). Rodents also demonstrate

reductions in GR binding in the hippocampus and hypothalamus following social defeat (Buwalda et al., 2001), in the hippocampus and PFC following maternal separation (Meaney et al., 1996) and in the hippocampus, hypothalamus, and PFC following chronic stress (Makino et al., 1995, Raone et al., 2007). Furthermore, early life stressors such as decreased maternal care which causes alterations in stress responses also leads to long-lasting epigenetic modifications of GR in the hippocampus (Weaver et al., 2004, Weaver, 2007). Specifically, rat pups that receive low maternal care have increased stress responses, associated with low levels and hypermethylation of exon1_7 of the GR promoter in the hippocampus (Weaver et al., 2004, Weaver, 2007), and decreased binding of the GR promoter to the transcription factor NGF1-A (Weaver et al., 2004, Weaver, 2007), suggesting an epigenetic mechanism by which social experience may cause long term adaptations to stress responses. Future studies will therefore determine if loss of sexual reward may cause increased stress responses via decreased GR expression and hypermethylation of GR exon I_7 in the hippocampus.

The second major finding of the current study was that the development of depression-like behavior was dependent on activation of CRFR1 in brain areas including the NAc and PFC, as CRFR1 antagonism during the final days of sex reward abstinence prevented the increase in passive stress coping behaviors. ICV infusion of a CRFR2 antagonist had a partial effect on development of depression-like behavior. Activation of both CRFR1 and CRFR2 has been implicated in depression-like behaviors (Shaham et al., 1998, Hammack et al., 2003, Wang et al., 2005, Henry et al., 2006, Wang et al., 2006, Wang et al., 2007). Other reports have shown that activation of CRFR1 can be pro-depressant and induces stress responses, while CRFR2 activation can have antidepressant

effects and cause attenuation of the stress response (Bale and Vale, 2004, Contarino and Papaleo, 2005). The current findings do not support opposite roles for CRFR1 and CRFR2 in the effects of sexual reward abstinence on development of depression, but rather a more dominant role for CRFR1. The greater role for CRFR1 may be based on the critical involvement of the NAc and PFC that both express high levels of CRFR1, but only low levels of CRFR2 (Van Pett et al., 2000). A potential mechanism by which CRF may mediate the development of depression-like behavior is via the dynorphin/kappa opioid system as CRFR1 receptor activation in either NAc or PFC, has been shown to activate the dynorphin/kappa opioid receptor system to induce depression-like behavior (Land et al., 2008, Tejeda et al., 2010). The dynorphin/kappa opioid receptor system has been shown to exert pro-depressant effects when activated and antidepressant effects when blocked (Mague et al., 2003, McLaughlin et al., 2003, Beardsley et al., 2005, Carlezon et al., 2006).

The ligand that causes CRFR1 activation is potentially CRF. CRF has a 10x higher affinity for CRFR1 than CRFR2 (Bale and Vale, 2004), with CRFR2 having a higher affinity for urocortin II and III (Bale and Vale, 2004), suggesting that CRF via activation of CRFR1 during the 28 day period of abstinence causes development of depression-like behavior. In addition, urocortin I has equal affinity for both CRFR1 and CRFR2 (Lovenberg et al., 1995, Perrin et al., 1995), and therefore cannot be ruled out as an additional contributor to the development of depression-like behavior following loss of sexual reward. The sources of CRF projections to the NAc or the mPFC are not clear. Both regions receive inputs from PVN (Silverman et al., 1981, van der Kooy et al., 1984) and the NAc also received inputs from the BnST and CeA, as part of the extended

amygdala (Koob, 1999). The current study was not able to identify which CRF neurons in PVN or extra hypothalamic sites were activated during sexual behavior, as cFos expression was not observed in CRF neurons following mating in either naïve or experienced males, suggesting that CRF neurons are not activated by sexual behavior. Previous studies have shown cFos activation in the PVN (Buwalda et al., 2012), BnST (Coolen et al., 1996) and amygdala (Coolen et al., 1996, Veening and Coolen, 1998) following sexual behavior. PVN CRF neurons have also been show to express cFos following other stimuli, including water deprivation (Benedetti et al., 2008) or lipopolysaccharide administration (Rorato et al., 2008). However, it should be noted that a lack of cFos activation does not indicate that sexual behavior does not cause activation of CRF neurons. The current study did identify an up-regulation of CRF mRNA in PVN, coinciding with the timepoint development of depression-like behavior is observed, suggesting that the PVN may be the source of CRF acting in NAc and mPFC to induce this negative affective state.

