Neuroplasticity in the Mesolimbic System Induced by Sexual Experience and Subsequent Reward Abstinence

Kyle Pitchers, The University of Western Ontario

Supervisor: Lique M. Coolen, The University of Western Ontario
A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Anatomy and Cell Biology
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NEUROPLASTICITY IN THE MESOLIMBIC SYSTEM INDUCED BY
SEXUAL EXPERIENCE AND SUBSEQUENT REWARD ABSTINENCE

(Spine Title: Sex, Drugs and Neuroplasticity)
(Thesis Format: Integrated Article)

By

Kyle Kevin Pitchers

Graduate Program in Anatomy and Cell Biology

A thesis submitted in partial fulfillment
of the requirements for degree of
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The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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The thesis by

Kyle Kevin Pitchers

entitled:

Neuroplasticity in the mesolimbic system induced by sexual experience and subsequent reward abstinence

is accepted in partial fulfillment of the requirements for degree of Doctor of Philosophy
ABSTRACT

Drugs of abuse are hypothesized to usurp brain reward circuits by causing neuroplasticity that contributes to the development and expression of addiction. However, little is known about how these brain circuits are altered in response to natural reward experience. Using male rodent sexual behavior, the goal of my thesis was to determine whether natural reward experience produces neural alterations in the mesolimbic dopamine system, that in turn, regulate incentive motivation for natural as well as drug reward. First, it was determined that male rat sexual experience causes facilitation of sexual behavior, sensitized amphetamine reward, and increased dendritic spines in the nucleus accumbens (NAc). Second, it was found that levels of the transcription factor deltaFosB were increased by sexual experience in several reward-related brain regions, notably the NAc. Blocking deltaFosB activity in the NAc using viral vector-mediated gene transfer attenuated long-term effects of sexual experience on sexual behavior, drug reward, and dendritic spines on NAc neurons. Then, we determined that NAc deltaFosB accumulation and sensitized drug reward caused by sexual experience were dependent on D1, but not D2, dopamine receptor activation during mating. Next, we demonstrated sex experience-induced neuroplasticity in the ventral tegmental area, consisting of an immediate but transient decrease in soma size of dopamine neurons, and reduced tonic dopamine input to NAc. This reduction in soma size was shown to be dependent on endogenous opioids, and critical for morphine reward tolerance, facilitation of sexual behavior, deltaFosB accumulation in the NAc, and mating cue-induced neuronal activity. Finally, sexual experience was found to cause short and long-term increases in NMDA and AMPA receptor subunit expression/trafficking respectively. Moreover, sexual experience caused
an immediate and long-lasting reduction in AMPA/NMDA ratio of synaptic currents in PFC-responding NAc shell neurons. Together, these studies begin to fill an important gap in our knowledge concerning the molecular/cellular basis for natural reward and extend our understanding of the underlying mechanisms of substance abuse.

**Keywords:** sexual behavior, neuroplasticity, deltaFosB, endogenous opioid, mesolimbic, nucleus accumbens, ventral tegmental area, medial prefrontal cortex, dopamine, glutamate, dendritic spines, substance abuse, psychostimulant, opiate, reward memory, incentive motivation.
CO-AUTHORSHIP

Chapter 1 entitled “Mixing pleasures: Review of the effects of drugs on sexual behavior in humans and animal models” was written by Kyle K. Pitchers and Karla S. Frohmader with inputs by Dr. Lique M. Coolen, experimental data included was completed by Kyle K. Pitchers, Karla S. Frohmader, and Margaret E. Balfour. Chapter 2 entitled “Neuroplasticity in the mesolimbic system induced by natural reward and subsequent reward abstinence” was written by Kyle K. Pitchers with inputs by all co-authors. Study design was by Kyle K. Pitchers and Dr. Lique M. Coolen with intellectual input by Drs. Neil M. Richtand, Lei Yu, and Michael N. Lehman. Experimental procedures and data analysis were performed by Kyle K. Pitchers and Margaret E. Balfour. Chapter 3 entitled “deltaFosB in the nucleus accumbens is critical for reinforcing effect of sexual reward” was written by Kyle K. Pitchers with input by all co-authors. Study design was by Kyle K. Pitchers and Dr. Lique M. Coolen with intellectual input by Drs. Michael N. Lehman and Eric J. Nestler. Experimental procedures and data analysis were performed by Kyle K. Pitchers and Karla S. Frohmader. Drs. Vincent Vialou and Eric J. Nestler, and Ezekiell Mouzon provided experimental tools (viral vectors). Chapter 4 entitled “DeltaFosB and dopamine receptor activity in the nucleus accumbens is critical for increased amphetamine reward and nucleus accumbens spine density following loss of natural reward in sexually experienced male rats” was written by Kyle K. Pitchers with input by all co-authors. Study design by Kyle K. Pitchers and Dr. Lique M. Coolen with intellectual input by Drs. Michael N. Lehman and Eric J. Nestler. Experimental procedures and data analysis were performed by Kyle K. Pitchers. Drs. Vincent Vialou and Eric J. Nestler provided viral vectors. Chapter 5 entitled “Endogenous opioid-
induced neuroplasticity of dopaminergic neurons in the ventral tegmental area mediates reinforcement of natural reward” was written by Kyle K. Pitchers with input by all co-authors. Study design was by Kyle K. Pitchers, Caroline M. Coppens, and Dr. Lique M. Coolen. Experimental procedures and data analysis were performed by Kyle K. Pitchers, Caroline M. Coppens, Jonathan Fuller, Sandy Van, and Karla S. Frohmader. Chapter 6 entitled “Natural reward experience alters AMPA and NMDA receptor distribution and function in the nucleus accumbens” was written by Kyle K. Pitchers with inputs by all co-authors. Study design by Kyle K. Pitchers and Dr. Lique M. Coolen with intellectual input by Drs. Susanne Schmid, Michael N. Lehman and Steven R. Laviolette, and Andrea Di Sebastiano. Experimental procedures and data analysis were performed by Kyle K. Pitchers, Xu Wang, Andrea R. Di Sebastiano, and Dr. Susanne Schmid.
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LIST OF ABBREVIATIONS

ABC, avidin-biotin-horseradish peroxidase complex
ACA, anterior cingulate area
AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate
Amph, d-amphetamine
AP-1, activator protein 1
BDNF, brain-derived neurotrophic factor
BLA, basolateral amygdala
BT, biotinylated tyramide
Cdk5, cyclic dependent kinase-5
CE, copulation efficiency
CPP, conditioned place preference
CPu, caudate putamen
D1R, D1 dopamine receptor
D2R, D2 dopamine receptor
DA, dopamine
DAB, 3,3’-diaminobenzidine tetrahydrochloride
DiI, 1,1'-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate
EL, ejaculation latency
EOP, endogenous opioids
ERK, extracellular-regulated protein kinase
GABA, gamma-aminobutyric acid
GFP, green fluorescent protein
HDAC, histone deacetylase
hr, hour
IF, infralimbic area
IL, intromission latency
IM, intromission
i.p., intraperitoneal
IR, immunoreactive
IRS2, insulin receptor substrate 2
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<td>locomotor activity chamber</td>
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<tr>
<td>LiCl</td>
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<td>M</td>
<td>mount</td>
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<td>Meth</td>
<td>methamphetamine</td>
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<td>ML</td>
<td>mount latency</td>
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<td>MOR</td>
<td>mu-opioid receptor</td>
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<td>mPFC</td>
<td>medial prefrontal cortex</td>
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<td>MPN</td>
<td>medial preoptic nucleus</td>
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<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<td>mTORC2</td>
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<td>NaAz</td>
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<td>phosphorylated MAP Kinase</td>
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<td>prelimbic area</td>
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<td>rAAV</td>
<td>recombinant adeno-associated virus</td>
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<td>s.c.</td>
<td>subcutaneous</td>
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<td>TrkB</td>
<td>tropomyosin-regulated kinase</td>
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<td>VTA</td>
<td>ventral tegmental area</td>
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CHAPTER 1: INTRODUCTION

Mixing pleasures: Review of the effects of drugs on sexual behavior in humans and animal models

1.1 INTRODUCTION

It is well recognized that drugs of abuse act on the circuits in the brain that regulate motivation and reward associated with natural behaviors, including sexual behavior, eating, drinking, aggression, and maternal behavior (Hyman, Malenka et al. 2006; Anselme 2009; Carlezon and Thomas 2009; Kalivas 2009). Drugs act on the receptors of the endogenous neurotransmitter systems that regulate these natural rewarding behaviors, such as dopamine, opioid, endocannabinoid, serotonin, and noradrenalin systems (Hull, Meisel et al. 2002; Pfaus 2009). The current review will summarize literature describing the effects of drugs of abuse on male and female sexual behavior in animal models. Next, potential sites where drugs may act to influence sexual behavior are reviewed, with emphasis on action in the mesolimbic system. Finally, we will review recent studies on how sexual behavior and sexual experience affect responses to drugs of abuse.

Currently, the neurobiological basis of this drug-sex nexus is not fully understood and there is a need for controlled human studies using validated methods to measure indicators of sexual function as well as non-human animal studies. The initial portion of this review will discuss studies on effects of systemic administration of drugs of abuse on sexual behavior in animals. Since rodents have been the model of choice in the majority of studies, the review is largely restricted to data in rodents.

1.2 Animal studies: Sexual performance and motivation

Studies in rodents have utilized various behavioral paradigms to allow measures of appetitive, precopulatory or proceptive (sexual motivation), and copulatory behaviors (sexual performance) in males and females. In male rats, approach to or active seeking of
receptive females are indicators of sexual motivation. Several paradigms are used to investigate this measure including recording of approach behaviors and amount of time spent investigating females that are presented behind a barrier. A paradigm used to determine alterations in sexual motivation is the bi-level chamber, consisting of two vertically interconnected levels that allows for measurement of anticipatory searching activity before the entrance of the female partner (Mendelson and Pfau 1989; Pfau, Damsma et al. 1990). Furthermore, latencies to first mount and intromission are also considered indicators of sexual motivation (Pfau, Damsma et al. 1990; Agmo 1997). Male sexual performance is indicated by percentages of animals that display mounts, intromissions, or ejaculations; as well as frequencies of these behaviors (Agmo 1997). In female rodents, copulatory behavior consist of lordosis behavior; the reflex dorsiflexion of the spine in response to stimulation of the flanks (Pfau, Giuliano et al. 2007).

Appetitive, precopulatory behaviors consist of solicitations, and hops and darts (Pfau, Giuliano et al. 2007). An elegant paradigm to investigate female appetitive behavior and thus female sexual motivation, is the paced mating paradigm, in which female rats have the ability to actively seek or avoid the male (Erskine 1989). The next section will review animal studies testing effects of acute or chronic systemic administration of drugs of abuse on male and female sexual behavior.

1.2.1 Psychostimulants

Amphetamine

The use of D-amphetamine (Amph) as a recreational drug is not common practice. However, a vast majority of research implements Amph administration in the rodent
animal model to study the effects of psychostimulants on sexual behavior. Previous research includes studies on the effects of both acute and repeated Amph administration on sexual motivation and performance in both male and female rats.

**Males:** Studies on effects of acute administration of Amph have yielded contradictory results and have either shown facilitation of sexual behavior (Bignami, 1966; Butcher et al., 1969) or have no effect (Agmo and Fernandez 1989; Agmo and Villalpando 1995). In particular, acute administration of Amph (i.p. 0.5 and 1 mg/kg) or amfonelic acid (0.25 and 0.5 mg/kg; i.p.), which like Amph increases dopaminergic neurotransmission, resulted in significant reductions in latencies to mount and intromit in sexually naïve male rats (Ågmo and Picker 1990). However, amfonelic acid administration resulted in significantly increased frequency of sniffing and rearing behavior while behaviors associated with sexual behavior (female pursuit and mounting attempts) were not altered (Ågmo and Picker 1990). Acute administration of Amph in a test for sexual motivation also failed to have effects (Agmo 2003). Therefore, Agmo and coworkers have suggested that increased dopaminergic neurotransmission caused by psychostimulants may indirectly stimulate sexual behavior in male rats by augmenting behavioral arousal.

In contrast, other studies have shown effects of repeated Amph pre-exposure on sexual motivation and performance in male rodents. Fiorino and Phillips (Fiorino and Phillips 1999; Fiorino and Phillips 1999) demonstrated facilitatory effects of repeated Amph (10 injections of 1.5 mg/kg Amph every 2 days) on sexual motivation and performance. Sexual behavior was tested in a drug-free state after a 21-day withdrawal
period during 10 mating tests, and males were sexually naïve at the start of testing. Higher percentages of Amph-pretreated sexually naïve males displayed mounts and intromission and with shorter latencies to mount and intromission compared to controls. No effects on ejaculatory behavior or other parameters of sexual performance were observed. The Amph-induced effects on sexual behavior were independent from drug-associated contextual cues and occurred regardless of whether mating was tested in the same or different environment of drug administration. Furthermore, anticipatory searching activity before the entrance of the female was assessed using a bi-level apparatus (Fiorino and Phillips 1999). Using this measure as an indicator of sexual motivation (Mendelson and Pfau 1989), it was shown that drug pretreatment also enhanced sexual motivation as the Amph group made significantly more level changes in anticipation of the receptive female compared to controls on the last mating test. These results suggested that Amph pretreatment enhanced the attribution of the incentive salience of the rewarding stimulus (the female).

Nocjar and Panskepp (2002) also reported effects of chronic Amph pretreatment on sexual motivation, but reported a complex interaction between drug history and drug-paired environment (Nocjar and Panksepp 2002). It was reported that rats pre-exposed to Amph injections and tested after 2 weeks of drug abstinence showed enhanced or decreased sexual pursuit dependent on the drug history. Animals treated on alternate days (5.0 mg/kg; i.p.; twice daily during 5 alternate days) displayed enhanced sexual pursuit, but only if drug was administered in an environment other than the home cage. In contrast, daily injections resulted in decreased sexual pursuit regardless of the drug-
history environment. In these studies, male rats were sexually naïve; hence, effects were independent of consummation of reward or learning of the incentive value of the female stimulus. Together, these studies demonstrate that Amph-sensitizing treatments can result in cross-sensitization of sexual behavior and cause increased sexual motivation and performance. However, the time of drug abstinence appears to be of influence as male rats that were tested 12 hours after withdrawal from an escalating dose schedule of Amph displayed decreased anticipatory activity (measured in the bi-level apparatus) and longer post ejaculatory intervals, reflecting a decrease in sexual motivation and initiation, without affecting other measures of sexual performance (Barr and Phillips 1999). Thus, drug history and drug history environment, as well as time of drug abstinence, are factors that influence effects of Amph on male rat sexual behavior.

Females: In female rats, acute or chronic administration of Amph seems to have opposite effects on female copulatory behavior and sexual motivation. Acute Amph decreased lordosis behavior and proceptive behaviors in hormone-primed female rats (Pednekar and Mascarenhas 1993; Guarraci and Clark 2003). Moreover, acute Amph affects sexual motivation: Guarraci and Clark (Guarraci and Clark 2003) conducted a study to test the effects acute doses of Amph on pacing mating behavior in ovariectomized and hormone-primed sexually experienced female rats. It was shown that an acute dose of Amph (0.5, 1, or 2 mg/kg; i.p.) increased the likelihood that a female would withdraw from a male after mounting and intromitting compared to saline-injected females and decreased the percentage of time females spent with the male. These results suggest decreased sexual motivation induced by acute Amph.
In general, chronic administration of Amph facilitates sexual function in female rats. Guarraci and Clark (Guarraci and Clark 2003) tested the effects of repeated intermittent Amph exposure on paced mating behavior. Amph-pretreatment resulted in decreased latencies for females to return to the male after sexual stimulation when tested 1 week following the last drug injection. Afonso et al. (Afonso, Mueller et al. 2009) showed that Amph-pretreated females tested following a 21-day drug abstinence period, displayed increased solicitations and proceptive behaviors (hops and darts, as well as pacing) without altering lordosis behavior. Effects of repeated Amph were independent from the drug history environment. These data together with those discussed above for males, suggest that Amph sensitization results in cross-sensitization of sexual behavior, with selective activation of appetitive sexual behavior as sexual performance was not enhanced.

**Methamphetamine**

Although there is abundant research looking at various aspects of Meth abuse on human sexuality, there are only few studies investigating the effects of Meth on sexual behavior under controlled settings. In male Japanese quails (Bolin and Akins 2009), chronic administration of Meth (1 or 3 mg/kg daily for 10 consecutive days) followed by a 10-day withdrawal period reduced sexual motivation but did not affect sexual performance. In contrast, Meth administration in females rats subchronically pretreated with Meth (total of 3 daily injections of 5 mg/kg) increased proceptive and receptive behaviors (Holder, Hadjimarkou et al. 2010). Recent studies from our laboratory have made progress in studying the effects of acute Meth on sexual performance, social interactions,
and inhibition of maladaptive sex-seeking behaviors (Frohmader, Bateman et al. 2010). A
dose-response curve was conducted to test the effects of Meth administration on sexual
performance in male rats. Sexually naïve and experienced males were administered Meth
(0, 1, 2, or 4 mg/kg; s.c.) 30 min before mating. Results showed that Meth administration
inhibited sexual behavior in a dose-dependent manner evidenced by decreased
percentages of males that mated as well as increased latencies to initiate sexual behavior.
Furthermore, sexual experience did not block the effects of Meth on sex (Frohmader,
Bateman et al. 2010). These effects were evident following either acute (Frohmader,
Bateman et al. 2010) or chronic administration (Frohmader, Lehman et al. 2011).

Cocaine

Data in animal studies have shown that acute and chronic cocaine induces penile
erections and facilitates male sexual behavior. Acute cocaine in sexually naive male rats
stimulates spontaneous penile erections when males are examined in all-male groups (7.5
mg/kg) (Andersen, Palma et al. 2000). In addition, cocaine stimulates spontaneous penile
erections and ejaculations when administered following 96 hours of paradoxical sleep
deprivation (7 mg/kg) (Andersen, Bignotto et al. 2003). Acute cocaine also decreased
intromission frequency and increased mount frequency before ejaculation in sexually
experienced males (7.5-15 mg/kg) (Ferrari and Giuliani 1997). Since increased mounting
can be indicative of increased appetitive behavior or alternatively of impaired copulation,
these data suggest that acute cocaine may have complex actions and not simply act as a
sexual stimulant. However, chronic treatment (15 mg/kg; 7 daily injections) caused a
slight decrease in mount and intromission frequency as well as ejaculation frequency
(Ferrari and Giuliani 1997), suggesting that chronic cocaine treatment facilitated sexual behavior. This effect was dose-dependent as acute or chronic treatment with a higher dose of cocaine (30 mg/kg) severely disrupted sexual behavior (Ferrari and Giuliani 1997). In support, chronic cocaine treatment in male Japanese quails (6 daily injections 10 mg/kg) displayed enhanced sexual motivation and performance when tested 10 days following last cocaine exposure (Levens and Akins 2004).

1.2.2 Opiates

*Morphine*

Although most the available research on the effects opiates on human sexual behavior is focused on heroin abuse, experimental research has exclusively focused on the effects of morphine on mating behavior. Acute and chronic administration of morphine severely inhibited sexual behavior in male rats and mating remained suppressed for at least 7 days after last morphine injection (Cui, Ren et al. 2004). Moreover, acute morphine administration (5-10 mg/kg) reduced spontaneous genital reflexes induced by cocaine in paradoxical sleep-deprived male rats (Andersen, Frussa-Filho et al. 2004). Finally, chronic treatment in male rats resulted in a reduction of successful impregnation of females, suggesting a potential effect of chronic morphine treatment on male fertility (Kalivas 2009). Mitchell and Stewart (1990) tested the effects of conditioned stimuli previously associated with systemic injections of morphine on sexual behavior in male rats. Males were subjected to a total of four conditioning trials, during which males received 10 mg/kg morphine and placed in the mating area for 1 hour (paired group) or in the home cage (unpaired group). Exposure to mating environment that males had learned
to associate with morphine resulted in facilitation of appetitive measures of sexual behavior, including increased anogenital exploration, pursuit activity, and shorter mount latencies. These data suggests that stimuli associated with opiate reward result in facilitation of sexual arousal or motivation. Furthermore, Nocjar and Panskepp (2002) reported that chronic morphine (10 mg/kg; 10 daily injections) enhanced preference for a receptive female placed in a stimulus box (only allowed interaction without copulation) when preference was tested following a 2-week, but not 3-day drug abstinence period. Hence, together, these studies show that acute or chronic morphine treatments impairs sexual performance and fertility; yet following chronic morphine exposure and a withdrawal period sexual motivation or arousal is enhanced. In contrast, high-dose methadone maintenance regimen was shown to decrease sexual motivation in male rats, consistent with reports of sexual dysfunction in methadone-maintained individuals (Leri, Sorge et al. 2007) suggesting that opiates may exert differential effects on male sexual behavior.

1.3 Brain sites of drug action

The endogenous neurotransmitter systems that are affected by drugs of abuse include dopamine, opioid, noradrenergic, serotonin, and endocannabinoid systems and are critically involved in regulation of sexual behavior as extensively reviewed (Hull, Meisel et al. 2002; Hull and Dominguez 2006; Pfau 2009). Hence, drugs can act in numerous brain areas to influence various neurotransmitter systems to affect different components of sexual behavior. Drugs may act in the medial preoptic area, the paraventricular nucleus and ventromedial nucleus of the hypothalamus to affect sexual performance and penile
erection in males or lordosis in females (Hull, Meisel et al. 2002; Hull and Dominguez 2006; Pfaus 2009). Drugs may also act at the level of the lumbosacral spinal cord to influence erectile and ejaculatory function (Truitt and Coolen 2002; Coolen and Hull 2004; Giuliano, Bernabe et al. 2004; Allard, Truitt et al. 2005; Coolen 2005; Hull and Dominguez 2006). However, the current review will focus primarily on the mesolimbic system as the main areas where drugs of abuse can affect sexual motivation and risk-taking behaviors.

1.3.1 Mesolimbic system

The mesolimbic dopamine (DA) circuit has been implicated in the regulation of motivational/appetitive, rewarding, and reinforcing aspects of natural rewarding behaviors including mating (Pfaus, Damsma et al. 1990; Kohlert and Meisel 1999; Balfour, Yu et al. 2004), aggression (Pucilowski, Plaznik et al. 1983), feeding (Hernandez and Hoebel 1988; Hernandez and Hoebel 1988; Noel and Gratton 1995; Martel and Fantino 1996; Avena, Bocarsly et al. 2008; Avena 2009), drinking (Yoshida, Yokoo et al. 1992), and social bonding (Young, Lim et al. 2001; Young and Wang 2004). The mesolimbic system is comprised of dopaminergic projection neurons in the ventral tegmental area (VTA) that innervate the nucleus accumbens (NAc), amygdala, and medial prefrontal cortex (mPFC) (Kelley 2004).