An unexpected finding of the current study was a reduction in CRF mRNA and protein in the CeA, both immediately following sexual behavior and after prolonged period of sex reward abstinence. These results indicate that sexual behavior does indeed influence CeA CRF neurons, by causing down regulation of CRF gene and protein expression in these cells. It is not entirely clear how this decreased CRF in the CeA may mediate depression-like behavior. The amygdala is an important brain area for regulation of emotions, particularly related to fear, stress and aversive stimuli (Eibl-Eibesfeldt and Sutterlin, 1990). In human depression, amygdala volume is reduced (Sheline et al., 1998). In contrast, in the maternal separation model of depression, CRF mRNA in the CeA is

increased (Menzaghi et al., 1993) and glucocorticoids have been shown to be stimulatory to CeA CRF mRNA levels (Schulkin et al., 1994, Shepard et al., 2000). Hence, the current findings that CRF is decreased in CeA during abstinence from sexual behavior suggest that there is no chronic elevation of glucocorticoids during the abstinence period. This is supported by a lack of difference in baseline cort and ACTH levels in sexually experienced and naïve males following the prolonged period of mating abstinence. But, whether CeA CRF plays a role in the development of depression following loss of sexual reward remains unclear.

A major finding of the current study, together with our previous findings, is that increased levels of CRF in the PVN and the development of depression-like behavior require a prolonged period of abstinence from sexual reward and are not induced immediately after sexual behavior. Development of depression-like behavior is completely prevented by twice weekly mating during the 28 days following daily sexual experience, demonstrating that abstinence from mating is critical. It is currently unknown what mechanisms underlie the effects of abstinence on subsequent alterations in behavior and neuropathology. One hypothesis for the pro-depressant effects of chronic stress is that stress reduces neurogenesis and numbers of spines in the hippocampus (Pham et al., 2003, McEwen, 2005, 2010, Veena et al., 2011, Schoenfeld and Gould, 2012). In apparent contrast, sexual behavior, either once or daily during a 14 day period, promotes neurogenesis and spinogenesis in adult male rats (Leuner et al., 2010), despite the fact that mating causes increased cort and ACTH release (Buwalda et al., 2001, Bonilla-Jaime et al., 2006, Leuner et al., 2010). Likewise, running or exposure to an enriched environment, which cause activation of the HPA axis also increase hippocampal

neurogenesis (Schoenfeld and Gould, 2012). However, it is important to note that shortly following mating, and thus at the timepoint that increased neurogenesis has been demonstrated, sexual behavior causes a dampening of the stress responses and reductions in anxiety-like behavior (Fernandez-Guasti et al., 1989, Saldivar et al., 1991, Waldherr and Neumann, 2007, Waldherr et al., 2010). Therefore, it is critical to examine the effects of prolonged periods of abstinence from sexual reward on neurogenesis and spinogenesis in the hippocampus, as well as in the NAc and mPFC. Recent studies form our laboratory have shown that a short period of abstinence from sexual behavior (7 days) caused increased numbers of spines in the NAc (Pitchers et al, 2010), but this returned to baseline after the prolonged 28 day period of abstinence (Pitchers, 2012), suggesting that spinogenesis is a dynamic and time-dependent process.

Another hypothesis for the effects of chronic stress on depression is that of stress sensitization: the view that previous exposures to stressors may cause an enhanced stress response upon re-exposure to a stressor and favor development of depression (Anisman and Merali, 2003, Anisman et al., 2003a). Anisman and colleagues showed that systemic infusions of the inflammatory cytokine, tumor necrosis factor-alpha (TNF-alpha) caused activation of the HPA axis (Anisman et al., 2003b). Moreover, re-exposure to TNF-alpha elicited a sensitized stress response (Anisman et al., 2003b). Of particular interest was their finding that re-exposure to TNF-alpha triggered the most sensitized and robust stress effect after a re-expousre interval of 28, but not 7 days (Anisman et al., 2003b). Thus, inflammatory cytokines may also play a role in the development of depression following abstinence from sexual reward. It has been well documented that humans with depression have elevated inflammatory cytokines (Maes, 1994, Maes and Smith, 1998, Schiepers et

al., 2005, Kim et al., 2008, Dinan et al., 2009, Pace and Miller, 2009, Dowlati et al., 2010). Moreover, it has been suggested that exercise is an effective treatment in depressed patients (Biddle, 1989, Beesley and Mutrie, 1997, Barbour et al., 2007, Greer and Trivedi, 2009, Carek et al., 2011, Rozanski, 2012) as it has anti-inflammatory actions (Das, 2004, Petersen and Pedersen, 2005, Gleeson et al., 2011, Rethorst et al., 2012). Recently it has been shown that TNF-alpha levels in serum of depressed patients may be a predictor for the antidepressant effects of exercise, with high TNF-alpha levels linked to better outcomes for exercise, but poor outcomes for antidepressant treatment (Rethorst et al., 2012). Thus, we hypothesize that sexual behavior and subsequent abstinence from sexual behavior may influence inflammatory and immune responses in the brain. Even though effects of sexual behavior on the brain immune system have not yet been reported, environmental enrichment has been shown to decrease inflammatory markers and cause a blunted pro-inflammatory response (Williamson et al., 2012). Moreover, exposure to females, without mating, has been shown to increase mast cells in the brains of male rats (Asarian et al., 2002). These mast cells have been implicated in the regulation of anxiety-related behaviors, suggesting that increased mast cells following exposure to female stimuli may mediate dampened anxiety responses (Nautiyal et al., 2008).

In conclusion, results of these studies demonstrate that loss of sexual reward, which causes depression-like behavior, also leads to underlying neuropathology of depression including HPA axis activity and increased CRF in the PVN. Moreover, these studies provide insight into the mechanism by which CRF may modulate depression-like behavior following loss of sexual reward, via CRFR1 in the NAc and PFC. We suggest

that these studies of the effects of loss of sexual reward demonstrate homology to pathology seen in human depression following loss of social reward and may thus provide further insights into this particular form of depression.

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CHAPTER 6: GENERAL DISCUSSION

6.1. Orexin and Sexual Reward

Results from Chapter 2 demonstrated that the hypothalamic neuropeptide orexin plays a role in arousal and anxiety related to sexual behavior in sexually naive male rats, but is not critical for male sexual performance or motivation (Di Sebastiano et al., 2010, Di Sebastiano and Coolen, 2012). Results from Chapter 3 demonstrated that orexin is a critical mediator of sexual reward (Di Sebastiano et al., 2011, Di Sebastiano and Coolen, 2012). Together, these studies formed a critical step in identifying the role of orexin in natural reward behavior (Di Sebastiano and Coolen, 2012) and specifically in sexual reward.

6.1.1 Future Directions - Orexin

In our previous studies, we demonstrated the role of orexin using cell specific lesions of orexin neurons in the PFA-DMH and LHA (Di Sebastiano et al., 2010). However, as the lesioning technique permanently eliminated orexin neurons, it is currently unknown if orexin is involved in the acquisition of stimulus-reward learning or the expression of this learned association. We tested the role of orexin in sexual reward using the CPP paradigm, which includes both a phase of acquisition of the associative memory of reward-cue associations and a phase of expression of the seeking of the reward-associated cue (Tzschentke et al, 2007). Future studies may utilize pharmacological manipulations of orexin neurons in the PFA-DMH and LHA either during the CPP conditioning trials; to determine if orexin is involved in acquisition of sexual reward induced CPP, or during the post-test to determine if orexin is involved in the expression of mating induced CPP. Studies may also utilize newly developed

technologies such as optogenetics, which rely on activation or inhibition of specific neurons via expression of light activated proteins, channelrhodopsin or halorhodopsin, respectively (Fenno et al., 2011). This technique has a higher temporal resolution than pharmacological techniques (Fenno et al., 2011), and as such, would allow for more precise activation or inactivation of orexin neurons during the acquisition or expression phases of mating induced CPP.