In male rats, the mesolimbic system is activated via release of endogenous opioids (EOP) in the ventral tegmental area (VTA) where they inhibit GABAergic interneurons, consequently causing the disinhibition of dopaminergic neurons and subsequent
dopamine efflux in the nucleus accumbens (NAc) (Balfour, Yu et al. 2004). The NAc encompasses the ventral portion of the striatum and is primarily composed of GABAergic medium spiny neurons (90-95%) (Meredith 1999) which project to the ventral pallidum and VTA; the other cells being GABAergic and cholinergic interneurons. The NAc is the primary target of mesolimbic DA release and is believed to integrate glutamate, GABA, opioid, and other neurotransmitters (including serotonin and substance P), for which the medium spiny neurons have been shown to express receptors (Martin, Blackstone et al. 1993; Mansour, Fox et al. 1995; Kwok, Tse et al. 1997; Choe, Shin et al. 2006; Berridge 2007). Specifically, DA signaling is hypothesized to modulate synaptic plasticity in the NAc by affecting the response to glutamate released primarily from the mPFC (Kelley 2004; Mangiavacchi and Wolf 2004). This integration of both excitatory (glutamate) and inhibitory (DA) signals is believed to play a role in attaching valence to stimuli, and regulating mood and goal-mediated behavior (Meredith 1999; Kelley 2004).

**Ventral tegmental area**

The VTA has been implicated in sexual motivation or the anticipation of sexual reward. Bilateral intra-VTA morphine injections facilitate sexual motivation in castrated male rats (Mitchell and Stewart 1990). Naloxone, an opioid receptor antagonist, infused into the VTA inhibits DA release into the NAc and blocks anticipatory sexual behavior (van Furth and van Ree 1996) and the formation of a conditioned place preference for sexual reward (Agmo and Berenfeld 1990), whereas the infusion of morphine facilitates behavior (Mitchell and Stewart 1990). Hence, opiates act in the VTA to facilitate sexual motivation. The VTA is a mesencephalic brain region primarily composed of GABAergic
interneurons and DA projection neurons, located adjacent to a functionally separate population of DA neurons in the substantia nigra. VTA dopaminergic neurons are under tonic inhibition by local GABAergic interneurons. Stimulation of the $G_{i/o}$-coupled mu-opioid receptor (MOR) results in the inhibition of these GABAergic neurons, which in turn leads to disinhibition of dopaminergic projection neurons and the release of DA into NAc (Matthews and German 1984; Johnson and North 1992; Klitenick, Deutch et al. 1992; Ikemoto, Kohl et al. 1997). MORs are expressed postsynaptically on both GABAergic cell bodies (Balfour, Yu et al. 2004) (Figure 1.1) and presynaptically in enkephalin-containing axon terminals in close proximity to GABA neurons (Svingos, Garzon et al. 2001). MORs are activated by endogenous ligands in male rats during sexual behavior or during anticipation of sexual behavior following exposure to conditioned contextual cues that predict mating (Balfour, Yu et al. 2004) (Figure 1.1). MOR activation in these studies was determined by visualization of receptor endocytosis, a powerful marker for endogenous ligand-induced activation of G-protein coupled receptors, including MOR (Sinchak and Micevych 2003; Coolen, Fitzgerald et al. 2004). Similar to, and presumably as a consequence of the activation of MOR, VTA DA neurons are activated (using c-Fos expression as marker of neural activation) during sexual behavior, or following exposure to predictive cues in male rats (Balfour, Yu et al. 2004) (Figure 1.2). In male birds, VTA DA neurons are involved in production of song specifically for courtship (Heimovics and Ritters 2008; Huang and Hessler 2008; Goodson, Kabelik et al. 2009). The VTA has also been shown to play role in female sexual behavior as progesterone and its metabolite $3\alpha, 5\alpha$-THP act in this brain area via
Figure 1.1. VTA MOR-containing neurons and DA neurons are activated by mating behavior as well as exposure to predictive cues that were learned to be associated with sexual behavior in male rats. Confocal images (A) illustrating MOR (red) located on GABA-IR (green) cell bodies (arrow) and MOR-IR fibers in close apposition to GABA-IR cells (triangle) in the VTA. Photomicrograph (B) illustrating mating-induced Fos-IR (black) and tyrosine hydroxylase-IR (brown) in the VTA. Double-labeled cells are indicated by arrows. Confocal images (C, D) illustrating MOR-IR in the VTA of a non-mated control animal (C) or following sexual behavior (D). Arrows indicate MOR-IR endosome like particles. Scale bars indicate 10 μm. E-G illustrate quantitative data showing activation of VTA MOR, VTA dopamine, and NAc shell neurons in eight experimental groups. Animals were either exposed to the home environment, lacking predictive cues (naïve home cage (NH) and experienced home cage (EH)), exposed to the environmental cues predictive of sexual behavior (experienced test cage (ET)), or environmental cues that were unpaired and not predictive of sexual behavior (NT). Activation was examined following sexual behavior (S) or in unmated controls (C). Mating (NHS, EHS, NTS, ETS) as well as exposure to contextual cues that are associated with sexual behavior (ETC) induce internalization of VTA MOR neurons (E), induce Fos in VTA TH-IR cells (F), and Fos in NAc shell neurons (G). Data are presented as mean ± SEM. Solid bars represent groups that mated on the test day, and open bars represent control groups that did not mate on the test day. The statistical relationship between the groups is indicated by lowercase letters; groups that share a common letter do not differ significantly. Modified from (Balfour, Yu et al. 2004).
D1 receptors to activate lordosis (Frye and Walf 2007) and VTA neurons are activated during female sexual behavior (Coria-Avila and Pfau 2007). Moreover, estradiol affects VTA DA neurons as well as basal and stimulated release of DA in the striatum (reviewed by (Becker 2009)).

Few studies have investigated the effect of VTA lesions on female sexual behavior, whereas there is no known literature concerning the effects of VTA lesions on male rodent sexual behavior. Non-specific lesions of the VTA decrease sexual behavior in female rats (Herndon, Paull et al. 1976). In contrast, 6-OHDA lesions show no effect on female rat sexual behavior (Nance 1983; Hansen, Harthon et al. 1991). More recently, it has been shown that 6-OHDA lesions in the VTA increase the lordosis quotient and total lordosis duration in female rats and hamsters respectively (Frye, Petralia et al. 2010). Concomitant effects on motor behavior with 6-OHDA lesions in the VTA and its projection sites have opposite effects in rats and hamsters, despite similar lesion effects on female sexual behavior (Frye, Petralia et al. 2010).

**Nucleus accumbens**

Microdialysis studies in male rats have shown that extracellular levels of DA in the NAc are escalated and maintained at an elevated level upon introduction of a receptive female, throughout mating, and return to baseline shortly after termination of copulation (Pfaus, Damsma et al. 1990; Damsma, Pfaus et al. 1992; Wenkstern, Pfaus et al. 1993; Pfaus and Everitt 1995; Fiorino and Phillips 1999). By contrast, DA and its metabolites do not increase significantly in male rats exposed to non-receptive females.
(Wenkstern, Pfaus et al. 1993). Interestingly, sexually experienced animals that failed to mate also showed no DA release into the accumbens (Pleim, Matohichik et al. 1990; Wang, Huang et al. 1995) suggesting a prerequisite for DA in engaging copulatory acts. The female-induced elevation in extracellular DA levels are low or absent as a male continues to copulate to satiety with the same female, but upon introduction of a novel female, the DA efflux is renewed, as is copulation (Phillips, Vacca et al. 2008). Sexual behavior by female hamsters and rats shows similar elevations in extracellular DA levels during mating (Meisel, Camp et al. 1993; Pfaus and Everitt 1995). Notably there is an effect of sexual history on mating-induced elevation of NAc DA levels. Sexually experienced female hamsters displayed an exaggerated elevation of DA levels, which persisted throughout mating compared to sexually naïve females and females with only three prior mating sessions (Meisel, Camp et al. 1993; Kohlert and Meisel 1999). The escalated extracellular DA in sexually experienced female hamsters is in line with a similar response following chronic administration of drugs of abuse (Robinson and Berridge 2003). Moreover, NAc DA levels in female rats only increase when the females receive copulatory stimulation at a preferred rate (Mermelstein and Becker 1995; Becker, Rudick et al. 2001). These accumbens dialysis studies support the notion that extracellular DA levels may reflect the motivational state of the female.

Limited studies have investigated the effect of NAc lesions on sexual behavior. Lesions of the NAc in female rats increase the rejection of male rats and increase the length of post-ejaculatory interval without affecting copulatory behavior of the male sexual partners (Rivas and Mir 1990; Rivas and Mir 1991). In male rats, excitotoxic
lesions of the NAc suggest that this brain area plays a role in mediating sexual arousal. Specifically, NAc lesioned male rats failed to intromit or rarely copulated to ejaculation, as well as displayed impaired noncontact erections when exposed to an inaccessible receptive female. However, when simultaneously exposed to inaccessible receptive and non-receptive females, these males spent more time in the proximity of receptive females compared to non-receptive females (Kippin, Sotiropoulos et al. 2004). 6-OHDA lesions in the NAc cause an increase in lordosis in both female hamsters and rats (Frye, Petralia et al. 2010). The effects of NAc lesions on human sexual behavior are relatively unknown. One human male case reported a loss of interest in pleasure and sex (Goldenberg, Cox et al. 2002). There have been a number of studies locally infusing DA pharmacological agents into the NAc to observe effects on sexual behavior. The DA agonist apomorphine was found to induce a small decrease in intromission latency (Scaletta and Hull 1990). To a greater extent Amph significantly decreased mount and intromission latencies (Everitt 1990). In contrast with systemic administration, intra-accumbens DA antagonists (haloperidol and raclopride) had no effect on behavior latencies (Everitt 1990). Moses et al. (1995) bilaterally infused a number of DA agonists and antagonists into the accumbens with little to no effect of copulatory behavior; findings that are supported by additional DA antagonist studies (Ahlenius and Larsson 1990; Everitt 1990; Pfaus and Phillips 1991). It has become increasingly clear that DA in the NAc is not critical for the consummatory aspect of male copulatory behavior (reviewed by (Paredes and Agmo 2004). However, a number of studies have looked at the anticipatory phase of male sexual activity using bi-level chambers (Mendelson and Gorzalka 1987). Using this paradigm, DA (D1 and D2 receptors) antagonists have been
found to significantly decrease the number of level changing (Pfaus and Phillips 1991) indicating a facilitatory role for DA in the anticipation for sexual behavior. In support, Fos expression is induced in the NAc of sexually experienced males by exposure to conditioned contextual cues that the males learned to associate with sexual behavior (Balfour, Yu et al. 2004). Hence, NAc neurons are activated in anticipation of sexual behavior (Figure 1.1).

The role of DA for female sexual behavior is not completely clear with DA agonists and antagonists administered systemically having both inhibitory and stimulatory influence on sexual receptivity (reviewed by (Melis and Argiolas 1995). However, DA receptor antagonist block conditioned preference for an environment associated with mating, albeit only if mating actually occurred in that environment (Meisel, Joppa et al. 1996) and not when conditioned place preference was induced by mating that occurred in a separate chamber (Martinez and Paredes 2001). In summary, there is an abundance of evidence using pharmacological agents suggesting DA in the NAc plays a limited role in copulation, yet may be critical for sexual motivation.

**Medial prefrontal cortex**

The prefrontal cortex is divided into three regions: dorsolateral, medial and orbitofrontal (Spinella 2007). The medial prefrontal cortex (mPFC) can be further broken down into anterior cingulate, prelimbic, infralimbic, and precentral regions (Heidbreder and Groenewegen 2003). Collectively, these regions and associated subcortical structures mediate motivational aspects of behavior and may play a role in sexual arousal (Sewards
People with lesions to the anterior cingulate region of mPFC show a reduction in initiation and motivated behaviors, including sex (Nemeth, Hegedus et al. 1988; Devinsky, Morrell et al. 1995; Mega, Cummings et al. 1997). Moreover, the mPFC has been found to play a critical role in reward and punishment (Tzschentke 2000). Human neuroimaging studies show that secondary reinforcers activate the mPFC (Knutson, Westdorp et al. 2000; Gehring and Knight 2002). Using anterograde tracer biotinylated dextran amine injected into mPFC, many brain areas have been identified to be innervated by the mPFC (i.e., nucleus accumbens) (Gorelova and Yang 1997; Balfour, Brown et al. 2006). A significant finding from Balfour et al. (2006) was that the majority of the sex activated Fos-positive cells in the VTA receive putative contacts from the mPFC. Anterior cingulate activity increases in estrous (but not anestrous) female sheep when exposed to males (Ohkura, Fabre-Nys et al. 1997).

As discussed above, in the presence of a female before initiation of contact, male rats experience a DA efflux in the NAc. There is similar increase in extracellular DA release in the mPFC in the presence of a potential mate (Fiorino and Phillips 1999). Further supporting its role in the initiation of behavior, the mPFC is involved in the anticipation and execution of a T maze task in sexually motivated males (Hernandez-Gonzalez, Prieto-Beracochea et al. 2007). In male rats, mPFC lesions have been found to increase mount and intromission latencies one week after operation, an effect which dissipated in time (Agmo, Villalpando et al. 1995). Similar impairments to sexual behavior were partially reversed with the central infusion of dopaminergic stimulants (Agmo and Villalpando 1995). Recent studies conducted by (Davis, Loos et al. 2010)
reported mPFC lesions had no effect on sexual behavior, yet show that mPFC lesions block the animal’s ability to inhibit sexual behavior in a copulation-contingent aversion paradigm. This effect seems to be independent of a learning impairment because lesion animals are capable of forming a conditioned place preference for sex and a conditioned place aversion for LiCl. These studies support a role for the mPFC in the initiation, persistence and compulsive seeking of sexual reward.

1.3.2 Neural activation by sex and drugs in the mesolimbic system

As discussed above, there is a large body of evidence that drugs of abuse and sexual behavior converge on the mesolimbic DA system (Berhow, Hiroi et al. 1996; Pierce and Kalivas 1997). Further evidence that drugs and sex are both acting in this system was provided by an examination of neural activation induced by sex behavior or drugs of abuse, using neuronal activity markers such as Fos and phosphorylation of map kinase (pERK) (Robertson, Pfaus et al. 1991; Valjent, Corvol et al. 2000; Balfour, Yu et al. 2004; Valjent, Pages et al. 2004; Valjent, Pascoli et al. 2005). However, it is unclear if sex and drugs are activating the same neurons in the mesolimbic system. Therefore, a recent study from our laboratory set out to address the question if Meth activates neurons in the brain that are also activated by sexual behavior in male rats (Frohmader, Wiskerke et al. 2010) with the goal to identify potential sites where Meth may be acting to influence sexual behavior. This study utilized dual immunohistochemical detection of two markers of neuronal activation that have separate temporal profiles. Specifically, male rats mated to one ejaculation and were killed one hour after copulation to investigate mating-induced Fos expression. In addition, 10 or 15 minutes before
perfusion, males received acute administration of Meth (4 mg/kg) to examine drug-induced pERK. Indeed, Meth activated the same neurons as were activated by sexual behavior in specific brain areas: NAc, mPFC, and basolateral amygdala (Frohmader, Wiskerke et al. 2010). Since these brain areas are particularly associated with regulation of motivation and compulsivity (see above), these data open up the possibility that methamphetamine acts in these brain sites to alter sexual motivation or contribute to compulsive risk-behaviors associated with sex.

1.3.3 Drug-induced neuroplasticity in the mesolimbic system

The mesolimbic DA circuit exists to mediate natural behaviors, yet it appears that repeated drug administration exerts its harmful effects by usurping the in-place reward system (Hyman, Malenka et al. 2006). A number of alterations at the levels of the gene, synapse, morphology and behavior have been identified following chronic drug exposure (Hyman, Malenka et al. 2006; Kalivas 2009). These changes are hypothesized to underlie the development and maintenance of drug addiction and the increase the susceptibility to drug relapse. The effect of repeated drug administration has been shown on many accounts to have an effect on subsequent drug exposure (Grimm, Hope et al. 2001; Lu, Grimm et al. 2004). The behavioral effects of repeated drug administration include a sensitized locomotor response to psychostimulants and opiates (Segal, Kuczenski et al. 1974; Kalivas and Stewart 1991), an enhanced drug-related reward value (Lett 1989; Shippenberg and Heidbreder 1995; Shippenberg, Heidbreder et al. 1996), and increased operant responses for cues associated with prior drug intake (Reti, Crombag et al. 2008). Moreover, there are several examples of cross sensitization between families of drugs
both with respect to a sensitized locomotor response (Cunningham and Kelley 1992; Bonate, Swann et al. 1997; Pierce and Kalivas 1997; Leri, Flores et al. 2003) and enhanced drug reward (Lett 1989). In addition, repeated drug administration results in long-lasting changes in dendritic spine density and morphology in the nucleus accumbens (Brown and Kolb 2001; Robinson, Gorny et al. 2002; Li, Kolb et al. 2003; Robinson and Kolb 2004), prefrontal cortex (Robinson and Kolb 1997; Robinson and Kolb 1999; Robinson, Gorny et al. 2002; Li, Kolb et al. 2003; Robinson and Kolb 2004) and ventral tegmental area (Sarti, Borgland et al. 2007), and induces alterations in gene expression (McClung and Nestler 2003; Bowers, McFarland et al. 2004; Lu, Dempsey et al. 2005; McClung and Nestler 2008). Finally, repeated drug administration alters synaptic strength at excitatory and inhibitory synapses on midbrain DA neurons (Kauer 2003; Saal, Dong et al. 2003; Liu, Pu et al. 2005; Nugent, Penick et al. 2007; Nugent, Hwong et al. 2008) and neurons in the NAc (Thomas, Malenka et al. 2000; Tzschentke 2001; Thomas and Malenka 2003).

Until recently, it was generally hypothesized that non-compulsive natural behaviors did not induce neuroplasticity (Chen, Bowers et al. 2008; Kalivas 2009), suggesting that the changes being observed throughout the mesolimbic system were specific for compulsive behavior (i.e., addiction); thus, not related to reinforcement in general. Yet, it is becoming increasingly clear that neuroplasticity occurs in the mesolimbic system in response to natural behaviors and not necessarily linked to compulsive seeking of reinforcers.
1.3.4 Effects of experience on the locomotor-inducing effects of amphetamine

Recent studies from our laboratory (Pitchers, Balfour et al. 2010) have demonstrated that sexual experience in male rats induces a robust sensitized locomotor response to a challenge dose of Amph (0.5 mg/kg; s.c.). This effect was observed as early as 1 day and up to 28 days after final mating session, hence cross-sensitization was long-lasting and did not require an abstinence period. Additional experiments were conducted to determine the role of the environment in which sexual experience was obtained (same or different than drug administration) and mating paradigm (consecutive or intermittent) and it was demonstrated that behavioral sensitization induced by sexual experience is independent of context and mating paradigm. Moreover, ejaculation was shown to be critical for behavioral sensitization. Thus, sexual experience caused long-lasting alterations to the locomotor responses induced by Amph, supporting the hypothesis that sexual experience caused neural adaptations in the mesolimbic system.

A behavioral sensitization study has also been conducted in female hamsters (Bradley and Meisel 2001). Behavioral sensitization was tested 1 week after the completion of gaining sexual experience. Females were placed in the behavioral apparatus for 10 minutes to record baseline activity. After 10 minutes, the female hamsters were taken out, injected with a low dose Amph challenge (1.0 mg/kg) and then placed back in the apparatus. Both sexually experienced and naive hamsters receiving Amph displayed a significant increase in number of crossovers in comparison to the initial 10 minutes before receiving drug dose. However, sexually experienced animals
showed increased locomotor response at a significantly shorter time interval after injection (20 minutes) compared to naïve (30 minutes).

1.3.5 Effects of sexual experience on drug reward

To date there has been little work conducted testing the effect of sexual experience on drug reward. A study recently conducted in our laboratory composed drug dose-reward curves in sexually experienced and naive animals, utilizing the CPP paradigm (Pitchers, Balfour et al. 2010). Indeed, sexually experience affected drug-induced CPP, such that sexually experienced males formed a significant CPP for lower doses (0.5 and 1.0 mg/kg) of Amph that did not induce CPP in sexually naïve animals. These results indicated an enhanced or sensitized Amph reward value. Interestingly, this enhancement of drug reward appeared dependent on a period of abstinence from sexual behavior, as no difference existed between sexually experience and naive animals when tested for Amph CPP after the last mating session, and effects of sexual experience were only evident in animals tested 10 days following last mating experience.

Together, these studies support the notion that repeated sexual behavior (sexual experience) in males and females causes alterations in the mesolimbic system that in turn alter the responses to Amph, and increase Amph reward. The latter has only been shown in males and appears dependent not only on sexual experience, but also on subsequent removal of sexual reward.
1.4 SUMMARY

In summary, we discussed the effects of commonly abused drugs including psychostimulants and opiates on sexual behavior in animal studies and vice versa - effects of sexual experience on drug responses. Of particular interest, a greater portion of this chapter was allotted to discuss brain sites where drugs of abuse and sexual behavior converge, and how sexual experience can affect drug responsiveness.

1.5 THESIS RATIONALE AND OBJECTIVES

1.5.1 Rationale

The mesolimbic system mediates naturally rewarding behaviors, including feeding (Avena 2009; Avena, Rada et al. 2009), drinking (Yoshida, Yokoo et al. 1992), maternal behavior (Numan 2007), social bonding (Young, Lim et al. 2001; Young and Wang 2004), and sexual behavior (Pfaus and Phillips 1991; Bradley and Meisel 2001). In particular, male rat sexual behavior is highly rewarding and reinforcing. With sexual experience, male sexual behavior becomes facilitated in terms of sexual motivation and performance (Balfour, Yu et al. 2004) and sex reward is altered (Tenk 2009). These findings suggest sexual experience provokes neuroplasticity within the reward circuit to mediate altered behavioral outcomes and potentially alter responses to non-natural or drug reward. Drugs of abuse converge upon on this mesolimbic system and are hypothesized to usurp its functioning in the development of addiction (Wise 1996; Wise 1998; Nestler 2002). However, the underlying cellular and molecular mechanisms of this sex-induced neuroplasticity are not well understood. Furthermore, sexual behavior, as a model of natural reward behavior, allows the investigation of neural alterations important
for reward and reinforcement in general, which may provide paramount in elucidating differences between natural (normal) reward reinforcement versus compulsive seeking of a reward.

1.5.2 Hypothesis

Natural reward induces neuroplasticity in the mesolimbic system, which is essential for the reinforcing and rewarding properties of natural reward. This neuroplasticity can be detected at the level of gene and protein expression, receptor expression/distribution, synaptic strength, neuronal morphology, and behavior. Moreover, natural reward-induced neuroplasticity will influence rewarding properties of drugs of abuse.

1.5.3 Objectives

1. To demonstrate neuroplasticity in the mesolimbic system caused by sexual experience by analyzing dendritic morphology in the nucleus accumbens and cross-sensitization of amphetamine-induced locomotor activity and amphetamine reward (Chapter 2).

2. To demonstrate the role of the transcription factor deltaFosB in sex experience-induced facilitation of sexual motivation and performance (Chapter 3).

3. To demonstrate the role of deltaFosB and dopamine receptor activation in the nucleus accumbens in mediating sex experience-induced increased dendritic spines in the nucleus accumbens and cross-sensitized amphetamine reward (Chapter 4).
4. To demonstrate the role of endogenous opioids released during sexual behavior in producing neuroplasticity in the ventral tegmental area dopamine neurons, and the functional consequences of this plasticity for experience-induced facilitation of sexual behavior and altered drug reward (Chapter 5).

5. To demonstrate the effects of sexual experience on the glutamatergic system in the nucleus accumbens by analyzing ionotropic glutamate receptor expression, distribution, and function (Chapter 6).
1.6 REFERENCES


Mangiavacchi, S. and M. E. Wolf (2004). "Stimulation of N-methyl-D-aspartate receptors, AMPA receptors or metabotropic glutamate receptors leads to rapid


CHAPTER 2:

Neuroplasticity in the mesolimbic system induced by natural reward and subsequent reward abstinence

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2.1 INTRODUCTION

The mesolimbic dopamine (DA) system, consisting of dopaminergic neurons in the ventral tegmental area (VTA) with projections to the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC), plays a critical role in the motivating and rewarding aspects of behavior including aggression (Pucilowski and Kostowski 1983), feeding (Hernandez and Hoebel 1988; Noel and Wise 1995; Martel and Fantino 1996; Martel and Fantino 1996; Avena, Rada et al. 2008; Avena, Rada et al. 2009), drinking (Yoshida, Yokoo et al. 1992), mating (Pfaus, Damsma et al. 1990; Kohlert and Meisel 1999; Balfour, Yu et al. 2004) and social bonding (Young, Lim et al. 2001; Young and Wang 2004). Drugs of abuse converge upon the mesolimbic DA system (Wise 1987; Di Chiara and Imperato 1988). Moreover, repeated drug administration can induce neuronal alterations in these pathways, that in turn play a putative role in increasing the susceptibility to drug relapse, or in the transition from drug use to drug addiction (Meredith, Ypma et al. 1995; Robinson and Kolb 1999; Robinson, Gorny et al. 2001). The behavioral effects of repeated drug administration include a sensitized locomotor response to psychostimulants and opiates (Segal and Mandell 1974; Post and Rose 1976; Kalivas and Stewart 1991), an enhanced conditioned drug reward (Lett 1989; Shippenberg and Heidbreder 1995; Shippenberg, Heidbreder et al. 1996), and an increased operant responses for cues associated with prior drug intake (Crombag, Bossert et al. 2008). Furthermore, repeated drug administration results in long-lasting changes in dendritic morphology and spine density throughout the mesolimbic circuit (Robinson and Kolb 1997; Robinson and Kolb 1999; Brown and Kolb 2001; Robinson, Gorny et al. 2002; Li, Kolb et al. 2003; Robinson and Kolb 2004; Sarti, Borgland et al. 2007), and
induces gene expression changes (McClung and Nestler 2003; Bowers, McFarland et al. 2004; Lu, Hope et al. 2005; McClung and Nestler 2008). Finally, repeated drug administration alters synaptic strength at excitatory and inhibitory synapses on midbrain DA neurons (Kauer 2003; Saal, Dong et al. 2003; Liu, Pu et al. 2005; Kauer and Malenka 2007; Nugent, Penick et al. 2007; Nugent and Kauer 2008), and neurons in the NAc (Thomas, Malenka et al. 2000; Thomas, Beurrier et al. 2001; Thomas and Malenka 2003). It is currently unclear whether similar alterations in the mesolimbic system occur with repeated exposure to natural rewards. Determining whether such alterations overlap with or are unique to drugs of abuse may lead to a better understanding of the cellular mechanisms underlying the differences between normal reward reinforcement versus compulsive seeking of a particular reward.

Supporting the hypothesis that stimuli other than drugs can cause neuronal alterations in the mesolimbic system are findings that stressful stimuli activate DA systems (Thierry, Tassin et al. 1976; Tidey and Miczek 1996; de Jong, Wasilewski et al. 2005), and cause psychomotor stimulant sensitization (Kalivas and Stewart 1991; Miczek, Covington et al. 2004; Yap, Covington et al. 2005; Mathews, Mills et al. 2008) and relapse in self-administration models (Robinson and Berridge 1993; Piazza and Le Moal 1998; Leri, Flores et al. 2002; Marinelli and Piazza 2002). However, few studies have investigated whether natural rewarding behaviors can also produce functional changes in the DA system (Meisel and Mullins 2006; Avena, Rada et al. 2008; Wallace, Vialou et al. 2008). Therefore, the hypothesis was tested that male sexual experience causes neuronal alterations within the mesolimbic DA system via analysis of effects of
sexual experience on locomotor sensitization, conditioned place preference, and dendrite morphology of NAc neurons. Furthermore, we hypothesized that an abstinence period from sexual behavior (sexual reward) is critical for the onset of these changes, based on recent observations that abstinence from drugs play a key role in the development of neural plasticity associated with repeated drug exposure (Nestler 2001; Wolf, Sun et al. 2004; Kauer and Malenka 2007; Thomas, Kalivas et al. 2008).

2.2 MATERIALS AND METHODS

2.2.1 Animals

Adult male Sprague Dawley rats (210-250 grams) were obtained from Harlan Laboratories (Indianapolis, IN, USA) or Charles River Laboratories (Senneville, QC, Canada) and housed in Plexiglas cages with tunnel tubes. Males were housed in same sex pairs throughout the experiments (experiments 2-5), except for experiment 1 in which males were single housed at the onset of the study. The temperature-regulated colony room was maintained on 12/12 hr light dark cycle with food and water available ad libitum except during behavioral testing. Stimulus females (210-220 grams) for mating behavior sessions were bilaterally ovariectomized and received a subcutaneous implant containing 5% estradiol benzoate and 95% cholesterol. Sexual receptivity was induced by administration of 500 µg progesterone in 0.1 ml sesame oil approximately 4 hours before testing. All procedures were approved by the Animal Care and Use Committees of the University of Cincinnati and the University of Western Ontario, and conformed to NIH and CCAC guidelines involving vertebrate animals in research.
2.2.2 Drug treatment

D-amphetamine (Amph) sulfate (Sigma, St. Louis, MO, USA) was dissolved in sterile 0.9% saline (SAL). Animals received Amph doses ranging 0.5-5.0 mg/kg body weight, calculated based upon the free base, in a volume of 1 mL/kg body weight. Control animals received SAL. All injections were given subcutaneously during the first half of the light phase (2-6 hours after lights on), immediately prior to placement into the behavioral apparatus.

2.2.3 Sexual behavior

In all experiments, sexually naïve male rats were randomly divided into groups that either gained sexual experience or remained naïve. For experience, all mating tests were conducted during the first half of the dark phase (3-8 hours after lights off) under dim red light. Animals that remained sexually naïve were handled and housed in the same rooms as sexually experienced males, hence exposed to similar levels of disturbance, environment novelty and distant female odors as experienced animals. For all experiments, groups of sexually experienced males were matched for sexual experience (based on numbers of ejaculations, and latencies to ejaculation and intromission during last mating session).

2.2.4 Locomotor activity

Locomotor activity was measured using custom-designed locomotor activity chambers (LACs), modeled on chambers designed by Segal and Kuczenski (Segal and Kuczenski 1987). Locomotor activity was measured using a 16x16 photobeam array (San Diego
Instruments, San Diego, CA, USA) and expressed as crossovers per minute(s). A crossover was recorded each time the animal entered any of the “active zones” of the chamber, depicted as shaded areas in Figure 2.1A (Pritchard, Logue et al. 2003).

2.2.5 Experiment 1

Experiments 1 and 2 utilized different paradigms to test the effects of intermittent mating and environment. In experiment 1, animals in the sexually experienced groups received 5 intermittent mating sessions spaced 3-4 days apart, during which they mated in their home cages with a receptive females for 3 copulatory series (including ejaculation) or 60 minutes, whichever came first. Animals that completed more than five cumulative copulatory series were considered sexually experienced. Sexually naïve animals did not receive female partners. One week following the last mating session, sexually experienced and naïve animals were subdivided into groups receiving Amph (0.5 mg/kg) or SAL for a total of four groups (Naïve Amphetamine: NA; Experienced Amphetamine: EA; Naïve Saline: NS; and Experienced Saline: ES; n = 6 each).

2.2.6 Experiment 2

This experiment differed from experiment 1 in three ways: 1. Animals mated to one ejaculation during consecutive days; 2. Animals mated in the same cage as in which they received Amph (in the LACs); 3. Locomotor activity following Amph was analyzed at three different times following sexual experience. The sexually experienced animals received 7 consecutive daily mating sessions in the LACs and locomotor activity was recorded during the 15 minutes between placement in LACs and introduction of female.
The sexually naïve animals were placed in the LACs for seven consecutive sessions without mating. The day following the final mating session (Day 8 of the experiment), animals were placed in the LACs immediately following injection of Amph (0.5 mg/kg) or SAL (Naïve Amphetamine: NA; Experienced Amphetamine: EA; Naïve Saline: NS; and Experienced Saline: ES; n = 8-9 each) and locomotor activity was recorded. The animals were tested in the LACs again one week following the final mating session (Day 14). Animals that received Amph on Day 8 received SAL on Day 14, and animals that received SAL on Day 8 received Amph on Day 14. Half of the naïve and experienced animals were sacrificed one day later for RNA extraction (data not included in this report). Twenty-eight days following the final mating session (Day 35), the remaining half of the animals (Naïve, n = 8; Experienced, n = 9) received Amph and locomotor activity was recorded.

**2.2.7 Data analysis: Locomotor activity**

Data were collected in 3-minute bins for 90 minutes following Amph or SAL injection. Results are shown as the mean ± SEM for each group and analyzed using two-way ANOVA (experiment 1, experiment 2 days 8-14: factors: sexual experience and drug treatment), or a t-test (experiment 2 day 35 and activity before mating sessions; factor: sexual experience). Post-hoc comparisons were made using Fisher LSD tests with significance set at p-value < 0.05.
2.2.8 Conditioned place preference (CPP) testing

2.2.8A Apparatus

CPP was performed in a three-compartment apparatus (Med Associates Inc., St. Albans, VT, USA) which consisted of two larger, outer chambers (28 x 22 x 21cm) distinguishable by visual and tactile cues, separated by a small central compartment (13 x 12 x 21cm). The apparatus was equipped with photo-beams for automated analysis of tracking and measuring locomotor activity.

2.2.8B Conditioning and testing

CPP conditioning and testing was conducted during the first half of the light period. A pre-test was conducted to determine each animal’s initial preference. No significant differences were detected between the times spent in either chamber. On the following day, the male rats were either confined to the Amph-paired chamber or to the SAL-paired chamber for 30 minutes. Rats received the opposite treatment the following day in a counterbalanced manner. A post-test that was procedurally identical to the pre-test was conducted on the final day.

2.2.8C Data analysis: CPP

CPP scores were calculated for each animal as the time spent (sec) in the paired chamber during the post-test minus the pre-test. Group means were calculated and compared to the SAL-treated group (E0) using unpaired t-tests. For all experiments significance was set at a p-value < 0.05.
2.2.9 Experiment 3

Animals in the sexually experienced groups received 5 consecutive daily mating sessions in test cages. Day 1 was assigned to the first mating day. Control males remained sexually naïve, but were placed into a clean test cage for 1 hour each day for 5 consecutive days. Animals were divided into groups receiving different doses of Amph (mg/kg; s.c.) (Naïve: N0.5, N1.0, N2.5 or N5.0, n = 7-8 each; Experience: E0, E0.5, E1.0, E2.5 or E5.0, n = 6-9 each). Pre-test occurred on day 14, conditioning trials on days 15 and 16, and post-test on day 17. This schedule allowed for 10 days of abstinence from sexual behavior before conditioning.

2.2.10 Experiment 4

Sexually experienced males gained sexual experience through 5 consecutive days of mating identical to Experiment 3. The key difference with experiment 4 was that CPP testing occurred while animals were gaining sexual experience, thus there was no period of abstinence from sexual behavior. Instead, conditioning trials began following the first 3 mating sessions (approximately 10 hrs after third mating session). Animals were divided into groups receiving different doses of Amph (mg/kg; s.c.) (Naïve: N0.5, N1.0, N2.5 or N5.0, n = 6-8 each; Experience: E0, E0.5, E1.0, E2.5 or E5.0, n = 7-11 each).

2.2.11 Experiment 5

2.2.11A Experimental design

Males in the sexually experienced groups were placed in a test cage with a receptive female and allowed to mate until one ejaculation or 60 minutes, whichever occurred first,
during 7 consecutive days. Control males remained sexually naïve, but were taken from their home cage and placed into the clean test cage for 30 minutes each day for seven consecutive days. Groups of experienced or naïve animals were sacrificed either one day (N1; n = 5; E1; n = 7) or 7 days (N7, E7; n = 5 each) following the last mating session or exposure to the test cage. Sexually experienced groups did not differ in experience.

2.2.11B Perfusion and tissue processing

One day or one week following the last mating session or exposure to test cage, animals were given an overdose of sodium pentobarbital (i.p.) and were perfused with 500 mL of saline. The brains were processed for Golgi-Cox staining using a method adapted from Pugh and Rossi (1993).

2.2.11C Data analysis: Golgi

Camera Lucida drawings were made of 5-7 neurons in the caudal NAc core and shell subregions in each animal. Cells were selected for which the entire or the majority of the dendritic branches were visible and easy to distinguish from neighboring cells. Dendritic branches were quantified by centrifugal order (Uylings and van Pelt 2002) and averages per animal were calculated. Dendritic spines were quantified on a 40 μm length of two second order dendrites per cell (4-7 cells per animal). Group means were compared using a two-way ANOVA (factors: sex experience and abstinence period) and Fisher LSD tests for post hoc analysis.
2.3 RESULTS

2.3.1 Experiment 1

The goal of Experiment 1 was to determine if sexual experience affects the locomotor response to Amph in male rats. Locomotor activity during a 90-minute period was measured in sexually experienced and naïve rats following treatment with 0.5 mg/kg Amph or SAL. Results from Experiment 1 are illustrated in Figure 2.1. Both sex experience ($F_{1,22} = 15.88; p = 0.0006$) and drug treatment ($F_{1,22} = 45.00; p < 0.0001$) had significant effects on locomotor activity and a two-way interaction between sexual experience and drug treatment was observed ($F_{1,22} = 14.27; p = 0.0010$). Specifically, both naïve and experienced animals showed a significantly increased locomotor response to Amph compared to the appropriate SAL controls. Moreover, sexually experienced rats displayed an increased locomotor response to Amph compared to naïve animals. Sexually experienced and naïve rats did not differ in their responses to SAL.

Analysis of the locomotor responses to Amph in smaller time intervals of 30 minutes and 3 minutes are illustrated in Figure 2.1, panels C-F. Sexually experienced males displayed an increased locomotor response to Amph compared to naïve rats throughout the 90-minute test period. Moreover, sexually experienced rats showed an increased locomotor response to Amph (EA) compared to their SAL controls (ES) throughout the 90-minute test period, while naïve animals only displayed a significantly higher locomotor response during the last 30-minute interval (Figure 2.1; p-values are listed in figure legend).
Figure 2.1. Locomotor response of sexually experienced and naïve animals to saline or Amph administration. A, Schematic diagram of the zone map used to measure locomotor activity. A crossover is recorded each time the animal enters one of the black zones. Mean ± SEM of total number of crossovers over 90 minutes (B) and in 30-minute intervals (C-E; C, 0-30 minutes. D, 31-60 minutes. E, 61-90 minutes). * indicates a statistically significant difference between Amph and saline. # indicates a statistically significant difference between experience and naive groups (p < 0.05). F displays crossovers per 3-minute interval over 90-minutes. Groups: NS = Naïve saline, NA = Naïve amphetamine, ES = Experienced saline, EA = Experienced amphetamine (statistical differences are not indicated) (p-values: B, NA vs. NS, p = 0.0422; EA vs. ES, p < 0.0001, EA vs. NA; p < 0.0001; C-E, EA vs. NA; p < 0.001, EA vs. ES; p < 0.001; E, NA vs. NS; p = 0.0024).
2.3.2 Experiment 2

The goal of experiment 2 was to test if sexual experience results in locomotor sensitization in animals that mated during consecutive days, and in the same environment as in which they are exposed to Amph. Exposure to the sex-paired environment caused increased locomotor activity during the 15 minutes prior to each mating session (Appendix A: supplemental figure 1), illustrating the learned association between sexual behavior and environment. In addition, experiment 2 investigated the temporal pattern of locomotor sensitization to Amph in sexually experienced male rats. The locomotor response to Amph or SAL was measured one day (Day 8; Figure 2.2A, B), one week (Day 14; Figure 2.2C, D) and one month (Day 35; Figure 2.2E, F) following the last mating session. As in experiment 1, sexually experienced rats displayed a greater locomotor response to Amph compared to naïve animals. Moreover, this effect was evident on all three testing days. Figure 2 illustrates locomotor activity during the last 60 minutes of the tests during which the most robust differences were observed, and data for first 30 minutes are shown in supplemental figure 2 (Appendix A). Naïve and experienced animals did not differ in their response to SAL on any of the testing days, and rats that received Amph displayed increased locomotor activity when compared to their SAL controls (Figure 2.2; p-values are listed in figure legend).
Figure 2.2. Temporal expression of sensitized Amph-induced locomotor response by sexually experienced animals. Locomotor response of sexually experienced and naïve animals to saline or Amph administered one day (Day 8; A, B), seven days (Day 14; C, D), or twenty-eight days (Day 35; E, F) following the last mating session. Mean ± SEM of total number of crossovers over the last 60 minutes divided into 30-minute intervals (31-60 minutes; A, C, E. 61-90 minutes; B, D, E). * indicates a statistically significant difference between AMPH and saline (NA vs NS: A; 0.0009, B; 0.0190, C; < 0.0001, D; 0.0002. EA vs ES: A; < 0.0001, B; 0.0002, C; < 0.0001, D; < 0.0001). # indicates a statistically significant difference between experience and naïve groups (p-values: EA vs NA: A; 0.0154, B; 0.0170, C; = 0.05, D; = 0.05, E; 0.0014, F; 0.0400).
2.3.3 Experiment 3
Experiment 3 investigated the effect sexual experience on conditioned Amph reward. Amph CPP was tested in sexually naïve and experienced males 10 days following the final mating session. Sexually experienced animals show an enhanced conditioned Amph reward. Specifically, sexually experienced males formed a strong preference for the Amph-paired chamber with the lower doses of 0.5 and 1.0 mg/kg but not with the higher doses 2.5 or 5.0 mg/kg. In contrast, sexually naïve males only formed a strong preference for the Amph-paired chamber with the higher doses, 2.5 and 5.0 mg/kg, and not the lower doses (Figure 2.3A; p-values are listed in figure legend).

2.3.4 Experiment 4
Experiment 3 demonstrated that sexual experience followed by a period of abstinence resulted in an enhanced conditioned Amph reward. Experiment 4 investigated whether the effect of sexual experience on conditioned Amph reward was dependent on this period of abstinence. Results indicated that sexually experienced animals did not show an increased conditioned reward value of Amph. Sexually experienced and naïve animals showed a strong preference for the Amph-paired chamber with the higher doses of 2.5 and 5.0 mg/kg. However, neither sexually experienced nor naïve males showed an increased CPP score with the lower doses of 0.5 and 1.0 mg/kg dose. The lowest dosage of 0.5 mg/kg even caused an aversion response, but this reached significance only in the sexually experienced animals for the Amph-paired chamber (Figure 2.3B; p-values are listed in figure legend).
Figure 2.3. Sensitized Amph reward in sexually experienced animals. Conditioned place preference of sexually experienced and naive animals in response to Amph either 10 days following (A) or during (B) mating sessions. Mean ± SEM of CPP score, defined as the time spent in the Amph-paired chamber in the post-test minus the pre-test (seconds). * indicates a statistically significant difference from males receiving saline (E0) (p < 0.05). (p-values: A, E0.5 p = 0.014; E1.0 p = 0.017; N2.5 p = 0.016, N5.0 p = 0.022; B, E0.5 p = 0.014, E2.5 p = 0.005, E5.0 p = 0.001; N2.5 p = 0.007, N5.0 p = 0.037).
2.3.5 Experiment 5

The purpose of Experiment 5 was to examine morphological alterations in the mesolimbic system, specifically the NAc, following sexual experience. Morphological alterations were evident one week (Figure 2.4H, J and L; p-values are listed in the figure legend), but not one day (Figure 2.4G, I and K), following the last mating session. In particular, significant increases in numbers of dendrites (indicative of increased dendritic branching) were detected in NAc core and shell (Figure 2.4H, J). In addition, numbers of dendritic spines were significantly increased in both the shell and core regions, one week, but not one day, after sex experience (Figure 2.4L).