In addition, it is unclear where in the brain orexin is acting to mediate sexual reward. Orexin is synthesized exclusively in the PFA-DMH and LHA (de Lecea et al., 1998, Sakurai et al., 1998), but has broad projections, and orexin receptors are widely distributed throughout the brain and spinal cord (Trivedi et al., 1998, Marcus et al., 2001). Orexin neurons project to both the NAc and VTA (Peyron et al., 1998, Fadel and Deutch, 2002, Baldo et al., 2003) and contact dopamine positive cells in the VTA (Fadel and Deutch, 2002). Furthermore, both orexin receptor 1 and orexin receptor 2 are located in the NAc and VTA, including on DA cells in the VTA (Korotkova et al., 2003, Narita et al., 2006). In the NAc orexin-A infusion stimulates feeding behavior (Thorpe and Kotz, 2005), suggesting a role for orexin in food reward, as the NAc plays an important role in hedonia related to feeding (Berridge, 1996, Pecina and Berridge, 2000). Orexin action in the VTA has also been shown to be important for food motivation and reward, as infusion of an orexin receptor 1 antagonist decreases seeking of high-fat, palatable food (Borgland et al., 2009). Much more is known about the role of orexin in the VTA in drug reward. Administration of an orexin receptor-1 antagonist into the VTA blocks morphine induced CPP (Harris et al., 2007) and intra-VTA administration of orexin-A has been shown to reinstate morphine CPP following extinction (Harris et al., 2005).

Thus, future experiments may use pharmacological or optogenetic manipulations of orexin receptors 1 or 2 in the VTA and NAc to determine if orexin action in these brain regions is critical for mediation of sexual performance, motivation or reward. As dopamine has been shown to play a role in reward-cue association learning (Robinson and Berridge, 1993, Berridge, 1996) and reward prediction (Schultz, 1997, 1999, 2006, 2010); transgenic studies may utilize mice that have a conditional knockout (Friedel et al., 2011) of orexin receptors that are located on dopamine neurons to determine if orexin acts via dopamine to mediate sexual reward.

6.2 Development of an animal model for depression following loss of sexual reward

Studies from Chapter 4 demonstrated that a 28 day period of abstinence from sexual behavior causes depression-like behavior including passive stress coping and anhedonia in male rats. Studies from Chapter 5 demonstrated that this prolonged loss of sexual reward induced depression-like behavior is also associated with underlying neuropathology of depression seen as increased corticotropin releasing factor (CRF) mRNA in the paraventricular nucleus of the hypothalamus (PVN) and increased corticosterone (cort) and ACTH responses to an acute stressor. Finally, it was shown that development of depression-like behavior following loss of sexual reward is mediated by CRF receptor 1 in mesolimbic brain regions, the NAc and PFC.

6.2.1 Future Directions: Interactions with vulnerability to addiction

In addition to causing depression-like behavior, abstinence from sexual reward also causes increased vulnerability for psychostimulant abuse in male rats (Pitchers et al., 2010). Given that depression and substance abuse have a high comorbidity (Volkow,

2004); it will be interesting to determine which neural alterations caused by abstinence from sexual reward lead to this behavioral pathology. As CRF is activated by drugs of abuse (Sarnyai et al., 2001) as well as by withdrawal and abstinence from drug taking (Koob and Kreek, 2007, Koob, 2008), CRF may also be an important mediator of the enhanced drug reward (Pitchers et al., 2012) and locomotor sensitization to amphetamine (Pitchers et al., 2010) seen following a period of abstinence from mating. Thus, future studies may examine the effects of CRF antagonists in limbic brain regions that express CRF receptors and are also involved in depression and addiction, such as the PFC and NAc (Van Pett et al., 2000, Krishnan and Nestler, 2008) during the final week of mating abstinence on development of conditioned place preference for a subthreshold dose of amphetamine and sensitization of the locomotor response to psychostimulants (Pitchers et al., 2010).

6.2.2 Glutamate

In the discussion of Chapter 5, I discussed the potential effects of prolonged sexual reward abstinence on neurogenesis, spinogenesis, and cytokines in the development of depression. However, many more transmitters and neural factors have been implicated in depression. Many studies have shown that glutamate, the main excitatory neurotransmitter in the central nervous system is altered in depressed patients, as they show increased plasma glutamate levels compared to healthy controls (Kim et al., 1982, Altamura et al., 1993, Mitani et al., 2006). Glutamate receptor trafficking is also decreased in the striatum (Kristiansen and Meador-Woodruff, 2005) and thalamus (Clinton and Meador-Woodruff, 2004) of depressed patients and enhanced membrane

expression of ionotropic 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid (AMPA) receptors is observed in the hippocampus following treatment with antidepressants (Martinez-Turrillas et al., 2007), suggesting a role for glutamate receptor trafficking in mediation of depression. Glutamate receptor trafficking and function is also altered by exposure to drugs of abuse (Thomas et al., 2008, Wolf and Ferrario, 2010). Studies from our lab have shown that a one month period of abstinence from mating also induces alterations in glutamate receptors, including increased surface expression of the AMPA glutamate receptor subunits, GluA1 and GluA2 in the NAc (Pitchers et al., 2012), and decreased AMPA/NMDA ratio in the NAc (Pitchers et al., 2012). These sex abstinence-induced changes in glutamate receptor expression and trafficking are partially similar to changes induced by abstinence from drugs of abuse. Abstinence from repeated cocaine exposure causes increased cell surface AMPA and NMDA receptor subunit expression in the NAc and different receptor subunits have been shown to be differentially affected by drug withdrawal (Conrad et al., 2008, Wolf and Ferrario, 2010). These changes are thought to contribute to alterations in the locomotor response to psychostimulants and increased drug seeking (Vanderschuren and Kalivas, 2000, Conrad et al., 2008, Wolf and Ferrario, 2010, Ferrario et al., 2011). Thus, as glutamate and glutamate receptors appear to play a critical role in depression and drug withdrawal, and have also been shown to be altered by a periods of abstinence from sexual behavior, future studies may test the functional role of these alterations in development and expression of depression-like behavior following loss of sexual reward as well as alterations in stress responses.

6.2.3 BDNF

Brain derived neurotrophic factor (BDNF), is the most prevalent growth factor in the central nervous system (Autry and Monteggia, 2012). Depressed humans shown reduced plasma BDNF (Duman and Monteggia, 2006) as well as decreased BDNF levels in the hippocampus (Karege et al., 2005, Castren et al., 2007). Chronic stress in rodents has been shown to decrease BDNF signaling and this is reversed by chronic treatment with antidepressants (Nestler et al., 2002, Duman and Monteggia, 2006). Furthermore, in the social defeat model of depression, VTA specific deletions of BDNF blocked development of depression-like behavior (Berton et al., 2006). Moreover, BDNF is also critically involved in drug addiction as well as withdrawal. A single infusion of cocaine increases BDNF levels in the NAc (Russo et al., 2009), and infusion of BDNF into the NAc leads to increased drug taking and seeking, and enhances drug craving after withdrawal (Graham et al., 2007). Moreover, infusion of BDNF into the VTA during withdrawal enhances drug seeking behavior (Corominas et al., 2007). Much less is known about the role of BDNF in sexual behavior; however it has been shown that mating behavior leads to upregulation of BDNF mRNA in the PFC (Kakeyama et al., 2003). Thus, as BDNF is involved in depression, and appears to facilitate drug seeking following drug withdrawal, BDNF may also be altered following abstinence from sexual reward, and mediate vulnerability for depression via the mesolimbic system. Future studies therefore may utilize pharmacological manipulations of BDNF during mating or the period of abstinence to test the functional role of BDNF on development of depression-like behavior and alterations in stress responses observed following loss of sexual reward.

6.3 Orexin interactions with CRF and the stress system

In addition to playing a critical role in mediation of reward, the hypothalamic neuropeptide orexin has also been shown to be involved in mediation of anxiety and depression-like behavior. Orexin-A infusion into the paraventricular nucleus of the thalamus of male rats causes decreased anxiety-like behavior on the elevated plus maze (Li et al., 2010). Orexin has also been shown to play a role in depression-like behavior, with activation of OxR1 having a pro-depressant effect and activation of OxR2 having an antidepressant effect (Scott et al., 2011), suggesting orexin differentially regulates depression-like behavior depending on which receptor is activated. Orexin has also been shown to interact with the stress system, as stimulation of orexin receptors increases CRF mRNA levels (Al-Barazanji et al., 2001, Singareddy et al., 2006), as well as ACTH (Ida et al., 1999, Ida et al., 2000a, Kuru et al., 2000) and corticosterone release (Ida et al., 2000b, Kuru et al., 2000). Orexin mRNA is also increased in response to acute restraint stress (Ida et al., 2000a). Furthermore, orexin neurons in the hypothalamus also express CRF receptors, and CRF application to brain slices has been shown to increase orexin signaling (Winsky-Sommerer et al., 2004). Thus, it is clear that there are interactions between orexin and the CRF system, which may play a role in mediation of stress responses.