2.4 DISCUSSION

This study demonstrates that sexual experience and the post-experience abstinence from sexual behavior induce functional and morphological alterations in the mesolimbic system of male rats. Functional changes were evident in the form of a sensitized locomotor response and an enhanced conditioned reward with Amph following sexual experience. The sensitized locomotor response was observed as early as 1 day and maintained up to 28 days after last mating session. By contrast, the enhanced conditioned Amph reward was only evident following an abstinence period from sexual behavior. Morphological alterations in both core and shell subregions of NAc were observed 7 days, but not 1 day, following the last mating session in sexually experienced animals. Together these data demonstrate that sexual experience induces plasticity in the mesolimbic system and that an abstinence period from mating is critical for the development of some, but not all mesolimbic system changes.
Figure 2.4. Dendritic morphology in the NAc of sexually experienced and naïve animals. Sexual experience caused an increase in numbers of dendrites and dendritic spines, illustrated by images (A, B) and camera lucida drawings (C, D) of representative NAc shell neurons as well as spine density on second order dendrites (E, F) in NAc shell of naïve (A, C, E) or experienced (B, D, F) animals, 7 days following last treatment. Scale bar indicates 50 (A, B) or 10 (E, F) µm. Quantitative analysis shows significant increases of numbers of dendrites per centrifugal order (G-J) and numbers of spines (K, L) in NAc core (G, H) and shell (I, J) in sexually experienced males (black bars) compared to naïve males (white bars), but only 7 days following last mating (H, J, L). Data are expressed as mean ± (SEM). * indicates a statistically significant difference from naïve males (all p-values < 0.001, except G: 0: 0.013, I: 0.002).
It is well recognized that natural rewarding behaviors and drugs of abuse act within the same neural pathways (Hyman, Malenka et al. 2006). Indeed, drugs of abuse have been demonstrated to affect the expression of rewarding behaviors (Della Maggiore and Ralph 2000; Avena and Hoebel 2003; Aragona, Detwiler et al. 2007), including male rat sexual behavior (Mitchell and Stewart 1990; Barr, Fiorino et al. 1999; Fiorino and Phillips 1999; Avena and Hoebel 2003). The alterations in sexual behavior and motivation caused by repeated drug administration are dependent on a withdrawal or abstinence period from drug, as well as the environment in which the drug was presented. The current study showed that exposure to sexual behavior alters responsiveness to drugs of abuse. It was determined that sexually experienced male rats are sensitized to the locomotor effects of Amph, and that this phenomenon is long-lasting and independent of an abstinence period from mating. Moreover, the sensitized locomotor response was independent of mating schedule or mating environment and was observed following either consecutive or intermittent mating sessions that occurred in the same or different environment as drug exposure. Studies conducted in female hamsters showed that sexually experienced female hamsters display a more rapid onset of Amph-induced locomotor response compared to sexually naïve controls (Bradley and Meisel 2001). However, rodents display sexual dimorphic responses to psychostimulants (Castner, Xiao et al. 1993; Becker, Molenda et al. 2001). Thus, the current studies expand the findings in female hamsters and demonstrate in male rats, the fast onset and the long duration of the enhanced locomotor responses to psychostimulants following sexual behavior.
It is unclear from the current studies which elements of sexual behavior contribute to the Amph locomotor sensitization and if social interactions are sufficient. Animals in experiment 2 that failed to reach the criteria for sexual experience (displayed mounts and intromissions, but did not copulate to 5 ejaculations during the mating sessions) did not show a sensitized response (Appendix A: supplemental figure 3). Therefore, an additional experiment was performed during which males were exposed to a receptive female without physical interaction, or displayed mounts and intromissions, neither of which resulted in sensitized locomotor responses to Amph (Appendix A: supplemental figure 4). Thus, social interactions do not appear to contribute to the effects of sexual experience on Amph sensitization, but rather copulation including ejaculation appears essential for this form of plasticity.

In addition to a sensitized behavioral response, sexual experience enhances the conditioned reward value of Amph, but only following abstinence from sexual reward. Previous work using CPP has shown that repeated exposures to psychostimulants or opiates augment drug-induced rewarding effects in line with the drug-induced locomotor sensitization (Lett 1989; Shippenberg and Heidbreder 1995; Shippenberg, Heidbreder et al. 1996). Repeated administration for 5 days of either cocaine (10 mg/kg), Amph (0.5 mg/kg) or morphine (5 mg/kg) sensitizes the rewarding effects of cocaine when tested 3 days following the cessation of drug pre-treatment. The sensitized effect was displayed by observing a conditioned preference with fewer conditioning trials (from 3 to 2) and with lower drug doses compared to SAL pre-treated control animals. The sensitized conditioned reward caused by repeated cocaine was found 7 days, but not 14 days, after
final pretreatment of cocaine (Shippenberg and Heidbreder 1995). A similar study utilizing 5 days of morphine (5.0 mg/kg) shows an augmented conditioned reward response to morphine when conditioning started 3, 10, or 21 days after drug pre-treatment. This augmented response was absent 1 day following morphine pre-treatment (Shippenberg, Heidbreder et al. 1996). Such findings suggest that a period of drug withdrawal of at least 3 days is required for the sensitized or cross-sensitized conditioned reward for both psychostimulants and opiates. Sexual experience, like repeated drug administration, may be instilling similar neuroadaptations in the mesolimbic system responsible for this sensitized drug responsiveness once the reward has been removed. It is currently unclear if reward abstinence is associated with stress and thus acts as a psychological stressor contributing to the observed alterations.

Clearly, there is interplay between the effects of natural and drug reward. Reward cross-sensitization suggests that the long-lasting effects of both sexual behavior and drugs are mediated by common cellular or molecular mechanisms. Therefore, it is hypothesized that the sex behavior-induced alterations regulate the reinforcing components of sexual behavior and thus may be critical for positive reinforcement of rewarding behaviors in general. However, a subsequent abstinence from sexual reward may induce a state of increased reward seeking, or vulnerability to the effects of addictive substances similar to the effects of abstinence an ‘incubation of drug craving’ (Grimm, Hope et al. 2001; Lu, Hope et al. 2005; Crombag, Bossert et al. 2008). In general, sexual behavior in male rodents does not causes compulsive seeking for sex, shown using
copulation-malaise associative conditioning experiments (Agmo 2002), although the influence of abstinence has not been tested.

Dendritic morphology has been examined in depth in the fields of learning and memory (Chang and Greenough 1982; Van Reempts, Dikova et al. 1992) and addiction (Nestler and Aghajanian 1997; Nestler 2001; Norrholm, Bibb et al. 2003), and is known to be influenced by environmental (Rosenzweig and Bennett 1996) and hormonal factors (Jansen, Cutter et al. 2003; Adams, Goodman et al. 2006). Since the synaptic inputs are predominantly on dendrites or dendritic spines, they are the most likely target of experience-induced neuroplasticity (Lamprecht and LeDoux 2004; Robinson and Kolb 2004). Natural fluctuations or administration of gonadal hormones have been found to cause dendritic changes within several hours (Gould, Woolley et al. 1990; Woolley, Gould et al. 1990; de Castilhos, Forti et al. 2008; Schwarz, Liang et al. 2008). Also, perturbations to the system, such stress (Dalla, Whetstone et al. 2009) or chronic cocaine (Norrholm, Bibb et al. 2003), cause dendritic alterations detectable within 24 hours. Here, changes to dendritic morphology of medium spiny neurons in both the NAc core and shell were not observed within 24 hours, and instead required a period of abstinence following sexual experience. The structural alterations induced by sexual experience and subsequent abstinence resemble those seen following repeated exposure to psychostimulants (Robinson and Kolb 1997; Robinson and Kolb 1999; Robinson, Gorny et al. 2001; Robinson and Kolb 2004). By contrast, DA depletion in the NAc results in a decreased number of dendrites and complexity in the shell (Meredith, Ypma et al. 1995; Robinson and Kolb 1999). Hence sexual experience-induced changes may be dependent
on endogenous DA action in the NAc. However, mating-induced morphological alterations were only evident 7 days following the last mating session and coincide with the enhanced conditioned Amph reward in sexually experienced animals. These data suggest that these increases in dendritic arborization and spines are not required for the expression of short-term locomotor sensitization to Amph, yet may play a role in the maintenance and long-term expression of the sensitization. Previous studies of repeated drug administration have also noted a disconnect between long-term sensitization and morphological alterations in the NAc (Vezina, Giovino et al. 1989; Spanagel and Shippenberg 1993; Cunningham, Finn et al. 1997; Robinson and Kolb 1999; Pulipparacharuvil, Renthal et al. 2008; Singer, Tanabe et al. 2009). It remains unclear what the functional relevance is of the morphological alterations, but it may play a role in the long term changes in function and gene expression.

In summary, the data presented here demonstrate that sexual behavior – a natural rewarding stimulus – can induce long-lasting neuroadaptations in the mesolimbic system. Our findings suggest that behavioral plasticity, particularly a sensitized locomotor response, is an immediate and long-term outcome of sexual experience. Moreover, an abstinence period may allow for neuroadaptations critical for observed morphological changes in the NAc and subsequent enhanced conditioned drug reward. This behavioral and neural plasticity follows a similar, but not identical, profile as seen in drug-sensitized animals. These data are of particular interest since we show that an abstinence from the natural reward induces a vulnerable state to drug administration. Understanding how both natural behaviors and drugs of abuse activate these systems causing neuroadaptations
may provide us with a better understanding of reinforcement and reward in general, and provide further insight into the mechanisms of drug addiction.
2.5 REFERENCES


CHAPTER 3:
DeltaFosB in the nucleus accumbens is critical for reinforcing effects of sexual reward

3.1 INTRODUCTION

Sexual behavior is highly rewarding and reinforcing for male rodents (Pfaus, Kippin et al. 2001; Balfour, Yu et al. 2004). Moreover, sexual experience alters subsequent sexual behavior and reward (Tenk, Wilson et al. 2009). With repeated mating experience, sex behavior is facilitated or “reinforced”, evinced by decreased latencies to initiate mating and facilitation of sexual performance (Pfaus, Kippin et al. 2001; Balfour, Yu et al. 2004). However, the underlying cellular and molecular mechanisms of sexual reward and reinforcement are poorly understood.

Sexual behavior and conditioned cues that predict mating have been shown to transiently induce expression of immediate-early gene c-fos in the mesolimbic system of male rats (Pfaus, Kippin et al. 2001; Balfour, Yu et al. 2004). Moreover, it was recently demonstrated that sexual experience induces long-lasting neuroplasticity in the male rat mesolimbic system (Frohmader, Pitchers et al. 2010; Pitchers, Balfour et al. 2010). In addition, in male rats, sexual experience has been shown to induce deltaFosB, a Fos family member, in the nucleus accumbens (NAc) (Wallace, Vialou et al. 2008). DeltaFosB, a truncated splice variant of FosB, is a unique member of the Fos family due to its greater stability (Ulery, Rudenko et al. 2006; Carle, Ohnishi et al. 2007; Ulery-Reynolds, Castillo et al. 2008) and plays a role in enhanced motivation and reward for drugs of abuse and the long-term neural plasticity mediating addiction (Nestler, Barrot et al. 2001). Typically, deltaFosB forms a heteromeric transcription factor complex (activator protein-1 (AP-1)) with Jun proteins, preferably JunD (Chen, Nye et al. 1995; Hiroi, Marek et al. 1998). Through inducible over-expression of deltaFosB, primarily
restricted to the striatum using bi-transgenic mice, a drug addicted-like behavioral phenotype is produced despite an absence of previous drug exposure (McClung, Ulery et al. 2004). This behavioral phenotype includes a sensitized locomotor response to cocaine (Kelz, Chen et al. 1999), increased preference for cocaine (Kelz, Chen et al. 1999) and morphine (Zachariou, Bolanos et al. 2006), and increased cocaine self-administration (Colby, Whisler et al. 2003).

Similar to drug reward, deltaFosB is up-regulated by natural rewarding behaviors and mediates the expression of these behaviors. Over-expression of deltaFosB in the NAc using rodent models increases voluntary wheel running (Werme, Messer et al. 2002), instrumental responding for food (Olausson, Jentsch et al. 2006), sucrose intake (Wallace, Vialou et al. 2008), and facilitates male (Wallace, Vialou et al. 2008) and female (Hedges, Chakravarty et al. 2009) sexual behavior. Thus, deltaFosB may be involved in mediating the effects of natural rewarding experiences. The current study expands on previous studies by specifically investigating the role of deltaFosB in the NAc in the long-term outcomes of sexual experience on subsequent mating behavior and neural activation in the mesolimbic system. First, it was established which brain regions implicated in the reward circuitry and sexual behavior express sex experience-induced deltaFosB. Next, the effect of sex experience-induced deltaFosB on mating-induced expression of c-Fos, a downstream target repressed by deltaFosB (Renthal, Carle et al. 2008), was investigated. Finally, the effect of manipulating deltaFosB activity in the NAc (gene over-expression and expression of a dominant negative-binding partner) on sexual
behavior and experience-induced facilitation of sexual motivation and performance was
determined using viral vector delivery technology.

3.2 MATERIALS AND METHODS

3.2.1 Animals
Adult male Sprague Dawley rats (200-225 grams) were obtained from Charles River
Laboratories (Senneville, QC, Canada). Animals were housed in Plexiglas cages with a
tunnel tube in same sex pairs throughout experiments. The colony room was temperature-
regulated and maintained on a 12/12 hr light dark cycle with food and water available ad
libitum except during behavioral testing. Stimulus females (210-220 grams) for mating
sessions received a subcutaneous implant containing 5% estradiol benzoate and 95%
cholesterol following bilateral ovariectomy under deep anesthesia (0.35g
ketamine/0.052g xylazine). Sexual receptivity was induced by administration of 500 µg
progesterone in 0.1 mL sesame oil approximately 4 hours before testing. All procedures
were approved by the Animal Care and Use Committees of the University of Western
Ontario and conformed to CCAC guidelines involving vertebrate animals in research.

3.2.2 Sexual behavior
Mating sessions occurred during the early dark phase (between 2-6 hours after onset of
the dark period) under dim red illumination. Prior to experiment onset, animals were
randomly divided into groups. During mating sessions male rats were allowed to copulate
to ejaculation or 1 hour, and parameters for sexual behavior were recorded including:
mount latency (ML; time from introduction of the female until the first mount),
intromission latency (IL; time from introduction of the female until the first mount with vaginal penetration), ejaculation latency (EL; time from the first intromission to ejaculation), post ejaculation interval (PEI; time from ejaculation to first subsequent intromission), number of mounts (M; pelvic thrusting without vaginal penetration), number of intromissions (IM; mount including vaginal penetration) and copulation efficiency (CE = IM/(M+IM)) (Agmo 1997). Numbers of mounts and intromissions were not included in the analysis for animals that did not display ejaculation. Mount and intromission latencies are parameters indicative of sexual motivation, while ejaculation latency, number of mounts and copulation efficiency reflect sexual performance (Coolen and Hull 2004).

3.2.3 Experiment 1: Expression of deltaFosB

3.2.3A Experimental design

Sexually naïve male rats were allowed to mate in clean test cages (60 x 45 x 50 cm) for 5 consecutive, daily mating sessions or remained sexually naïve. Supplemental table 1 (Appendix B) outlines the behavioral paradigm for experimental groups: naïve no sex (NNS; n = 5), naïve sex (NS; n = 5), experienced no sex (ENS; n = 5) and experienced sex (ES; n = 4). NS and ES animals were sacrificed 1 hour following ejaculation on the final day of mating to investigate mating-induced c-Fos expression. NNS animals were sacrificed concurrently with ENS animals 24 hours after final mating session to examine sex experience-induced deltaFosB. Sexually experienced groups were matched for sexual behavior before subsequent testing. No significant differences were detected between groups for any behavioral measures within the appropriate mating session and sex
experience-induced facilitation of sexual behavior was displayed by both experienced groups (Appendix B: supplemental table 2). Controls included sexually naïve males handled concurrently with mating animals ensuring exposure to female odors and vocalizations without direct female contact.

3.2.3B Perfusion, tissue processing, and immunohistochemistry

For sacrifice, animals were deeply anesthetized using sodium pentobarbital (270 mg/kg; i.p.) and perfused intracardially with 50 mL of 0.9% saline, followed by 500 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were removed and post-fixed for 1 hr at room temperature (RT) in the same fixative, then immersed in 20% sucrose and 0.01% sodium azide in 0.1 M PB and stored at 4°C. Coronal sections (35 μm) were cut with a freezing microtome (H400R, Micron, Germany), collected in four parallel series in cryoprotectant solution (30% sucrose and 30% ethylene glycol in 0.1 M PB) and stored at -20°C. Free floating sections were washed extensively with 0.1 M phosphate-buffered saline (PBS; pH 7.3-7.4) between incubations. Sections were exposed to 1% H2O2 for 10 min at room temperature to destroy endogenous peroxidases, then blocked in PBS+ incubation solution, which is PBS containing 0.1% bovine serum albumin (catalog item 005-000-121; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 0.4% Triton X-100 (catalog item BP151-500; Sigma-Aldrich) for 1 hr. Sections were then incubated overnight at RT in a pan-FosB rabbit polyclonal antibody (1:5K; sc-48 Santa Cruz Biotechnology, Santa Cruz, CA, USA). The pan-FosB antibody was raised against an internal region shared by FosB and deltaFosB. The deltaFosB-immunoreactive (IR) cells were specifically deltaFosB-positive because at the post-stimulus time (24 hr)
all detectable stimulus-induced FosB is degraded (Perrotti, Hadeishi et al. 2004; Perrotti, Weaver et al. 2008). In addition, in this experiment animals mating on the final day (NS, ES) were sacrificed 1 hr after mating, thus prior to FosB expression. Western Blot analysis confirmed the detection of deltaFosB at approximately 37 kDa. After primary antibody incubation, sections were incubated for 1 hr in biotin-conjugated goat anti-rabbit IgG (1:500 in PBS+; Vector Laboratories, Burlingame, CA, USA) and then 1 hr in avidin-biotin-horseradish peroxidase (ABC elite; 1:1K in PBS; Vector Laboratories, Burlingame, CA, USA). Following this incubation sections were processed in one of the following ways:

*Single peroxidase labeling*

Sections of NNS and ENS animals were used for a brain analysis of sexual experience-induced deltaFosB accumulation. Following ABC incubation, the peroxidase complex was visualized following treatment for 10 minutes to a chromogen solution containing 0.02% 3,3’-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO, USA) enhanced with 0.02% nickel sulfate in 0.1 M PB with hydrogen peroxide (0.015%). Sections were washed thoroughly in 0.1 M PB to terminate the reaction and mounted onto coded Superfrost plus glass slides (Fisher, Pittsburgh, PA, USA) with 0.3% gelatin in ddH₂O. Following dehydration, all slides were cover-slipped with DPX (dibutyl phthalate xylene).
Dual immunofluorescence

Sections from all four experimental groups containing NAc and mPFC were used for analysis of deltaFosB and c-Fos. Following ABC incubation, sections were incubated for 10 min with biotinylated tyramide (BT; 1:250 in PBS + 0.003% H₂O₂ Tyramide Signal Amplification Kit, NEN Life Sciences, Boston, MA, USA) and for 30 min with Alexa 488-conjugated streptavidin (1:100; Jackson Immunoresearch Laboratories, West Grove, PA, USA). Sections were then incubated overnight with a rabbit polyclonal antibody specifically recognizing c-Fos (1:150; sc-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by a 30 min incubation with goat anti-rabbit Cy3-conjugated secondary antibody (1:200; Jackson Immunoresearch Laboratories, West Grove, PA, USA).

Following staining, the sections were washed thoroughly in 0.1 M PB, mounted onto coded glass slides with 0.3% gelatin in ddH₂O and cover-slipped with an aqueous mounting medium (Gelvatol) containing the anti-fading agent 1,4-diazabicyclo(2,2)octane (DABCO; 50 mg/ml, Sigma-Aldrich, St. Louis, MO, USA). Immunohistochemical controls included omission of either or both primary antibodies, resulting in absence of labeling in the appropriate wavelength.

3.2.3C Data analysis: Brain analysis of deltaFosB

Two experimenters blind to treatment performed the brain wide scan on coded slides. DeltaFosB-IR cells throughout the brain were semi-quantitatively analyzed using a scale to represent the number of deltaFosB-IR cells as outlined in Table 3.1. In addition, based on semi-quantitative findings, numbers of deltaFosB-IR cells were counted using standard areas of analysis in brain areas implicated in reward and sexual behavior using a
camera lucida drawing tube attached to a Leica DMRD microscope (Leica Microsystems GmbH, Wetzlar, Germany): NAc (core (C) and shell (S); 400x600μm) analyzed at three rostral-caudal levels (Balfour, Yu et al. 2004); ventral tegmental area (VTA; 1000x800μm) analyzed at three rostral-caudal levels (Balfour, Yu et al. 2004) and VTA tail (Perrotti, Bolanos et al. 2005); prefrontal cortex (anterior cingulate area (ACA); prelimbic cortex (PL); infralimbic cortex (IL); 600x800μm each); caudate putamen (CP; 800x800μm); and medial preoptic nucleus (MPN; 400x600 μm) (Appendix B: supplemental figures 1-3). Two sections were counted per subregion, and averaged per animal for calculation of the group mean. Sexually naïve and experienced group averages of deltaFosB-IR cells were compared for each subregion using unpaired t-tests.

3.2.3D Data analysis: DeltaFosB and c-Fos

Images were captured using a cooled CCD camera (Microfire, Optronics) attached to a Leica microscope (DM5000B, Leica Microsystems; Wetzlar, Germany) and Neurolucida software (MicroBrightfield Inc.) with fixed camera settings for all subjects (using 10x objectives). Number of cells expressing c-Fos-IR or deltaFosB-IR in standard areas of analysis in NAc core and shell (400x600μm each; Appendix B: supplemental figure 1) and ACA of the mPFC (600x800μm; Appendix B: supplemental figure 3) were manually counted by an observer blinded to the experimental groups, in 2 sections per animal using Neurolucida software (MBF Bioscience, Williston, VT, USA) and averaged per animal. Group averages of c-Fos or deltaFosB cells were compared using two-way ANOVA (Factors: sexual experience and sex activity) and Fisher LSD for post hoc comparisons at a significance level of 0.05.
3.2.4 Experiment 2: DeltaFosB expression manipulation

3.2.4A Viral vector-mediated gene transfer

Sexually naive male Sprague Dawley rats were randomly divided into groups prior to stereotaxic surgery. All animals received bilateral microinjections of recombinant adeno-associated viral (rAAV) vectors encoding GFP (control; n = 12), wild-type deltaFosB (n = 11) or a dominant-negative binding partner of deltaFosB termed deltaJunD (n = 9) into the NAc. DeltaJunD decreases deltaFosB mediated transcription by competitively heterodimerizing with deltaFosB before binding the AP-1 region within gene promoters (Winstanley, LaPlant et al. 2007). Virus titer was determined by qPCR and evaluated in vivo prior to study onset. Titer was 1-2 x 10^{11} infectious particles per mL. RAAV vectors were injected in a volume of 1.5 μL/side over 7 minutes (coordinates: AP +1.5, ML ±1.2 from bregma; DV -7.6 from skull surface according to Paxinos and Watson, 1998) using a Hamilton syringe (5 μL; Harvard Apparatus, Holliston, MA, USA). The vectors produce no toxicity greater than control infusions alone (Winstanley et al, 2007; for details of AAV preparation, see Hommel et al., 2003). Behavioral experiments started 3 weeks after vector injections allowing for optimal and stable viral infection (Wallace, Vialou et al. 2008). Transgene expression in murine species peaks at 10 days and remains elevated for at least 6 months (Winstanley, LaPlant et al. 2007). At the end of the experiment, the animals were transcardially perfused and NAc sections were immuno-processed for GFP (1:20K; rabbit anti-GFP antibody; Molecular Probes) using an ABC-peroxidase-DAB reaction (as described above) to histologically verify injection sites using GFP as a marker (Appendix B: supplemental figure 4). DeltaFosB and deltaJunD vectors also contain a segment expressing GFP separated by an internal ribosomal entry site, allowing
for injection site verification by GFP visualization in all animals. Only animals with
injection sites and spread of virus restricted to the NAc were included in statistical
analyses. Spread of virus was generally limited to a portion of the NAc and did not
spread rostral-caudally throughout the nucleus. Moreover, spread of virus appeared
mostly restricted to either shell or core. However, the variation of injection sites and
spread within the NAc did not influence effects on behavior. Finally, GFP injections did
not affect sexual behavior or experience-induced facilitation of sexual behavior compared
to non-surgery animals from previous studies (Balfour, Yu et al. 2004).

3.2.4B Sexual behavior

Three weeks following viral vector delivery, animals mated to one ejaculation (or for 1
hour) for 4 consecutive, daily mating sessions to gain sexual experience (experience
sessions) and were subsequently tested for long-term expression of experience induced
facilitation of sexual behavior 1 and 2 weeks (test sessions 1 and 2) after the final
experience session. Sexual behavior parameters were recorded during all mating sessions
as described above. Statistical differences for all parameters during each mating session
were compared within and between groups using two-way repeated measures ANOVAs
(Factors: treatment and mating session) or one-way ANOVAs (ejaculation latency,
number of mounts and intromissions; Factor: treatment or mating session) followed by
Fisher LSD or Newman-Keuls tests for post hoc comparisons at a significance level of
0.05. Specifically, the facilitative effects of sexual experience on mating parameters were
compared between experience session 1 (naïve) and experience sessions 2, 3, or 4 each,
as well as between experimental groups within each experience session. Moreover, to
analyze effects of treatment (vector) on long-term facilitation of sexual behavior, mating parameters were compared between experience session 4 and test session 1 and 2 within each treatment group, and compared between experimental groups within each test session.

3.3 RESULTS

3.3.1 Sexual experience causes deltaFosB accumulation

Initially, a semi-quantitative investigation of deltaFosB accumulation throughout the brain in sexually experienced males compared to sexually naïve controls was conducted. A summary of overall findings is provided in Table 3.1. DeltaFosB-IR analysis was furthered by determining the numbers of deltaFosB-IR cells in several limbic-associated brain regions using standard areas of analysis. Figure 3.1 demonstrates representative images of DAB-Ni staining the NAc of sexually naïve and experienced animals.

Significant deltaFosB up-regulation was found in mPFC subregions (Figure 3.2A), NAc core and shell (3.2B), CPu (3.2B) and VTA (3.2C). In NAc, significant differences existed at all rostral-caudal levels in the NAc core and shell, and data shown in Figure 2 is the average over all rostro-caudal levels. In contrast, there was no significant increase in deltaFosB-IR in the hypothalamic medial preoptic nucleus (NNS: Avg 1.8 ± 0.26; ENS: Avg 6.0 ± 1.86).
Table 3.1. Summary of deltaFosB expression in sexually naïve and experienced animals. Semi-quantitative analysis of deltaFosB expression in sexually naïve and experienced animals (n=5 each group). Numbers of deltaFosB-IR cells were rated using the following scale: - (absent; 0-1 cell), + (low; 2-10 cells), ++ (10-50 cells), +++ (50-100 cells) and ++++ (highest; >100 cells).

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Control</th>
<th>Sexual Experience</th>
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<tbody>
<tr>
<td>Prefrontal Cortex</td>
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<tr>
<td>Anterior Cingulate Area</td>
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<td>+++</td>
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<tr>
<td>Prelimbic Area</td>
<td>++</td>
<td>+++</td>
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<tr>
<td>Infrahlimbic Area</td>
<td>++</td>
<td>+++++</td>
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<tr>
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<td>Core</td>
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<tr>
<td>Shell</td>
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<tr>
<td>Caudate Putamen</td>
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<tr>
<td>Ventral Pallidum</td>
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<td>Globus Pallidum</td>
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<td>-</td>
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<tr>
<td>Olfactory Tubercle</td>
<td>+</td>
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<tr>
<td>Piriform Cortex</td>
<td>+</td>
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<td>Bed Nucleus of the Stria</td>
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<tr>
<td>Medial Preoptic Area</td>
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<tr>
<td>Anterior</td>
<td>-</td>
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<td>Lateral</td>
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<tr>
<td>Posterior</td>
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<td>Supraociasmatic Nucleus</td>
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<tr>
<td>Dentate Gyrus</td>
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<tr>
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<tr>
<td>CA3</td>
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<tr>
<td>Ventral Tegmental Area</td>
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<tr>
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<tr>
<td>Periaqueductal Grey</td>
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Figure 3.1. DeltaFosB cells in the NAc. Representative images showing deltaFosB-IR cells (black) in the NAc of naïve no sex (A) and experience no sex (B) groups. aco: anterior commissure. Scale bar indicates 100 μm.

Figure 3.2. Number of deltaFosB-IR cells in the mesolimbic system. Number of deltaFosB-IR cells in: A. infralimbic (IL), prelimbic (PL) and anterior cingulate cortex (ACA) subregions of the medial prefrontal cortex; B. Nucleus accumbens core and shell, and caudate putamen (CP); C. Rostral, middle, caudal and tail sections from the ventral tegmental area. Data are expressed as group means (± SEM). * indicates significant difference from sexually naïve animals (p = 0.037 - < 0.001) (n = 5 each group).
3.3.2 Sexual experience attenuates mating-induced c-Fos

The effect of sexual experience on deltaFosB levels in the NAc were confirmed using fluorescence staining techniques. In addition, effects of sexual experience on expression of c-Fos were analyzed. Figure 3.3 demonstrates representative images of deltaFosB-(green) and c-Fos (red)-IR cells in all experimental groups (A, NNS; B, NS; C, ENS; D, ES). Sexual experience significantly increased deltaFosB expression in NAc core (Figure 3.4A: $F_{1,15} = 12.0; p = 0.003$) and shell (Figure 3.4C: $F_{1,15} = 9.3; p = 0.008$). By contrast, mating 1 hour prior to perfusion, did not have an effect on deltaFosB expression (Figure 3.4A,C) and no interaction between sexual experience and mating immediately prior to perfusion was detected. There was an overall effect of mating prior to perfusion on c-Fos expression in both the NAc core (Figure 3.4B: $F_{1,15} = 27.4; p < 0.001$) and shell (Figure 3.4D: $F_{1,15} = 39.4; p < 0.001$). Moreover, an overall effect of sexual experience was detected in the NAc core (Figure 3.4B: $F_{1,15} = 6.1; p = 0.026$) and shell (Figure 3.4D: $F_{1,15} = 1.7; p = 0.211$) and an interaction between sexual experience and mating prior to perfusion was detected in the NAc core ($F_{1,15} = 6.5; p = 0.022$), with a trend in the shell ($F_{1,15} = 1.7; p = 0.211; F_{1,15} = 3.4; p = 0.084$). Post hoc analyses demonstrated mating-induced c-Fos expression in core and shell of sexually naïve males (Figure 3.4B, D). However, in sexually experienced males, c-Fos was not significantly increased in NAc core (Figure 3.4B) and significantly attenuated in the shell (Figure 3.4D). Thus, sexual experience caused a reduction of mating-induced c-Fos expression. P-values for specific pair-wise comparisons are in the figure legends.
Figure 3.3. DeltaFosB and c-Fos cells in the NAc. Representative images showing deltaFosB (green) and c-Fos (red) in NAc for each experimental group. Scale bar indicates 100 µm.
Figure 3.4. Sex experience-induced deltaFosB and mating-induced c-Fos. Numbers of deltaFosB (Core, A; Shell, C; ACA, E) or c-Fos (Core, B; Shell, D; ACA, F) immunoreactive cells for each group: NNS (n = 5), NS (n = 5), ENS (n = 5) or ES (n = 4). Data are expressed as group means (± SEM). * indicates significant difference between sexually naïve or experienced sex groups with the appropriate no sex control group (NS vs NNS, ES vs ENS; all p-values p < 0.001; except H: ES vs ENS, p = 0.008); # indicates significant difference between sexually naïve and experienced sex or no sex groups respectively (ES vs NS; p = 0.049 - < 0.001; ENS vs NNS: p = 0.028 - < 0.001).
The effect of sexual experience on mating-induced c-Fos levels was not restricted to the NAc. A similar attenuation of c-Fos expression was observed in the ACA in sexually experienced animals compared to sexually naïve controls. Sexual experience had a significant effect on deltaFosB expression in the ACA (Figure 3.4E: $F_{1,15} = 154.2; p < 0.001$). Mating prior to perfusion did not have an effect on deltaFosB expression (Figure 3.4E) but significantly increased c-Fos (Figure 3.4F: $F_{1,15} = 203.4; p < 0.001$) in the ACA. Moreover, mating-induced c-Fos expression in the ACA was significantly decreased by sexual experience (Figure 3.4F: $F_{1,15} = 15.8; p = 0.001$). A two-way interaction between sexual experience and mating prior to perfusion was detected for c-Fos expression (Figure 3.4F: $F_{1,15} = 15.1; p < 0.001$). P-values for specific pair-wise comparisons are in the figure legends. Finally, there was no significant reduction in mating-induced c-Fos expression in the medial preoptic nucleus (NS: Avg 63.5 ± 4.0; ES: Avg 41.4 ± 10.09), an area where mating experience did not cause a significant increase in deltaFosB expression, indicating that mating-induced c-Fos expression was not affected in all brain areas.

3.3.3 DeltaFosB in the NAc mediates reinforcement of sexual behavior

To explore a potential molecular mechanism for reinforcement of sexual behavior as demonstrated by experience-induced facilitation of sexual behavior, effects of local manipulation of deltaFosB levels and its transcriptional activity were determined. Sexual experience during the four consecutive experience sessions had a significant effect on mount latency (Figure 3.5A: $F_{1,23} = 13.8; p = 0.001$), intromission latency (Figure 3.5B: $F_{1,23} = 18.1; p < 0.001$), and ejaculation latency (Figure 3.5C: GFP, $F_{11,45} = 3.8; p =$
0.006). GFP control animals displayed the expected experience-induced facilitation of sexual behavior and displayed significantly lower latencies to first mount, first intromission and ejaculation during experience session 4 compared to experience session 1 (Figure 3.5A-C; see figure legend for p-values). This experience-induced facilitation of sexual behavior was also observed in the deltaFosB group for mount and intromission latencies, but there was not a significant difference detected in the ejaculation latency (Figure 3.5A-C). In contrast, deltaJunD animals displayed a stunted facilitation; even though latencies for mounts, intromissions, and ejaculations did decrease with repeated mating sessions, none of these parameters reached statistical significance when compared between experience sessions 1 and 4 (Figure 3.5A-C).

Between group comparisons for each experience session show that deltaJunD had significantly longer latencies to mount, intromit and ejaculate during experience sessions compared to deltaFosB and GFP (Figure 3.5A-C). In addition, both sexual experience and treatment had significant effects on copulation efficiency (Figure 3.5F: sexual experience, F_{1,12} = 22.5; p < 0.001; treatment, F_{1,12} = 3.3; p = 0.049). DeltaFosB males had increased copulation efficiency during experience session 4 compared to experience session 1 (Figure 3.5F). In addition, deltaFosB animals had significantly fewer mounts preceding ejaculation during experience session day 4, compared to experience session 1 (Figure 3.5D: F_{10,43} = 4.1; p = 0.004), and that deltaJunD males had significantly more mounts preceding ejaculation, thus significantly decreased copulation efficiency, than either of the other two groups (Figure 3.5D, F). Thus, GFP and deltaFosB animals
displayed experience-induced facilitation of initiation of sexual behavior and sexual performance, whereas deltaJunD animals did not.

To test the hypothesis that deltaFosB expression is critical for long-term expression of experience-induced facilitation of sexual behavior, animals were tested 1 week (test session 1) and 2 weeks (test session 2) after the final experience session. Indeed, facilitated sexual behavior was maintained in both GFP and deltaFosB groups as none of the behavioral parameters differed between test sessions 1 or 2 and the final experience session 4, within GFP and deltaFosB groups (Figure 3.5A-C; except for ejaculation latency and copulation efficiency in test session 1 for deltaFosB animals). Significant differences between deltaJunD animals and GFP or deltaFosB groups were detected in both test sessions for all sexual behavior parameters (Figure 3.5A-F). There were no differences detected between or within groups when comparing numbers of intromissions, PEI, or percentages of animals that ejaculated (100% of males in all groups ejaculated during last four mating sessions).

3.4 DISCUSSION
The current study demonstrated that sexual experience causes an accumulation of deltaFosB in several limbic-associated brain regions, including the NAc core and shell, mPFC, CPu and VTA. In addition, sexual experience attenuated mating-induced expression of c-Fos in the NAc and ACA. Finally, deltaFosB in the NAc was shown to be critical in mediating facilitation of mating during acquisition of sexual experience and the long-term expression of experience-induced facilitation of sexual
Figure 3.5. DeltaFosB activity in the NAc is required for facilitation of sexual behavior and its maintenance. Sexual behavior of GFP (n = 12), deltaFosB (n = 11), and deltaJunD (n = 9) animals: mount latency (A), intromission latency (B), ejaculation latency (C), number of mounts (D), number of intromissions (E) and copulation efficiency (F). Data are expressed as group means (± SEM). * indicates significant difference from GFP (p = 0.047 - 0.007); + indicates significant difference from deltaFosB (p = 0.036 - 0.005); and # indicates a statistical difference between mating sessions 1 and 4 (GFP: p = 0.025 - < 0.001; deltaFosB: p = 0.036 - < 0.001).
behavior. Specifically, reducing deltaFosB-mediated transcription attenuated experience-induced facilitation of sexual motivation and performance, while over-expression of deltaFosB in the NAc caused an enhanced facilitation of sexual behavior, in terms of increased sexual performance with less experience. Together, the current findings support the hypothesis that deltaFosB is a critical molecular mediator for the long-term neural and behavioral plasticity induced by sexual experience.

The current findings extend previous studies showing sex experience-induced deltaFosB in the NAc in male rats (Wallace, Vialou et al. 2008) and female hamsters (Hedges, Chakravarty et al. 2009). Wallace et al. (2008) showed that rAAV-deltaFosB over-expression in the NAc enhanced sexual behavior in sexually naïve animals during the first mating session, as evidenced by fewer intromissions to ejaculation and shorter post-ejaculatory intervals, but had no effect in sexually experienced males (Wallace, Vialou et al. 2008). In contrast, the current study demonstrated no effects of deltaFosB over-expression in sexually naïve males during the first test, but rather during and following the acquisition of sexual experience. DeltaFosB over-expressors demonstrated increased sexual performance (increased copulation efficiency) compared to GFP animals. In addition, the current study tested the role of deltaFosB by blocking deltaFosB-mediated transcription using a deltaJunD-expressing viral vector. Prevention of experience-induced increase in deltaFosB expression inhibited experience-induced facilitation of sexual motivation (increased mount and intromission latencies) as well as sexual performance (increased ejaculation latency and number of mounts) and subsequent long-term expression of facilitated sexual behavior. Hence, these data are the first to
indicate an obligatory role for deltaFosB in the acquisition of experience-induced facilitation of sexual behavior. Moreover, these data show that deltaFosB is also critically involved in the long-term expression of experience-induced facilitated behavior. We propose that this long-term expression of facilitated behavior represents a form of memory for natural reward; hence deltaFosB in NAc is a mediator of reward memory. Sexual experience also increased deltaFosB levels in the VTA and mPFC, areas implicated in reward and memory (Balfour, Yu et al. 2004; Phillips, Vacca et al. 2008). Future studies are required to elucidate a potential significance of deltaFosB up-regulation in these areas for reward memory.

DeltaFosB expression is highly stable, thus it has great potential as a molecular mediator of persistent adaptations of the brain following chronic perturbations (Nestler, Barrot et al. 2001). DeltaFosB has been shown to gradually increase in the NAc over multiple cocaine injections and persist for up to several weeks (Hope, Kosofsky et al. 1992; Hope, Nye et al. 1994). These changes in NAc deltaFosB expression are associated with drug reward sensitization and addiction (Nestler, Barrot et al. 2001; McClung and Nestler 2003; Chao and Nestler 2004; McClung, Ulery et al. 2004; Nestler 2004; Nestler 2005; Zachariou, Bolanos et al. 2006; Nestler 2008). In contrast, the role of deltaFosB in mediating natural reward has been understudied. Recent evidence has surfaced suggesting that deltaFosB induction in the NAc is involved in natural reward. DeltaFosB levels are similarly increased in the NAc following sucrose intake and wheel running. The over-expression of deltaFosB in the striatum using bi-transgenic mice or viral vectors in rats causes an increase in sucrose intake, enhanced motivation for food and increased
spontaneous wheel running (Werme, Messer et al. 2002; Olausson, Jentsch et al. 2006; Wallace, Vialou et al. 2008). The current data substantially add to these reports and further support the notion that deltaFosB is a critical mediator for reward reinforcement and natural reward memory.

DeltaFosB may mediate experience-induced reinforcement of sexual behavior via induction of plasticity in the mesolimbic system. Indeed, sexual experience causes a number of long-lasting changes to the mesolimbic system (Frohmader, Pitchers et al. 2010; Pitchers, Balfour et al. 2010). At the behavioral level, a sensitized locomotor response to amphetamine and an enhanced amphetamine reward have been shown in sexually experienced male rats (Pitchers, Balfour et al. 2010); an altered locomotor response to amphetamine has also been observed with female hamsters (Bradley and Meisel 2001). Furthermore, increases in number of dendritic spines and complexity of dendritic arbors have been found following an abstinence period from sexual experience in male rats (Pitchers, Balfour et al. 2010). The current study suggests deltaFosB may be a specific molecular mediator of the long-term outcomes of sexual experience. In agreement, deltaFosB has recently been shown to be important for inducing dendritic spine changes in response to chronic cocaine administration (Maze, Covington et al. 2010).

It is not clear which upstream neurotransmitter(s) is responsible for inducing deltaFosB in the NAc, but DA has been proposed as a candidate (Chen, Nye et al. 1995). Virtually all drugs of abuse, including cocaine, amphetamine, opiates, cannabinoids, and
ethanol, as well as natural rewards, increase deltaFosB in the NAc (Werme, Messer et al. 2002; Perrotti, Bolanos et al. 2005; Wallace, Vialou et al. 2008). Both drugs of abuse and natural rewards increase synaptic DA concentration in the NAc (Hernandez and Hoebel 1988; Hernandez and Hoebel 1988; Damsma, Pfaus et al. 1992; Jenkins and Becker 2003). DeltaFosB induction by drugs of abuse has been shown in DA receptor containing cells and cocaine-induced deltaFosB is blocked by a D1 DA receptor antagonist (Chen, Nye et al. 1995). Hence, DA release is hypothesized to stimulate deltaFosB expression and thereby mediate reward-related neuroplasticity. Further supporting the idea that deltaFosB levels are DA-dependent is the finding that brain areas where sexual experience altered deltaFosB levels do receive strong dopaminergic input from the VTA, including the medial prefrontal cortex and basolateral amygdala. However, in contrast, deltaFosB is not increased in the medial preoptic area even though this area receives dopaminergic input, albeit from hypothalamic sources (Miller and Lonstein 2009). Future studies are needed to test if mating-induced deltaFosB expression and the effects of sexual experience on sexual motivation and performance are dependent on DA action. The role for DA in sexual reward in male rats is currently not completely clear (Agmo and Berenfeld 1990; Pfaus 2009). There is ample evidence that DA is released in the NAc during exposure to a female or mating (Damsma, Pfaus et al. 1992) and DA neurons are activated during sexual behavior (Balfour, Yu et al. 2004). However, systemic injections of DA receptor antagonist do not prevent sexual reward-induced conditioned place preference (Agmo and Berenfeld 1990) and the hypothesis that DA is critical for experience-induced reinforcement of mating is untested.
It is also unclear as to what are the downstream mediators of deltaFosB effects on sexual behavior. DeltaFosB has been shown to act as both a transcriptional activator and repressor through an AP-1 dependent mechanism (McClung and Nestler 2003; Peakman, Colby et al. 2003). Numerous target genes have been identified, including the immediate early gene c-fos (Morgan and Curran 1989; Hope, Kosofsky et al. 1992; Hope, Nye et al. 1994; Zhang, Zhang et al. 2006; Renthal, Carle et al. 2008), cdk5 (Bibb, Chen et al. 2001), dynorphin (Zachariou, Bolanos et al. 2006), sirtuin-1 (Renthal, Kumar et al. 2009), NF-κB subunits (Ang, Chen et al. 2001), and the AMPA glutamate receptor GluR2 subunit (Kelz, Chen et al. 1999). The current results demonstrate that mating-induced c-Fos levels were reduced by sexual experience in brain areas with increased deltaFosB (NAc and ACA). The suppression of c-Fos appears dependent on the period since last mating and repeated mating sessions, as in previous studies, such a decrease in c-Fos was not detected in male rats tested 1 week following the final mating session (Balfour, Yu et al. 2004) or after sexual experience consisting of only a single mating session (Lopez and Ettenberg 2002). Moreover, the current finding is consistent with the evidence that deltaFosB represses the c-fos gene after chronic amphetamine exposure (Renthal, Carle et al. 2008). In line with these findings, the induction of several immediate early gene mRNAs (c-fos, FosB, c-jun, junB, and zif268) was reduced following repeated cocaine injections in comparison to acute drug injections (Hope, Kosofsky et al. 1992; Hope, Nye et al. 1994), and amphetamine-induced c-fos was suppressed following withdrawal from chronic amphetamine administration (Jaber, Cador et al. 1995; Renthal, Carle et al. 2008). The functional relevance of the down-regulation of c-Fos expression after chronic drug treatment or sexual experience remains
unclear, and has been suggested to be an important homeostatic mechanism to regulate an animal’s sensitivity to repeated reward exposure (Renthal, Carle et al. 2008).