The studies in this thesis demonstrated a critical role for orexin in mediation of sexual reward (Di Sebastiano et al., 2011), and for CRF in mediation of development of depression-like behavior following loss of sexual reward. Therefore, orexin and CRF may be acting together to mediate development of depression-like behavior following loss of sexual reward as well as alterations in stress responses.

6.4 Overall Conclusions

Results of the studies in this thesis further elucidate the neural circuitry of sexual reward. Furthermore, results of these studies provide insight into the mechanism by which loss of natural rewards can influence disorders related to reward processing such as substance abuse and depression.

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APPENDIX A

2010-211::2:

AUP Number: 2010-211
AUP Title: Neural regulation of rewarding behavior and substance abuse
Approval Date: 03/22/2010

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2010-211 has been approved.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
   Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

APPENDIX B

PI: Coolen, Lique
Protocol Number: #10531
Approval Period: 03/02/2011 - 03/02/2014
Funding Agency: Canadian Institutes of Health Research
Title: Effects of Sexual Experience on Drug Responsiveness
DRDA Number:

Date: 03/03/2011

Dear Principal Investigator,

The University of Michigan Committee on Use and Care of Animals (UCUCA) has reviewed your application to use vertebrate animals (Application #10531). This project has been approved. The proposed animal use procedures are in compliance with University guidelines, State and Federal regulations, and the standards of the "Guide for the Care and Use of Laboratory Animals."

When communicating with the UCUCA Office please refer to the Approval Number #10531. The approval number must accompany all requisitions for animals and pharmaceuticals.

The approval date is 03/02/2011. The approval period is for three years from this date. However, the United States Department of Agriculture (USDA) requires an annual review of applications to use animals. Therefore, each year of this application prior to the anniversary of its approval date, you will be notified via email to submit a short annual review. Your continued animal use approval is contingent upon the completion and return of this annual review. You will also be notified 120 days prior to the expiration of the approval period so that your renewal application can be prepared, submitted and reviewed in a timely manner in the eSirius program and an interruption in the approval status of this project avoided.

UCUCA approval must be obtained prior to changes from what is originally stated in the protocol. An amendment must be submitted to the UCUCA for review and approved prior to the implementation of the proposed change.

The University's Animal Welfare Assurance Number on file with the NIH Office of Laboratory Animal Welfare (OLAW) is A3114-01, and most recent date of accreditation by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC, Intl.) is November 06, 2009.

If you receive news media inquiries concerning any aspect of animal use or care in this project, please contact James Erickson, News and Information Services. If you have security concerns regarding the animals or animal facilities, contact Bill Bess, Director of Public Safety.

Sincerely,

Richard Keep, Ph.D.
Professor, Neurosurgery
Chairperson, University Committee on Use and Care of Animals

APPENDIX C

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CURRICULUM VITA

Name: Andrea R. Di Sebastiano

Educational Background

PhD **Sept. 2007 – Present,**
University of Western Ontario
Department of Anatomy and Cell Biology
Supervisor: Dr. Lique M. Coolen

BMSc (Hons.) **Sept. 2003 – May 2007,**
University of Western Ontario
Department of Physiology and Pharmacology

Teaching Experience

Laboratory Coordinator
September 2009 - April 2010, University of Western Ontario
Systemic Human Anatomy, Department of Anatomy and Cell Biology

Teaching Assistant
September 2007 - April 2009, University of Western Ontario
Systemic Human Anatomy, Department of Anatomy and Cell Biology

Undergraduate Student Mentor
Jacob N. Davis, September 2009 – May 2010, University of Western Ontario
Sabrina Yong-Yow, September 2007 – May 2008, University of Western Ontario
Department of Physiology and Pharmacology

NSERC Summer Student Mentor
Marissa Tsoi, May 2009 – August 2010, University of Western Ontario

Scholarships and Awards

2011-present: Canadian Institute of Health Research Doctoral Research Award
2007-present: Western Graduate Research Scholarship
2011: Ontario Graduate Scholarship (Declined)
2011: Travel Award for the Society for Behavioral Neuroendocrinolgy Meeting
2009-2011: Ontario Graduate Scholarship in Science and Technology (OGSST)
2010: Gabriel G. Altman Award for Best Poster Presentation in the Department of Anatomy and Cell Biology.
2009: Murray Barr Research Day Award for Best Poster Presentation in the Department of Anatomy and Cell Biology.
2005-2007: Dean's Honor List
2003: The University of Western Ontario Entrance Scholarship

Publications

Di Sebatiano AR, Coolen LM (2012). Orexin and Natural Reward: Feeding, Maternal and Male Sexual Behavior. Progress in Brain Research, In Press, Invited Review.