In conclusion, the current study demonstrates that deltaFosB in the NAc plays an integral role in sexual reward memory, supporting the possibility that deltaFosB is important for general reward reinforcement and memory. The findings from the current study further elucidate our understanding of cellular and molecular mechanisms that mediate sexual reward and motivation, and add to a body of literature showing that deltaFosB is an important player in development of addiction, by demonstrating a role for deltaFosB in natural reward reinforcement.
3.5 REFERENCES


CHAPTER 4:

DeltaFosB and dopamine receptor activity in the nucleus accumbens is critical for increased amphetamine reward and nucleus accumbens spine density following loss of natural reward in sexually experienced male rats
4.1 INTRODUCTION

Natural reward behaviors and drug reward converge on a common neural pathway, the mesolimbic dopamine (DA) system, in which the nucleus accumbens (NAc) plays a central, integrative role (Kelley 2004; Frohmader, Pitchers et al. 2010; Frohmader, Wiskerke et al. 2010; Morales and Pickel 2012). Drugs of abuse induce neuroplasticity in this mesolimbic system, which plays a putative role in the transition from drug use to drug addiction (Di Chiara and Imperato 1988; Imperato, Mele et al. 1992; Robinson and Kolb 1997; Robinson and Kolb 1999; Robinson and Kolb 2004). These alterations induced by repeated drug administration involve sensitized drug behaviors, including a sensitized amphetamine (Amph)-induced locomotor response (Segal and Mandell 1974; Post and Rose 1976; Kalivas and Stewart 1991) and enhanced drug reward (Lett 1989; Shippenberg and Heidbreder 1995; Shippenberg, LeFevour et al. 1998; Crombag, Bossert et al. 2008), increased dendritic spine density (Robinson and Kolb 2004; Sarti, Borgland et al. 2007), gene expression changes (McClung and Nestler 2003; McClung, Ulery et al. 2004), altered receptor trafficking (for review (Wolf and Ferrario 2010)), altered synaptic strength (Saal, Dong et al. 2003; Kauer and Malenka 2007; Nugent, Penick et al. 2007; Nugent, Hwong et al. 2008), and decreased intrinsic neuronal excitability (Kourrich and Thomas 2009). It has become increasingly clear that natural and drug rewards affect the mesolimbic system in both similar and different ways that allow for an interplay between natural reward, specifically sex reward and drugs of abuse (Fiorino and Phillips 1999; Bradley and Meisel 2001; Frohmader, Pitchers et al. 2010; Liu, Aragona et al. 2010; Pitchers, Balfour et al. 2010; Liu, Young et al. 2011).
Sexual behavior is a highly rewarding and reinforcing behavior (Pfaus, Kippin et al. 2001; Bradley, Haas et al. 2005; Tenk, Wilson et al. 2009). Repeated sexual behavior (sexual experience) leads to long-term facilitation and reinforcement of sexual behavior, indicated by decreased latencies to initiate behavior, increased sexual performance (Pitchers, Frohmader et al. 2010; Pitchers, Schmid et al. 2012), and altered expression of mating-induced conditioned place preference (CPP; (Tenk, Wilson et al. 2009)). In addition, sexual experience causes sensitized drug-related behavior, including cross-sensitization to Amph-induced locomotor activity (Bradley and Meisel 2001; Pitchers, Balfour et al. 2010) and enhanced Amph reward (Pitchers, Balfour et al. 2010). These findings suggest that sexual experience causes plasticity in the mesolimbic system to mediate these sensitized behavioral responses to rewards (Frohmader, Pitchers et al. 2010). Indeed, repeated experience with sexual reward causes neural plasticity in the NAc similar to that induced by psychostimulant exposure, including increased number of dendritic spines (Meisel and Mullins 2006; Pitchers, Balfour et al. 2010), altered ionotropic glutamate receptor trafficking, and decreased synaptic strength in PFC-responding NAc shell neurons (Pitchers, Schmid et al. 2012). Moreover, periods of abstinence from sexual reward were found to be critical for enhanced Amph reward and increased number of spines in the NAc which were observed 1 week, but not immediately after sexual experience (Pitchers, Balfour et al. 2010) as well as trafficking of AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionate) receptors which was only evident after prolonged abstinence periods (Pitchers, Schmid et al. 2012).
The goal of the current study was to determine the molecular mechanisms mediating plasticity in the mesolimbic system and enhanced drug reward caused by a reward abstinence period following sexual experience. Specifically, the role of the transcription factor deltaFosB was investigated because it has been reported to be critical for the effects of both natural and drug reward (Nestler, Barrot et al. 2001; Werme, Messer et al. 2002; Olausson, Jentsch et al. 2006; Nestler 2008; Wallace, Vialou et al. 2008; Hedges, Chakravarty et al. 2009; Pitchers, Frohmader et al. 2010). DeltaFosB, the truncated form of FosB, is a unique member of the Fos family of transcription factors due to its greater stability (Ulery, Rudenko et al. 2006; Carle, Ohnishi et al. 2007; Ulery-Reynolds, Castillo et al. 2008). It is expressed at relatively low levels in response to an acute stimulus, but its stability allows it to accumulate in cells with repeated stimuli, where it persists after the cessation of the stimulus; thus, suggestive of a role in long-term neuroplasticity. DeltaFosB is up-regulated by most drugs of abuse (Perrotti, Weaver et al. 2008), and is critical for sensitized drug behavior (Kelz, Chen et al. 1999; Colby, Whisler et al. 2003; Zachariou, Bolanos et al. 2006) and spine density increases in the NAc (Maze, Covington et al. 2010). Finally, repeated psychostimulant administration caused persistent increases in deltaFosB, dependent on functional D1 dopamine receptors (D1R; (Zhang, Zhang et al. 2002)), and increased spine density in D1R-containing medium spiny neurons in the NAc (Lee, Kim et al. 2006; Kim, Teylan et al. 2009).

DeltaFosB is also up-regulated by natural rewarding behaviors and its overexpression increased voluntary wheel running (Werme, Messer et al. 2002), instrumental responding for food (Olausson, Jentsch et al. 2006), sucrose intake (Wallace, Vialou et al. 2008), and sexual behavior in male (Wallace, Vialou et al. 2008; Pitchers, Frohmader et al. 2010).
and female rodents (Hedges, Chakravarty et al. 2009). Moreover, deltaFosB in NAc is essential for the experience-induced alterations of natural reward behavior, as functional blockade of deltaFosB activity in NAc attenuated facilitation and reinforcement of sexual behavior in sexually experienced male rats (Pitchers, Frohmader et al. 2010). However, the question remains if deltaFosB is critical for cross-sensitization between natural and drug rewards.

Together, these findings lead to the hypothesis that the cross-sensitizing effects of sexual experience followed by a period of reward abstinence on enhanced Amph reward are mediated by a D1R-dependent increase deltaFosB activity in the NAc via increased NAc spine density. The current study determined the temporal profiles of sex experienced-induced changes in deltaFosB expression, dendritic spines in the NAc, and Amph-CPP following prolonged periods of abstinence from sexual reward (7 or 28 days). Next, using viral vector-mediated gene transfer a dominant-negative binding partner for deltaFosB was used to test the functional relevance of deltaFosB activity for sex experience-induced enhanced Amph reward and increased NAc dendritic spines. Finally, it is shown that sex-induced deltaFosB and enhanced drug reward were dependent on mating-induced D1R activity in the NAc.
4.2 MATERIALS AND METHODS

4.2.1 Animals

Adult male (225-250 grams upon arrival) and female (210-220 grams) Sprague Dawley rats were obtained from Charles River Laboratories (Senneville, QC, Canada or Wilmington, MA, USA) and were housed in Plexiglas cages in same sex pairs throughout experiments. The colony room was temperature- and humidity-regulated and maintained on a 12/12 hr light dark cycle with food and water available \textit{ad libitum} except during behavioral testing. Female partners for mating sessions received a subcutaneous implant containing 5% estradiol benzoate (Sigma-Aldrich, St. Louis, MO, USA) and 95% cholesterol following bilateral ovariectomy under deep anesthesia (isofluorine; 2% MAC; MWI, Boise, ID, USA) administered using a Surgivet Isotec4 gas apparatus (Smiths Medical Vet Division, Markham, Ontario, Canada). Sexual receptivity was induced by administration of 500 µg progesterone (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 mL sesame oil (Sigma-Aldrich, St. Louis, Missouri) approximately 4 hours before testing. All procedures were approved by the Animal Care and Use Committees of the University of Western Ontario and the University of Michigan and conformed to Canadian Council on Animal Care and National Institutes of Health guidelines involving vertebrate animals in research.

4.2.2 Drugs

D-Amph sulfate (Sigma-Aldrich, St. Louis, MO, USA), D1R antagonist R(+)-SCH-23390 hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), and D2 receptor (D2R) antagonist S-(-)-eticlopride hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in
sterile saline (0.9%). Animals received an Amph dose of 0.5 mg/kg body weight, calculated on the basis of the free base, in a volume of 1 mL/kg body weight. All Amph injections were given subcutaneously (s.c.) during the first one-half of the light phase (2-6 hours after lights on), immediately before placement into the behavioral apparatus. Animals received a DA receptor antagonist (either D1R or D2R) at a dose of 10 µg/µL in 1.0 µL per side into the NAc at 15 minutes prior to introduction of receptive female. Control injections were saline.

4.2.3 Sexual Behavior

Mating sessions occurred during the early dark phase (between 2-6 hours after onset of the dark period) under dim red illumination. During mating sessions male rats were allowed to copulate to ejaculation or 1 hour (whichever occurred first) in clean test cages (60 x 45 x 50 cm). Sexual behavior parameters (i.e., latency to first mount, intromission and ejaculation, and number of mounts and intromissions) were recorded (Agmo 1997). For all experiments, sexually experienced groups were matched for sex behavior (total number of ejaculations and latency to ejaculation during each mating session). Following the fifth mating session, males remained housed with same sex partners, but were not allowed to mate during sex abstinence periods of 1, 7, or 28 days. Animals that remained sexually naïve were handled and housed in the same rooms as sexually experienced males. In addition, naïve controls were placed in clean test cages for an hour each mating day, but were not given access to a receptive female. Hence, naïve controls were exposed to similar levels of disturbance, environment novelty, distant female noises and odors as experienced males, but without direct contact with females.
4.2.4 Temporal studies: DeltaFosB expression, spine density, and Amph reward

The goal of the initial set of experiments was to determine the temporal profiles of the effects of sexual experience on deltaFosB up-regulation, increased spine density on NAc neurons, and enhanced Amph reward. Previously, we demonstrated that sexual experience increased deltaFosB 1 day after last mating, and increased NAc spines and enhanced Amph CPP 10 days (but not 1) after last mating (Pitchers, Balfour et al. 2010). Here, it was tested if these effects persist following longer periods of sex abstinence (28 days).

4.2.5 Temporal expression of deltaFosB up-regulation following sexual experience

4.2.5A Perfusion, tissue processing, and immunohistochemistry

Sexually experienced males were perfused at 3 different sex abstinence periods after final mating session: 1 day (1D), 7 days (7D), or 28 days (28D; n = 4 each). Sexually naïve males were perfused at the same time points after last handling session (n = 4 each). Animals were deeply anaesthetized using sodium pentobarbital (390 mg/kg; i.p.) and perfused intracardially with 50 mL of 0.9% saline, followed by 500 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were removed and post-fixed for 1 hr at room temperature (RT) in the same fixative, then immersed in 20% sucrose and 0.01% sodium azide (NaAz) in 0.1 M PB and stored at 4°C. Coronal sections (35 μm) were cut with a freezing microtome (H400R, Microm, Walldorf, Germany), collected in four parallel series in cryoprotectant solution (30% sucrose and 30% ethylene glycol in 0.1M PB) and stored at -20°C. Free floating sections were washed extensively with 0.1M phosphate-buffered saline (PBS; pH 7.35) between incubations. Sections were
exposed to 1% H$_2$O$_2$ for 10 min at RT to destroy endogenous peroxidases, then blocked in PBS+ incubation solution, which is PBS containing 0.1% bovine serum albumin (catalog item 005-000-121; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 0.4% Triton X-100 (catalog item BP151-500; Sigma-Aldrich) for 1 hr. Sections were then incubated overnight at RT in pan-FosB rabbit polyclonal antibody (1:5K; sc-48 Santa Cruz Biotechnology, Santa Cruz, CA, USA). The pan-FosB antibody was raised against an internal region shared by FosB and deltaFosB, and has been previously characterized to specifically visualize deltaFosB cells at the time points used in this study (>1 day after stimulus) (Perrotti, Hadeishi et al. 2004; Perrotti, Weaver et al. 2008; Pitchers, Frohmader et al. 2010). After primary antibody incubation, sections were incubated for 1 hr in biotin-conjugated goat anti-rabbit IgG (1:500 in PBS+; Vector Laboratories, Burlingame, CA, USA) and then 1 hr in avidin-biotin-horseradish peroxidase (ABC elite; 1:1K in PBS; Vector Laboratories, Burlingame, CA, USA). Following ABC incubation, the peroxidase complex was visualized following treatment for 10 minutes to a chromogen solution containing 0.02% 3,3’-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO, USA) enhanced with 0.02% nickel sulfate in 0.1M PB with hydrogen peroxide (0.015%). Sections were washed thoroughly in 0.1M PB to terminate the reaction and mounted onto coded Superfrost plus glass slides (Fisher, Pittsburgh, PA, USA) with 0.3% gelatin in ddH$_2$O. Following dehydration, all slides were cover-slipped with DPX (dibutyl phthalate xylene).
4.2.5B Data analysis

Number of deltaFosB-IR cells was determined in the NAc shell and core within standard areas of analysis (400 x 600 µm) using a camera lucida drawing tube attached to a Leica DMRD microscope (Leica Microsystems GmbH, Wetzlar, Germany) (Pitchers, Frohmader et al. 2010). Two sections were counted per NAc subregion and averaged per animal for calculation of group mean. All counts for each animal were expressed as a fold change of the naïve control group at the appropriate time point. Means were compared between sexually experienced and naïve groups for each subregion at each individual time point using unpaired t-tests with a significance level of p-value < 0.05.

4.2.6 Temporal expression of NAc spines changes following sexual experience

4.2.6A Perfusion, tissue processing, and immunohistochemistry

Sexually naïve and experienced animals were euthanized either 7D or 28D after final mating session producing 4 groups: naïve 7D (n = 3), experienced 7D (n = 3), naïve 28D (n = 4) and experienced 28D (n = 4). Rats were deeply anaesthetized using sodium pentobarbital (390mg/mL; i.p.) and perfused intracardially with 50mL saline (0.9%), followed by 500mL of 2% paraformaldehyde in 0.1M PB. Brains were stored in 0.1M PB with 0.01% NaAz at 4°C. Brains were coronally sectioned (100 µm thick) using a vibratome (Microm, Walldorf, Germany) at RT in 0.1M PB. Sections were then stored in 0.1M PB with 0.01% NaAz at 4°C until labeled with DiI.
4.2.6B Preparation of DiI coated “bullets”

Coating of tungsten particles (1.3 µm diameter, Bio-Rad, Hercules, CA, USA) with the lipophilic carbocyanine dye DiI (1,1’-dioctadecyl-3,3,3’3’-tetramethylindocarbocyanine perchlorate; Invitrogen, Grand Island, NY, USA) was adapted from (Forlano and Woolley 2010). Briefly, 7.5 mg carbocyanine fluorescent DiI was dissolved in 220 µL methylene chloride (Fisher Scientific, Fair Lawn, NJ, USA). DiI was applied to 90 µg of 1.3 µm tungsten particles and distributed evenly on a glass slide. Tungsten particles were allowed to dry (5 minutes), and collected in 3 mL deionized H₂O and sonicated for 15 minutes. Polyvinylpyrrolidine (0.75 µL, PVP, Sigma-Aldrich, St. Louis, MO, USA) was added to DiI solution. The DiI/PVP suspension was drawn into the Tefzel tubing (Bio-Rad) under slow rotation (5 minutes). Next, excess solution was removed and tubing was dried for 15 minutes under 0.4 LPM nitrogen gas flow. Tubing was cut into approximately 1 cm segments (bullets) and stored at RT in a light-proof container with desiccant.

4.2.6C Diolistic labeling

DiI-coated tungsten particles were delivered into the tissue at 160-180 psi using the Helios Gene Gun system (Bio-Rad) through a filter with 3.0 µm pore size (BD Biosciences, Sparks, MD, USA). A 40 mm spacer was attached to the Gene Gun to ensure a consistent placement and distance between the Gene Gun and brain section. DiI was allowed to diffuse through neuronal membranes in 0.1M PB for 24 hr while light-protected at 4°C. Next, slices were post-fixed in 4% paraformaldehyde in PB for 3 hr at RT. After a brief washing in PB, sections were mounted in frame-sealed chambers (Bio-
Rad) with gelvatol containing the anti-fading agent 1,4-diazabicyclo(2,2)octane (DABCO; 50 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) (Lennette 1978).

4.2.6D Confocal imaging

A Zeiss LSM 510M confocal microscope (Carl Zeiss Microscopy, LLC, NY, USA) was used to image the DiI labeled neurons. DiI was excited using the Helium/Neon 543 nm laser. Cells localized in the NAc shell and core were used for spine quantification on second order dendrites. For each animal, 2-5 neurons in each NAc subregion were used to locate a region of interest on a dendrite for spine quantification. The DiI-positive neuron was captured using a 40x water-immersion objective with 0.7 zoom (Zeiss; NA = 1.2, WD = 0.28mm) with a xy pixel distribution of 1024 x 1024 and at 2 μm intervals along the z-axis. For each neuron, 2-4 dendrites were analyzed in order to quantify a total dendritic length of 40 - 100 μm. Dendritic segments were captured using 40x water-immersion objective at 0.25 μm intervals along the z-axis, resulting in complete three-dimensional image stacks of the dendritic segment (reconstructed using Zeiss LSM image software).

4.2.6E Spine analysis

The three dimensional image underwent deconvolution using Autoquant X software (Media Cybernetics, Bethesda, MD, USA) using adaptive (blind) and theoretical PSF setting as recommended by the software. Following deconvolution, spine density was quantified using the Filament module of Imaris software package (version 7.0, Bitplane, South Windsor, CT, USA). Numbers of dendritic spines were averaged within each
dendrite and expressed per 10 μm. Averages for each neuron and then for each animal were calculated to determine group means. Statistical differences were determined between sexually naïve and experienced animals using unpaired t-tests with significance set at p-value < 0.05.

4.2.7 Temporal expression of sensitized Amph reward following sexual experience

4.2.7A Experimental design

Following sexual experience, animals were habituated to handling and received saline injections on each of the 3 days prior to conditioning. Amph-CPP was investigated in sexually experienced and naïve animals 7D or 28D after final mating session or handling producing 5 experimental groups that received either 0.5 mg/kg Amph or saline (Sal) in the paired chamber: Naïve Sal (n = 8; 7D after handling), Naïve Amph (n = 9; 7D after handling), Exp Sal (n = 7; n = 3 tested 7D after mating; n = 4 tested 28D after mating), 7D Exp Amph (n = 9; 7D after mating), and 28D Exp Amph (n = 11; 28D after mating). The Naïve Sal and Exp Sal groups receive Sal paired with both chambers, thus served as negative controls. The Naïve-Amph and 7D Exp Amph group served as positive controls confirming our previous finding of a sensitized Amph reward in sexually experienced, but not naïve males (Pitchers, Balfour et al. 2010).

4.2.7B CPP apparatus and methods

The CPP experimental design was identical to that described in (Pitchers, Balfour et al. 2010). Briefly, CPP was performed in an unbiased three-compartment apparatus (Med Associates, St. Albans, Vermont, USA), which consisted of two larger outer chambers
(28 x 22 x 21 cm) distinguished by visual and tactile cues, separated by a small central chamber (13 x 12 x 21 cm). The apparatus was equipped with photo-beams for automated analysis of tracking animal location. CPP conditioning and testing was conducted during the first half of the light period. A pre-test was conducted to determine each animal’s initial chamber preference. On the following day, each rat was either confined to the paired chamber (Amph, 0.5 mg/kg or saline) or unpaired chamber (saline) for 30 min immediately following appropriate injection. Rats received the opposite treatment the following day in a counterbalanced manner. A post-test, procedurally identical to the pre-test, was conducted on the final day to determine final chamber preference.

4.2.7C Data analysis

CPP scores were calculated for each animal as the time spent (in seconds) in the paired chamber during the post-test minus the pre-test and group means were calculated. Males in the Exp Sal group tested either 7D or 28D after last mating did not differ in CPP scores and were combined into one Exp Sal group (n = 7). Statistical analysis was conducted to test for effects of Amph within naïve or experience groups. An unpaired t-test was used to compare Naïve Sal and Naïve Amph. A one-way ANOVA and Holm-Sidak method with significance set at a p-value < 0.05 was used to compare sexually experienced groups (7D Exp Amph, 28D Exp Amph, and Naïve Amph).
4.2.8 Functional significance of deltaFosB activity in increased Amph reward and NAc dendritic spines

4.2.8A Viral vector surgery

Sexually naïve male rats were randomly divided into groups prior to stereotaxic surgery. Male rats were anaesthetized with an intraperitoneal injection (0.1 ml/kg) of ketamine (87 mg/ml) and xylazine (13 mg/ml), and placed into a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). Animals received bilateral microinjections of recombinant adeno-associated viral (rAAV) vectors encoding GFP (green fluorescent protein; n = 19) or deltaJunD (dominant negative binding partner of deltaFosB; n = 18) into the NAc (coordinates: AP +1.5, ML ±1.2 from Bregma; DV -7.6 from skull surface according to Paxinos & Watson 1998). RAAV vectors were injected in a volume of 1.5 µL/side over 7 minutes using a Hamilton syringe (5 µL; Harvard Apparatus, Holliston, MA, USA). Behavioral testing commenced 3-4 Weeks following vector injections allowing for optimal and stable viral infection. Animals received sexual experience or remained naïve to create 4 groups: sexually naïve GFP, sexually experienced GFP, sexually naïve deltaJunD, and sexually experienced deltaJunD.

4.2.8B CPP analysis

All animals were tested for CPP using Amph (0.5 mg/kg) at 7D after final mating or handling session. In this experiment, all animals received Amph in the paired chamber and saline in the unpaired chamber. CPP scores were calculated for each animal as the time spent (seconds) in the Amph-paired chamber during the post-test minus the pre-test. Group means were calculated and comparisons were made within vector treatment (GFP
or deltaJunD) between sexually experienced and naïve groups using unpaired t-tests with significance set at a p-value < 0.05.

4.2.8C NAc Dendritic spine density analysis

Within 24-30 hours following the completion of the CPP post-test, all animals were euthanized by pentobarbital overdose and perfused intracardially (as described above). Tissue processing, diolistic labeling, confocal imaging, and spine analysis were conducted as described above. Analysis of dendritic spines was restricted to the shell because viral vector injections were directed at this area. Statistical differences between groups were determined using a two-way ANOVA (factors: sexual experience and viral vector) with post hoc comparisons made using the Holm-Sidak method with significance set at a p-value < 0.05.

4.2.8D Injection verification

NAc brain sections were taken from each animal at the conclusion of the experiment to histologically verify injection sites using GFP as a marker of virus infection. DeltaJunD expressing vectors also contain a segment expressing GFP separated by an internal ribosomal entry site, allowing for injection site verification by GFP visualization in all animals. NAc sections were immunoprocessed for GFP (1:20K; rabbit anti-GFP antibody; Molecular Probes, Eugene, OR, USA). Only animals with spread of virus restricted to the NAc were included in statistical analyses (final n-values: sexually naïve GFP, n = 9; sexually experienced GFP, n = 10; sexually naïve deltaJunD, n = 9; sexually
experience deltaJunD, n = 9). Spread of virus was generally limited to the shell portion of the NAc with limited spread to the core (Figure 4.3A).

**4.2.9 Role of DA receptor activation during mating in sex experience-induced sensitized Amph reward and deltaFosB up-regulation**

**4.2.9A Experimental design**

To determine whether the D1R or D2R activation in the NAc during mating is required for sex experience-induced deltaFosB up-regulation and sensitized Amph-CPP, animals underwent local infusion of either D1 receptor or D2 receptor antagonist (or saline) into the NAc immediately prior to each of 4 daily consecutive mating sessions.

**4.2.9B Cannulation surgery**

Male rats were anaesthetized with an intraperitoneal injection (0.1 mL/kg) of ketamine (87 mg/mL) and xylazine (13 mg/mL), and placed into a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). Bilateral 21-gauge guide cannulas (Plastics One, Roanoke, VA, USA) were lowered through small drill holes in the skull into the brain towards the NAc at AP +1.7, ML ±1.2 from bregma; −6.4 DV from skull surface according to Paxinos & Watson 1998. Cannulas were secured with dental acrylic that adhered to three screws set into the skull. Animals were given a two week recovery period, and were handled daily for habituation to infusion procedures.
4.2.9C DA receptor antagonism and sexual behavior

Males were allowed to mate during 4 consecutive days as described above. Fifteen minutes before introducing the receptive female, the male rats received bilateral microinjections of SCH-23390 (D1R antagonist; 10 µg/µL per hemisphere; 1.0 µl volume; dissolved in 0.9% saline), eticlopride (D2R antagonist; 10 µg/µL per hemisphere; 1.0 µl volume; dissolved in 0.9% saline), or saline (1.0 µl per hemisphere). Bilateral infusions were administered at a flow rate of 1.0 µl/min over a 1 min interval followed by an additional 1 min with the injection cannula left in place for drug diffusion. The injection cannula was then replaced with the dummy cannula and dust cap, and the male was placed in the mating cage for sexual behavior testing.

4.2.9D Amph-CPP

All animals were tested for Amph-CPP using a dose 0.5 mg/kg (as described above) at 7D after final mating (Exp) or handling session (Naïve). Four experimental groups were tested: Naïve Saline (n = 7), Exp Saline (n = 9), Exp D1R Ant (n = 9), Exp D2R Ant (n = 8). Animals did not receive any intra-NAc infusions during the CPP paradigm. CPP scores were calculated for each animal as the time spent (in seconds) in the Amph-paired chamber during the post-test minus the pre-test. Group means were calculated and between group comparisons were made between all groups using a one-way ANOVA and Holm-Sidak method with significance set at a p-value < 0.05.
4.2.9E DeltaFosB and cannula verification

Within 24-30 hours following the completion of the CPP post-test, all animals were euthanized by pentobarbital overdose and perfused intracardially. NAc sections were immunoprocessed and analyzed for deltaFosB-IR (as described above). Cannula placements were verified to be targeted at the NAc shell and core. In a small minority of animals, tissue damage caused by the cannula placements prevented accurate and reliable deltaFosB cell counts causing exclusion from analysis (Figure 4.4 A-C, final group sizes: Naïve Saline, n = 6; Exp Saline, n = 7; Exp D1R Ant, n = 9; Exp D2R Ant, n = 8).

4.3 RESULTS

4.3.1 Sex experienced-induced deltaFosB up-regulation is long-lasting

Previously, it was demonstrated that sexual experience caused an accumulation of deltaFosB throughout the mesolimbic pathway, notably in the NAc (Wallace, Vialou et al. 2008; Pitchers, Frohmader et al. 2010). In this past study and the majority of drug-related studies, deltaFosB levels were measured within 1 day of cessation of stimulus (i.e., (Perrotti, Weaver et al. 2008)). Here, we determine whether deltaFosB accumulation in the NAc induced by sexual experience persisted after prolonged periods of reward abstinence, and was evident 1 day, 7 days, or 28 days after final mating session. Indeed, the number of deltaFosB-IR cells in the NAc shell and core were significantly higher than sexually naïve controls at all time points (Figure 4.1A: shell; 1D, p = 0.022; 7D, p = 0.015; Figure 4.1B: core; 1D, p = 0.024; 7D, p < 0.001; 28D, p < 0.001) except in the NAc shell following 28 days abstinence (p = 0.280).
Figure 4.1. Sexual experience caused an immediate and persistent increase in number of deltaFosB-IR cells. Fold change of number of deltaFosB-IR cells in the NAc shell (A) and core (B) in sexually experienced (black) animals compared to sexually naïve (white) controls. Data are expressed as group means ± SEM. * indicates significant difference compared to naïve controls (p < 0.05). Representative of images of Naïve 1D (C), Exp 1D (D), Exp 7D (E), and Exp 28D (F). Abbreviation: ac = anterior commissure. Scale bar is 100 µm.
4.3.2 Sex experienced-induced increase in dendritic spines is no longer detected at 28 days

Pitchers et al (2010) previously reported using Golgi impregnation techniques that sexual experience followed by 7 days, but not 1 day of reward abstinence period caused significantly increased dendritic branching and number of dendritic spines in the NAc shell and core. The current findings using a diolistics labeling method confirmed that sexual experience followed by a 7 day sex abstinence period increased numbers of dendritic spines in the NAc shell (Figure 4.2A: p = 0.035) and core (Figure 4.2A: p = 0.048). However, this increased spine density was transient and was no longer detected after a prolonged sex abstinence period of 28 days in either NAc subregion (Figure 4.2B).

4.3.3 Sex experienced-induced sensitized Amph reward is long-lasting

It was previously demonstrated that sexual experience followed by a period of abstinence resulted in enhanced Amph reward (Pitchers, Balfour et al. 2010). Specifically, sexually experienced animals formed a significant CPP for lower doses of Amph (0.5 or 1.0 mg/kg) that did not induce CPP in sexually naïve controls. The current findings confirmed and extended these past results by demonstrating enhanced Amph reward in sexually experienced animals both following a 7 day as well as a 28 day sex abstinence period (Figure 4.2D). Specifically, sexually experienced animals with either 7 or 28 day abstinence period spent significantly greater time in the Amph-paired chamber during the post-test compared to sexually experienced negative controls that received saline in the both chambers (Figure 4.2D: Exp Sal vs 7D Exp AMPH, p = 0.032; vs 28D Exp AMPH, p = 0.021). Confirming previous findings, sexually naïve animals (7D and 28D
Figure 4.2. Sexual experience caused an increase in number of dendritic spines in the NAc and sensitized Amph reward. A, B. The number of dendritic spines in the NAc shell and core of 7D (C) or 28D (D) of sexually naïve (white) and experienced (black) animals. Data are expressed as group means ± SEM. # indicates significant difference compared to naïve controls (p < 0.05). C, Representative dendritic segments from Naïve 7D and Exp 7D groups used to quantify spine density. Scale bar is 3 µm. D, The amount of time spent in the paired chamber (Amph or saline) during the post-test minus the pre-test (CPP score) for sexually naïve (white) or experienced (black) animals tested either 7 days (7D) or 28 days (28D) after final mating after session. * indicates significant difference compared to sexually experienced saline controls (p < 0.05).
combined) did not spend more time in the Amph-paired chamber during the post-test and did not differ in preference from the sexually naive saline control group (Pitchers, Balfour et al. 2010).

4.3.4 DeltaFosB activity is critical for sex experience-induced sensitized AMPH reward

The results so far demonstrate that sexual experience caused long-lasting accumulation of deltaFosB in NAc neurons correlating with enhanced Amph reward. To determine whether increased deltaFosB activity is critical for enhanced Amph reward, deltaJunD, the dominant negative binding partner of deltaFosB, was up-regulated via viral vector-mediated gene transfer in the NAc. In neurons, deltaJunD binds to deltaFosB and consequently, prevents binding of the activator protein 1 (AP-1; typically heterodimer of deltaFosB and JunD) to AP-1 binding site promoter regions, hence rendering deltaFosB inactive as a transcriptional regulator (Hiroi, Marek et al. 1998). Results showed that attenuation of deltaFosB activity prevented the effects of sexual experience and 7 days sex reward abstinence on enhanced Amph reward. Whereas, sexually experienced GFP control animals formed a CPP for Amph (0.5 mg/kg) as indicated by a significantly greater CPP score compared to sexually naïve GFP controls (Figure 4.3B, p = 0.018). Sexually experienced deltaJunD animals did not form a significant CPP for Amph, and were not different from sexually naïve deltaJunD animals. Moreover, sexually experienced deltaJunD males spent less time in the Amph-paired chamber compared to sexually experienced GFP males, although this only reached a trend for statistical significance (Figure 4.3B; p = 0.079).
4.3.5 DeltaFosB is critical for sex experience-induced increased dendritic spines

Data showed deltaFosB activity is required for the increased spine density following sex experience and 7 days sex reward abstinence. A two-way ANOVA showed significant effects of both sexual experience ($F_{1,34} = 31.768, p < 0.001$) and viral vector ($F_{1,34} = 14.969, p = 0.001$), as well as an interaction between these two factors ($F_{1,34} = 10.651, p = 0.005$). Specifically, sexually experienced GFP control animals had a greater number of spines compared to sexually naïve GFP controls (Figure 4.3D: $p < 0.001$) confirming our previous finding (Pitchers, Balfour et al. 2010). In contrast, sexually experienced deltaJunD animals were not significantly different from sexually naïve deltaJunD groups, and were significantly lower compared to sexually experienced GFP animals (Figure 4.3D: $p < 0.001$). Thus, deltaJunD in the NAc blocked the effects of sexual experience and reward abstinence on NAc dendritic spines.

4.3.6 D1R antagonist blocks sex experience-induced deltaFosB up-regulation

Next, it was determined whether D1R or D2R activity in the NAc during mating was required for sex experience-induced deltaFosB expression by infusing either D1R or D2R antagonist into the NAc prior to each mating session. Neither D1R nor D2R infusions into the NAc affected initiation or expression of sexual behavior during any mating session. In particular, antagonists did not affect sexual behavior on day 1 (first) or day 4 (last) compared to saline controls (Figure 4.4D-F). Moreover, all groups demonstrated facilitated behavior with experience as evinced by shorter ejaculation latencies on day 4 compared to day 1 (Figure 4.4F: Saline, $p = 0.018$; D1R Ant, $p < 0.001$; D2R Ant, $p = 0.006$).
**Figure 4.3. Attenuating deltaFosB activity in the NAc blocked sensitized AMPH reward and increase in number of NAc spines in sexually experienced animals.** A, Representative image of GFP expression in an animal receiving an injection of rAAV-deltaJunD directed at the nucleus accumbens. Abbreviations: ac = anterior commissure, LV = lateral ventricle. Scale bar is 250 µm. B, The amount of time spent in the Amph-paired chamber during the post-test minus the pre-test (CPP score, sec) for sexually naïve (white) and experienced (black) animals that either received an injection of GFP control vector or deltaJunD vector. C, Representative images of dendritic segments from sexually experienced GFP and deltaJunD used to quantify spine density. Scale bar is 3 µm. D, The number of dendritic spines in the NAc of sexually naïve (white) and experienced (black) animals that either received an injection of GFP control vector or deltaJunD vector. Data are expressed as group means ± SEM. * indicates significant difference compared to naïve controls (p < 0.05). # indicates significant difference from GFP experienced controls (p < 0.05).
Figure 4.4. Dopamine receptor antagonists infused into the NAc did not affect sexual behavior. Coronal NAc sections (A, +2.2; B, +1.7; C, +1.2 from bregma) indicating intra-NAc injection sites for all animals. Cannulas were bilateral, but are represented unilaterally for ease of presentation of all animals (Naïve Sal, white; Exp Saline; dark grey; Exp D1R Ant, light grey; Exp D2R Ant, black). Abbreviations: ac = anterior commissure, LV = lateral ventricle, CPu = caudate putamen. Mount latency (D), intromission latency (E) and ejaculation latency (F) for all sexually experienced groups (Saline, white; D1R Ant, grey; D2R Ant, black). Data represent mean ± SEM. * indicates significant difference between day 1 and day 4 within treatment.
Analysis of number of deltaFosB-IR cells in the NAc 7 days after last mating session confirmed the results above in saline controls as sexual experience caused a significant up-regulation of deltaFosB compared to sexually naïve controls (Figure 4.5A: shell, p < 0.001; 4.5B: core, p < 0.001). However, D1R, but not D2R antagonist, abated this up-regulation of deltaFosB. In the NAc shell, D1R antagonist treated sexually experienced males did not have a significant increase in deltaFosB-IR cells compared to sexually naïve controls (Figure 4.5A: p = 0.110) and deltaFosB expression was significantly lower compared to sexually experienced saline males (Figure 4.5A: p = 0.002). In the NAc core, although deltaFosB was significantly increased in D1R antagonist treated males compared to naïve saline controls (Figure 4.5B: p = 0.031), this up-regulation was significantly lower compared to sexually experienced saline treated males (Figure 4.5B: p = 0.012). D2R antagonist treatment did not affect deltaFosB induction as sexually experienced males that received D2R antagonist had a significantly greater number of deltaFosB-IR cells compared to naïve saline controls (Figure 4.5A: shell, p < 0.001; Figure 4.5B: core, p < 0.001) and D1R antagonist-treated males (Figure 4.5A: shell, p < 0.001; Figure 4.5B: core, p = 0.013), and did not differ from sexually experienced saline males.

4.3.7 D1R antagonist treatment blocks sensitized sex experience-induced enhanced Amph reward

D1R blockade during mating also blocked sex experience-induced enhanced Amph reward after 7 days sex abstinence. Similar results described above, sexually experienced animals that received saline in the NAc during mating sessions spent a significantly greater amount of time in
Figure 4.5. Blocking D1R in the NAc attenuates the increase in number of deltaFosB-IR cells in the NAc of sexually experienced animals. Fold change of number of deltaFosB-IR cells in the NAc shell (A) and core (B) in sexually experienced (black) animals compared to sexually naïve (white) controls. Data are expressed as group means ± SEM. * indicates significant difference compared to naïve controls (p < 0.05). # indicates significant difference compared to saline and D2R Ant experienced animals (p < 0.05). Representative of images of Naïve Sal (C), Exp Sal (D), Exp D1R Ant (E) and Exp D2R Ant (F). Abbreviation: ac = anterior commissure. Scale bar is 100 µm.
the Amph-paired chamber compared to sexually naïve males (Figure 4.6: p = 0.025). In contrast, sexually experienced animals that received D1R antagonist into the NAc did not form a CPP for Amph. They were not different from sexually naïve controls, and spent significantly less time in the Amph-paired chamber compared to saline (Figure 4.6: p=0.049) or D2R antagonist (Figure 4.6: p = 0.038) infused sexually experienced males.

D2R antagonist infusions did not affect the enhanced Amph reward as sexually experienced animals with NAc D2R antagonist infusions formed a significant Amph-CPP compared to naïve saline controls (Figure 4.6: p = 0.040) and D1R antagonist experienced animals (Figure 4.6: p = 0.038), and did not differ from sexually experienced saline males.

4.4 DISCUSSION

In the current study, we demonstrated that activation of D1R in the NAc during sexual behavior causes a long-lasting up-regulation of deltaFosB. In turn, this D1R-mediated deltaFosB up-regulation in the NAc was shown to be critical for enhanced spine density in the NAc and enhanced reward for Amph even though these outcomes of sexual experience are dependent on a period of abstinence from sexual reward (Pitchers, Balfour et al. 2010). Hence, natural reward-induced deltaFosB in the NAc is critical for cross-sensitized effects on psychostimulant reward, potentially via spinogenesis in the NAc during a period of reward abstinence.
Figure 4.6. Blocking D1 receptors in the NAc abolishes sensitized Amph reward in sexually experienced animals. The amount of time spent in the Amph-paired chamber during the post-test minus the pre-test (CPP score, sec) for sexually naïve (white) and experienced (black) animals that received saline, D1R antagonist, or D2R antagonist. Data are expressed as group means ± SEM. * indicates significant difference compared to naïve saline controls (p < 0.05). # indicates significant difference from D1R Ant experienced animals (p < 0.05).
It has long been known that DA is released in the NAc during natural reward behavior, including sexual behavior. Upon introduction of a receptive female, extracellular DA in the NAc is increased and remains elevated as mating commences (Pfaus, Damsma et al. 1990; Damsma, Pfaus et al. 1992; Fiorino and Phillips 1999). The current study showed that infusing DA receptor antagonists into the NAc during mating did not have an effect on the initiation or performance of sexual behavior, which is consistent with the notion that DA is not involved in the expression of reward behavior per se, but rather for learning the incentive salience of sex-related cues (i.e., mating environment) (Berridge and Robinson 1998). Mating and cues that predict sexual reward cause activation of neurons within the mesolimbic DA reward system, including the DAergic cells in the ventral tegmental area and their targets: NAc and medial prefrontal cortex (Balfour, Yu et al. 2004; Balfour, Brown et al. 2006). Repeated sexual experience induces deltaFosB in the NAc, which mediates the experience-induced reinforcement of sexual behavior (Pitchers, Frohmader et al. 2010). Current results show that mating-induced deltaFosB up-regulation is dependent on D1R activation in the NAc during mating. This finding is consistent with previous studies that have shown repeated psychostimulant administration persistently increased deltaFosB in NAc medium spiny neurons expressing D1R (Lee, Kim et al. 2006; Kim, Teylan et al. 2009) and deltaFosB up-regulation in the NAc was dependent on D1R (Zhang, Zhang et al. 2002). In addition, sensitized drug responses typically observed in a drug experienced animal have been produced by the overexpression of deltaFosB in dynorphin- (D1R) expressing neurons in the striatum in the absence of prior drug exposure (Kelz, Chen et al. 1999). Thus, both
natural and drug rewards increase deltaFosB in the NAc via a D1R-dependent mechanism to sensitize reward behaviors.

Moreover, the current findings demonstrate that deltaFosB is a critical mediator of the cross-sensitization between natural reward experience and psychostimulant reward. DeltaFosB activity in the NAc has previously been implicated with sensitized drug responses as deltaFosB overexpression in the NAc sensitizes the locomotor response to cocaine after prior acute or repeated administration (Kelz, Chen et al. 1999), increases sensitivity to cocaine and morphine CPP (Kelz, Chen et al. 1999; Zachariou, Bolanos et al. 2006), and causes self-administration of lower doses of cocaine (Colby, Whisler et al. 2003). The current study shows that blockade of D1R or deltaFosB activity in the NAc during mating abolished sex experience-induced sensitization of Amph reward. Thus, natural and drug rewards not only converge on the same neural pathway, but natural and drug reward converge on the same molecular mediators (Nestler, Barrot et al. 2001; Wallace, Vialou et al. 2008; Hedges, Chakravarty et al. 2009; Pitchers, Frohmader et al. 2010), possibly in the same neurons in the NAc (Frohmader, Wiskerke et al. 2010), to influence the incentive salience and the “wanting” of both rewards (Berridge and Robinson 1998).

The current study demonstrated that an abstinence period from natural (sexual) reward is required for the sensitization of Amph reward. We hypothesize that deltaFosB during this abstinence period, affects neuronal function by altering downstream gene expression to initiate spinogenesis and alter synaptic strength. Indeed, blocking deltaFosB
activity in the NAc during mating prevented increased spine density in the NAc detected after reward abstinence. DeltaFosB is a transcription factor that can act as a transcriptional activator or repressor to influence the expression of a myriad of target genes that may in turn influence spine density and synaptic strength in the NAc (Nestler 2008). More specifically, deltaFosB activates cyclic dependent kinase-5 (cdk5) (Bibb, Chen et al. 2001; Kumar, Choi et al. 2005), nuclear factor kappa B (NK-κB) (Russo, Wilkinson et al. 2009), and GluA2 subunit of the glutamate AMPA receptor (Vialou, Robison et al. 2010) and represses transcription of the immediate early gene c-fos (Renthal, Carle et al. 2008; Pitchers, Frohmader et al. 2010) and histone methyltransferase G9 (Maze, Covington et al. 2010). Cdk5 regulates cytoskeletal proteins and neurite outgrowth (Sasaki, Cheng et al. 2002; Hallows, Chen et al. 2003; Chao and Nestler 2004) and cdk5 inhibitor roscovitine blocks drug-induced spine density (Norrholm, Bibb et al. 2003; Taylor, Lynch et al. 2007). Moreover, activating NF-κB increases the number of dendritic spines in the NAc, whereas inhibition NF-κB decreased basal dendritic spines and blocks the cocaine-induced increase in spines (Russo, Wilkinson et al. 2009). Russo et al (2009) hypothesized that repeated cocaine increases deltaFosB in the NAc, which alters NAc spine density through multiple targets (i.e., cdk5, NK-κB) and that the overall consequence is sensitized drug reward (Russo, Mazei-Robison et al. 2009). However, this hypothesis is based on data (described above) from direct gene manipulation studies in which spine density and cocaine reward were analyzed following a short period (< 7 days) of cocaine abstinence (Russo, Wilkinson et al. 2009; LaPlant, Vialou et al. 2010; Maze, Covington et al. 2010). The role of an abstinence period for drugs of abuse has been well established to play a critical role in the incubation of craving (Lu, Hope et al.
2005), but has not been investigated in regards to natural rewards. The current data confirmed that sex experience followed by prolonged periods of natural reward abstinence causes enhanced Amph reward for at least 28 days after mating. Enhanced Amph reward was correlated with increased spine density in NAc after a 7 day, but not at a 28 day abstinence period; hence these data show that spinogenesis may contribute to the initial development or short-term expression of sensitized Amph reward, but is not critical for continued expression of enhanced drug reward.

In conclusion, the current study highlights the cross-sensitization of drug reward by a natural reward and its dependence on a reward abstinence period. Moreover, the neuroplasticity in the system underlying this sensitized drug response was mediated by deltaFosB via D1R activation in the NAc. Therefore, data suggest that loss of a natural reward following reward experience may make individuals vulnerable to development of drug addiction and that one mediator of this increased vulnerability is deltaFosB. It remains important to investigate neural plasticity caused by natural rewards and reward abstinence, in order to reach a better understanding of the molecular and cellular mediators of drug addiction.
4.5 REFERENCES


CHAPTER 5:

Endogenous opioid-induced neuroplasticity of dopaminergic neurons in the ventral tegmental area mediates reinforcement of natural reward
5.1 INTRODUCTION

Male rodent sexual behavior, and ultimately ejaculation, is a natural rewarding and reinforcing behavior (Tenk, Wilson et al. 2009). As is characteristic of reward behaviors, incentive salience of the rewarding stimulus (Berridge and Robinson 1998), and initiation and performance of sexual behavior are increased by previous mating experience (Sheffield, Wulff et al. 1951; Kagan 1955; Lopez, Olster et al. 1999; Kippin and Pfau 2001; Balfour, Yu et al. 2004). It is well established that the mesolimbic dopamine (DA) system is critical for sexual reward and motivation (Eibergen and Caggiula 1973; Balfour, Yu et al. 2004; Frohmader, Pitchers et al. 2010; Pitchers, Frohmader et al. 2010).