Pitchers KK, Schmid S, **Di Sebastiano AR,** Wang X, Laviolette SR, Lehman MN, Coolen LM (2012). Natural reward experience alters AMPA and NMDA receptor distribution and function in the nucleus accumbens. PLoS ONE *7(4): e34700.*

Di Sebastiano AR, Wilson-Pérez HE, Lehman MN, Coolen LM (2011). Lesions of orexin neurons block conditioned place preference for sexual behavior in male rats. Horm. Behav. 59 (1): 1-8.

Di Sebastiano AR, Yong-Yow S, Wagner L, Lehman MN, Coolen LM (2010). Orexin mediates initiation of sexual behavior in sexually naïve male rats, but is not critical for sexual performance. Horm Behav. 58 (3): 397-404. Featured on the cover.

Davis JF, Loos M, **Di Sebastiano AR**, Brown JL and Coolen LM (2010). Lesions of the medial prefrontal cortex cause maladaptive sexual behavior in male rats. Biol Psychiatry; 67(12):1199-204.

Abstracts

Di Sebastiano AR, Coolen LM (2012). Loss of sexual reward causes depression-like behaviors including passive stress coping and anhedonia in male rats. Society for Behavioral Neuroendocrinology, June 2012, Madison, WI.

Di Sebastiano AR, Coolen LM (2011). Depression-like behavior following abstinence from sexual behavior in male rats is mediated by activation of corticotropin releasing factor receptors. Society for Neuroscience Meeting, November 2011, Washington DC.

Di Sebastiano AR, Coolen LM (2011). Depression-like behavior following abstinence from sexual behavior in male rats is mediated by activation of corticotropin releasing factor receptors. Anxiety and Depression: 21st Neuropharmacology Conference, November 2011, Falls Church, VA.

Di Sebastiano AR, Coolen LM (2011). Abstinence from sexual behavior induces depression in male rats by activation of corticotropin releasing factor receptors. Society for Behavioral Neuroendocrinology Meeting, June 2011, Queretaro, Mexico.

Di Sebastiano AR, Wang X, Coolen LM (2010). Sexual reward abstinence causes depression-like behavior and changes in expression of corticotropin releasing factor in male rats. Society for Neuroscience Meeting, November 2010, San Diego, CA.

Pitchers KK, Wang X, **Di Sebastiano AR**, Coolen LM (2010). The Role of mGlur5 signaling in the nucleus accumbens in sexual behavior of male rats. Society for Neuroscience Meeting, November 2010, San Diego, CA.

Di Sebastiano AR, Wang X, Coolen LM (2010). Role of Corticotropin Releasing Factor in Sexual Behavior and Experience in the Male Rat. Society for Behavioral Neuroendocrinology Meeting, July 2010, Toronto, Ontario, Canada.

Di Sebastiano AR, Schmid S, Pitchers KK, Coolen LM. (2009). Natural reward induces long-term neural plasticity in the nucleus accumbens. Society for Neuroscience Meeting, October 2009, Chicago, IL.

Di Sebastiano AR, Yong-Yow S, Coolen LM (2008). The role of orexin in sexual behavior and sexual reward of the male rat. Society for Neuroscience Meeting, November 2008, Washington DC.

Di Sebastiano AR, Yong-Yow S, Coolen LM (2008). The role of orexin in male rat sexual behavior. Society for Behavioral Neuroendocrinology Meeting, July 2008, Groningen, The Netherlands.

Di Sebastiano AR, Coolen LM (2008). Orexin is activated by male rat sexual behavior and by contextual cues related to sexual reward. Southern Ontario Neuroscience Association Meeting, May 2008, London, Ontario, Canada.

Seminars:

The University of Western Ontario, Sex, Stress and Depression . Department of Anatomy and Cell Biology Seminar Series (2011).

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