The mesolimbic DA system consists of DAergic neurons in the ventral tegmental area (VTA) that project to the nucleus accumbens (NAc), medial prefrontal cortex (mPFC) and basolateral amygdala (Kelley and Cador 1988; Kelley and Berridge 2002; Kelley 2004; Kelley, Baldo et al. 2005; Balfour, Brown et al. 2006). DA is released into the NAc upon presentation of a receptive female and during mating (Pfaus, Damsma et al. 1990; Pfaus and Phillips 1991; Damsma, Pfaus et al. 1992; Wenkstern, Pfaus et al. 1993).

Moreover, DA neurons in the VTA are activated during mating as well as following exposure to conditioned cues predictive of sexual reward (Balfour, Yu et al. 2004; Frohmader, Wiskerke et al. 2010).

Endogenous opioids (EOP) act in the VTA to modulate the mesolimbic DA system through activation of mu-opioid receptors (MOR) during mating or following exposure to conditioned cues predicting sexual reward (Balfour, Yu et al. 2004). G_{i/o}-coupled MOR are located on GABAergic interneurons and when activated, they cause

Exogenous opiates have also been shown to act on the mesolimbic system to cause functional and morphological changes in this system (Wise 1989; Koob 1992; Koob, Maldonado et al. 1992; Shippenberg, Bals-Kubik et al. 1993; Nestler 2004; Koob and Le Moal 2005). In particular, chronic morphine or heroin self-administration reduces the soma size of DA neurons, but not non-DA neurons in the VTA (Sklair-Tavron, Shi et al. 1996; Spiga, Serra et al. 2003; Chu, Zuo et al. 2007; Russo, Bolanos et al. 2007), decreases the level of neurofilament proteins (Beitner-Johnson, Guitart et al. 1992), increases excitability of DA cells (Mazei-Robison, Koo et al. 2011), and reduces axoplasmic transport and DA output from the VTA to the NAc (Beitner-Johnson and Nestler 1993; Mazei-Robison, Koo et al. 2011). These mesolimbic system changes are essential for the expression of morphine reward tolerance and dissipate within a month of drug abstinence (Russo, Bolanos et al. 2007).
It is currently unclear whether plasticity in the VTA DA neurons as seen with exogenous opiate exposure can also be caused by the release of EOP during natural rewarding behaviors. Here, we demonstrate natural reward causes neuroplasticity similar to the changes caused by drugs of abuse, such that sexual experience in male rats reduces the soma size of VTA DA neurons, a process dependent on EOP action in the VTA, and cause cross-tolerance to morphine reward. Furthermore, we test the hypothesis that EOP-induced alterations in VTA DA neurons are critical for the reinforcement of natural rewarding behavior and the learning of incentive value of cues associated with natural reward. Finally, we demonstrate the effects of EOP-dependent VTA DA neuron plasticity on sex experience-induced neural plasticity and cue-induced activity in the NAc.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Adult male Sprague-Dawley rats (200-225 g) were obtained from Charles River (Senneville, QC, Canada) and housed in pairs in artificially lighted rooms on a 12 hr light/dark cycle (experiments 1, 2, 4-7: lights off at 10 am; experiment 3: lights off at 5 pm). Food and water were available ad libitum except during behavioral testing. Stimulus females were ovariectomized and implanted subcutaneously with 5% 17-β-estradiol benzoate Silastic capsules (1.98 mm inner diameter, 0.5 cm length, Dow Corning Corporation, MI, USA). Injections of progesterone (subcutaneous, 500 μg in 0.1 ml of sesame oil) were administered 3-6 hours prior to testing to induce sexual receptivity. All procedures were approved by the Animal Care and Use Committees of the University of Western Ontario and the University of Michigan and confirmed to the Canadian Council.
5.2.2 Sexual behavior

Sexually naïve males were assigned to either of two experimental conditions: sexually naïve or sexually experienced. Experienced animals were allowed to mate five times either on consecutive days or twice weekly (supplemental experiment 2) with receptive females in rectangular test cages (60 x 45 x 50 cm) until display of ejaculation or up to one hour (whichever came first). Cages were thoroughly cleaned with 70% ethanol solution between mating sessions. Sexual behavior was performed during the dark phase (2-6 hours after onset of dark). Only animals that ejaculated during three or more of the five mating sessions were considered sexually experienced and included in experiments. All mating sessions were observed and sexual behavior was recorded. The number of mounts (M) and intromissions (IM), mount latency (ML; time from introduction of the female to first mount), intromission latency (IL; time from introduction of the female to first intromission), and ejaculation latency (EL; time from first intromission to ejaculation) were recorded (Agmo 1997). Naïve animals were placed in a clean test cage for one hour concurrently with sexually experienced males mating in the same room, such that that they were exposed to distant female odors, similar levels of disturbance, and environmental novelty as experienced males.
5.2.3 Experiment 1: Time course of VTA DA soma size changes

5.2.3A Experimental design

To study the time course of changes in DA neuron soma size in the VTA, sexually experienced and naïve animals were euthanized at 1, 7, or 31 days (n = 5-8 per group) after the last day of mating (experienced) or handling (naïve).

5.2.3B Perfusion, tissue processing, and immunohistochemistry

Animals were deeply anaesthetized using sodium pentobarbital (270 mg/kg; i.p.) and perfused intracardially with 50 ml of 0.9% saline, followed by 500 ml of 4% paraformaldehyde in 0.1M sodium phosphate buffer (PB). Brains were removed and post fixed for 1 hr at room temperature (RT) in the same fixative, then immersed in 20% sucrose and 0.01% sodium azide in 0.1M PB for storage at 4°C. Coronal sections were cut at 35 µm on a freezing microtome (H400R, Microm, Walldorf, Germany) and were collected in four parallel series in cryoprotectant solution (30% sucrose, 30% ethylene glycol in 0.1M PB) then stored at 20°C. All incubations were performed at RT with gentle agitation and copious rinses with 0.1M phosphate buffered saline (PBS; pH 7.35) between incubations. Sections were exposed to 1% H$_2$O$_2$ for 10 minutes to destroy endogenous peroxidases, then blocked for 1 hr in incubation solution (PBS+: PBS containing 0.4% Triton X-100 (Sigma-Aldrich; St. Louis, MO, USA) and 0.1% bovine serum albumin (Jackson Immuno Research Laboratories, West Grove, PA, USA). Next, sections were incubated overnight at RT in a mouse polyclonal tyrosine hydroxylase (TH)-antibody (1:20 000; Chemicon; Billerica, MA, USA). After primary antibody incubation, sections were incubated in Alexa 555 conjugated goat anti-mouse antibody
(1:100 in incubation solution; Molecular Probes, Eugene, OR, USA) for 30 minutes. Finally, sections were washed with 0.1 M PB, mounted on Superfrost Plus glass slides, dried and coverslipped with gelvatol containing the anti-fading agent 1,4-diazabicyclo(2,2)octane (DABCO; 50 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) (Lennette 1978).

5.2.3C Data analysis: DA neuron soma size
Images of TH-positive immunofluorescently-stained neurons in the VTA were taken at 40x magnification at three rostral to caudal levels (according to (Balfour, Yu et al. 2004)). There were no differences detected between cells at the different levels. The soma size of DAergic neurons was analyzed using Image J (Rasband, 1997-2008). Mean area, perimeter, and circularity were measured as described by Sklar-Tavron et al. (1996). An average of 25 cells per animal (combined all 3 VTA levels) was analyzed. Only cells with a clearly visible nucleus were included. For statistical analysis, the mean soma size for each animal was calculated and then compared between groups using a two-way ANOVA (factors: sexual experience (sex experienced or sex naïve) and time (1, 7 or 31 days)) followed by post hoc comparisons using the Holm-Sidak method with a significance level of 0.05.

5.2.4 Experiment 2: Mu-opioid receptor antagonist (systemic) and DA soma size
To determine whether MOR played a role in sex experience-induced changes in DA neuron soma size, MOR were blocked during sexual behavior. Half of the animals gained sexual experience, while the other half were handled but remained sexually naive. Within
the sexually experienced and naïve animals, animals were treated with the non-selective MOR antagonist NLX (10 mg/kg, s.c.; Sigma, dissolved in 0.9% saline) or saline 30 minutes before introduction of the female (experienced) or prior to handling (naïve); thereby creating 4 experimental groups: sexually naïve saline (Naïve Sal), sexually naïve naloxone (Naïve NLX), sexually experienced saline (Exp Sal), and sexually experienced naloxone (Exp NLX) (n = 5-8 per group). All animals were sacrificed via intracardial perfusion 7 days after last mating session. Sectioning, immunohistochemistry, and data analysis (two-way ANOVA; factors: sex experience and drug treatment) for DA soma size were conducted as described above in experiment 1.

5.2.5 Experiment 3: Morphine reward
5.2.5A Experimental design

Previously, Russo et al. (2007) showed chronic morphine induces tolerance to morphine reward. Since sexual experience and chronic morphine cause similar decreases in soma size of DA neurons in the VTA, the functional relevance of the sex-induced morphological changes were tested for morphine reward. Sexually experienced and naïve animals were divided into 6 different experimental groups (n = 9-13 per group) - based on sexual behavior (sexually naïve or experienced) and morphine dose (0.5, 5.0 or 10.0 mg/kg; i.p.) - and were tested for morphine-induced conditioned place preference (CPP).

5.2.5B Morphine-CPP

The CPP apparatus (MED associates, St. Albans, Vermont, USA) consisted of three distinct chambers separated by guillotine doors. Two large conditioning chambers (28 cm
long x 21 cm wide x 21 cm high) with white walls and a metal grid floor or black walls and a metal rod floor were separated by a smaller middle chamber (12 cm long x 21 cm wide x 21 cm high) with gray walls and floor. Between each session, the apparatus was thoroughly cleaned with 70% ethanol solution to minimize lingering olfactory cues. To determine individual preferences, a pre-test was conducted during which animals were given free access to the entire apparatus for 15 minutes. As a group animals did not show significant preference for a chamber. Rats that showed a substantial preference for one of the chambers (>200 sec difference between time spent in each of the chambers; < 5% of animals) during the pre-test were excluded from the study. This pre-test was conducted on the first day of the 5 days of mating sessions. Conditioning took place one day after the last mating session and groups were matched for sexual performance during the last mating session. During conditioning, drug was paired to either initially preferred or non-preferred chamber using an unbiased paradigm (Tzschentke 2007) and animals were confined to the chambers for 30 minutes. Animals were injected with saline (i.p.) in the morning (9 am – noon) and confined to the saline-paired chamber (control). In the afternoon (1 pm – 4 pm), animals were injected with morphine (i.p., 0.5 mg/kg, 5.0 mg/kg or 10.0 mg/kg; morphine sulphate dissolved in 0.9% saline, Johnson Matthey, UK) and confined to the morphine-paired chamber. Animals were subjected to two conditioning days. The next day (three days after last day of mating) a post-test, procedurally identical to the pre-test, was conducted. For statistical analysis, time spent in the morphine-paired chamber during the post-test was compared to the time spent in the saline-paired chamber during the post-test for sexually naïve or experienced males within each dosage using a paired t-test. A p-value of less than 0.05 was considered statistically
significant. Additional control groups of sexually naïve and experienced animals received saline in both paired and unpaired chambers to serve as negative controls. No differences in time spent between chambers were detected in any group.

5.2.6 Experiment 4: Experience-induced facilitation of sexual behavior

Sexual experience results in the facilitation of sexual behavior that is maintained for up to 1 month (Pitchers, Schmid et al. 2012). To analyze the effect of blocking MOR on experience-induced facilitation of sexual behavior, sexually experienced animals received either NLX or saline prior to the five consecutive mating sessions (n = 12 each) as described above in experiment 2. One week after the last mating session, a final mating test was conducted during which all animals were allowed to mate until one ejaculation or up to one hour. No NLX or saline treatment was administered before mating on the final test day. Figure 5.3A outlines the experimental design.

Parameters of mating were compared to determine whether NLX affected either sex experience-induced facilitation of mating (day 1 vs day 5) or maintenance of this facilitation (day 5 vs test) using a two-way ANOVA (factors: treatment (saline versus NLX) and day (day 1, day 5 or test)) and Holm-Sidak method for post hoc comparisons. For all statistical tests a probability value less than 5% was considered to be statistically significant.
5.2.7 Experiment 5: Intra-VTA MOR antagonist – Experience-induced facilitation of sexual behavior

5.2.7A Experimental design

To determine whether the MOR in the VTA, specifically, were responsible for the effects of sexual experience-induced changes on sexual behavior, animals underwent local infusion of NLX or saline into the VTA prior to 5 daily mating sessions. Behavioral paradigm was identical to experiment 4. Sexually experienced animals were allowed to mate during 5 consecutive days until one ejaculation or up to one hour. Fifteen minutes before introducing the receptive female, the male rats received bilateral infusions of either NLX (10 µg/µl per hemisphere; 0.5 µl volume; dissolved in 0.9% saline) or saline (0.5 µl per hemisphere). Bilateral microinjections were administered at a flow rate of 0.5 µl/min, over a 1 min interval, followed by an additional 1 min with the injection cannula left in place for optimal diffusion. The injection cannula was then replaced with the dummy cannula and dust cap. One week after the final day of mating (test day), all animals mated once more to ejaculation without an infusion of NLX or saline. Figure 5.3A outlines the experimental design. Data analysis was conducted as described in Experiment 4.

5.2.7B Cannulation surgery

Male rats were anaesthetized with an intraperitoneal injection (0.1 ml/kg) of ketamine (87 mg/ml) and xylazine (13 mg/ml) and placed into a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). Bilateral 21-gauge guide cannulas (Plastics One, Roanoke, VA, USA) were lowered through small drill holes in the skull into the brain
towards the VTA at -4.8 mm AP, ± 0.75 mm ML from Bregma and -7.8 mm DV from the top of the skull according to Paxinos & Watson (1998). Cannulas were secured with dental acrylic that adhered to three screws set into the skull. Animals were given a two week recovery period, and were handled daily for habituation to handling and injection procedures used during behavioral testing.

5.2.7C Cannula placement verification

The placement of the cannulas was examined using TH-immunostaining in order to confirm the VTA was accurately targeted. Only animals with proper placements were included in analyses (final group sizes: experienced saline n = 8; experienced NLX n = 6). Three additional animals that received intra-VTA NLX injections directed outside the VTA were grouped together in a ‘missed’ injection group. The ‘missed’ group was analyzed separately to serve as anatomical controls and Mann-Whitney U test was used to compare behavior on final test day with NLX and saline treated experienced males. Figures 5.3 H-J demonstrate the middle of injection sites for all animals.

5.2.8 Experiment 6: Sex experience-induced deltaFosB expression

5.2.8A Experimental design

To analyze the effect of blocking MOR on sex experience-induced deltaFosB up-regulation, animals received NLX or saline either systemically or intra-VTA (VTA) and were perfused 7 days following last mating or handling session to analyze deltaFosB expression in a number of reward-related brain regions. The experimental design was identical to experiment 2: NLX or saline was administered systemically 30 minutes prior
to introduction of a receptive female or control manipulation during 5 consecutive days; thus, creating 4 experimental groups: Naïve Sal (n = 7), Naïve NLX (n = 7), Exp Sal (n = 8), Exp NLX (n = 8). A different set of animals underwent VTA cannulation surgery (as outlined above) and NLX or saline was infused locally into VTA 15 minutes prior to introduction of the female during 5 consecutive days producing 2 experimental groups (VTA Exp Sal, VTA Exp NLX; n = 7 each).

5.2.8B Perfusion, tissue processing, and immunohistochemistry

All animals were sacrificed via transcardial perfusion 7 days after last mating session. Sectioning and immunohistochemistry were conducted as described above in experiment 1. Sections were then incubated overnight at 4°C in a pan-FosB rabbit polyclonal antibody (1:5K; sc-48 Santa Cruz Biotechnology, Santa Cruz, CA, USA). The pan-FosB antibody was raised against an internal region shared by FosB and deltaFosB, and has been previously characterized to specifically visualize deltaFosB cells at the time points used in this study (>1 day after stimulus) (Perrotti, Hadeishi et al. 2004; Perrotti, Bolanos et al. 2005; Pitchers, Frohmader et al. 2010). After primary antibody incubation, sections were incubated with biotin-conjugated goat anti-rabbit IgG (1 hr; 1:500 in PBS+; Vector Laboratories, Burlingame, CA, USA), avidin-biotin-horseradish peroxidase (1 hr; ABC elite; 1:1K in PBS; Vector Laboratories, Burlingame, CA, USA), and 3,3’-diaminobenzidine tetrahydrochloride (10 minutes; 0.02%, DAB; Sigma-Aldrich, St. Louis, MO) enhanced with nickel sulfate in (0.02% in 0.1 M PB) with hydrogen peroxide (0.015%). Sections were washed thoroughly in 0.1 M PB to terminate the reaction and mounted onto coded Superfrost plus glass slides (Fisher, Pittsburgh, PA, USA) with 0.3%
gelatin in ddH₂O. Following dehydration, all slides were cover-slipped with DPX mountant (dibutyl phthalate xylene; Sigma-Aldrich, St. Louis, MO).

5.2.8C Data analysis
DeltaFosB-immunoreactive (-IR) cells were counted in a number of brain regions using a camera lucida drawing tube attached to a Leica DMRD microscope (Leica Microsystems GmbH, Wetzlar, Germany): NAc [core (C) and shell (S); 400 × 600 µm; ventral tegmental area (VTA; 1000 × 800 µm); medial prefrontal cortex [mPFC; anterior cingulate area (ACA); prelimbic cortex (PL); infralimbic cortex (IL); 600 × 800 µm each]; caudate putamen (CP; 800 × 800 µm), basolateral amygdala (BLA; 900 x 1200 µm) and medial preoptic nucleus (MPN; 400 × 600 µm) (Balfour, Yu et al. 2004; Frohmader, Wiskerke et al. 2010; Pitchers, Frohmader et al. 2010). Two sections were counted per brain region and averaged per animal for calculation of group means. Counts for each animal were expressed as a ratio of the mean of saline naïve controls (systemic) or VTA Exp Sal (intra-VTA). Data were analyzed using two-Way ANOVA (factors: sex experience (sex experience or sex naïve) and drug treatment (NLX or Sal)) followed by post hoc comparisons using the Holm-Sidak method for animals receiving systemic administration and data were analyzed using t-tests to compare groups that received VTA infusions. Significance level was set at a p-value < 0.05.
5.2.9 Experiment 7: Cue-induced pERK expression

5.2.9A Experimental design

Exposure to the cage in which males acquired mating experience has been shown to cause MOR activation in the VTA and neural activity in VTA and NAc (Balfour, Yu et al. 2004). Hence, the mating environment serves as a conditioned cue predictive of sexual reward. The current study tested if MOR activation during sexual experience is required for subsequent conditioned cue-induced neural activation. The experimental design was identical to experiment 2. NLX or saline was administered systemically (i.p.) 30 minutes prior to introduction of a receptive female or control manipulation; thus creating 4 experimental groups: Naïve Sal, Naïve NLX, Exp Sal, and Exp NLX. One week after final mating session, half the animals in each group were exposed to the mating cage (conditioned cue in Exp animals) or handling cage (non salient cue in Naïve animals), while the other half was not exposed to any cues. This experimental paradigm produced 8 groups: Naïve Sal, Naïve Sal cue, Naïve NLX, Naïve NLX cue, Exp Sal, Exp Sal cue, Exp NLX, Exp NLX cue (n = 4 each except Naïve NLX, n =3). Animals were perfused 10-15 minutes after cue exposure. Control animals were perfused concurrently.

5.2.9B Perfusion, tissue processing, and immunohistochemistry

Sectioning and immunohistochemistry were conducted as described in Experiment 1 and 6. Here, we used a rabbit polyclonal antibody against p42 and p44 MAP Kinases ERK1 and ERK2 (pERK; 1:4 000; Cell Signaling Technology, Danvers, MA). The primary antibody has been extensively characterized in the literature (Roux and Blenis 2004; Murphy and Blenis 2006; Frohmader, Wiskerke et al. 2010). Moreover, omission of the
primary antibody prevented all immunoreactivity and Western Blot analysis of rat brain tissue revealed two bands at the appropriate molecular weights.

5.2.9C Data analysis

pERK-IR cells were counted in the same brain regions and using the same methods as deltaFosB in experiment 6. Group averages within sexually experienced or naïve groups were compared using Two-way ANOVA (factors: drug treatment (NLX or Sal) and cue (cue or no cue)) followed by post hoc comparisons using the Holm Sidak or Mann-Whitney ran sum tests where appropriate with a significance level of p < 0.05. In the NAc shell of sexually experienced animals, there was a strong trend towards significance of the factors and thus, pairwise comparisons were conducted to compare saline (Sal) and saline cue (Sal Cue) groups only.

5.2.10 Images

Digital images were captured using a cooled CCD camera (Macrofire, Optronics, Goleta, CA, USA) attached to a Leica microscope (DM5000B, Leica Microsystems, Wetzlar, Germany) with fixed camera settings. Images were imported into Adobe Photoshop 9.0 software (Adobe Systems, San Jose, CA, USA). Images were not altered in any way except for adjustment of brightness and contrast.
5.3 RESULTS

5.3.1 Experiment 1: Sex experience-induced changes in VTA DA cells

Sexual experience resulted in a decrease in VTA DA soma size (Figure 5.1C, D). Sexual experience significantly reduced area and perimeter of the soma of VTA TH-IR cells (area: $F_{(1,32)} = 11.13, p < 0.002$; perimeter, $F_{(1,32)} = 9.594, p = 0.04$). Pairwise comparisons revealed that area and perimeter of DA cells were significantly reduced 1 day and 7 days after the last day of sexual behavior in sexually experienced animals compared to sexually naïve controls (Figure 5.1C: area, $p = 0.014$ (1 day), $p = 0.004$ (7 days); Figure 5.1D perimeter, $p = 0.028$ (1 day), $p = 0.005$ (7 days)). The effect of sexual behavior dissipated as the soma size of DA neurons reverted to baseline 31 days after the last mating session (Figure 5.1C: area, $p = 0.829$; Figure 5.1D: perimeter, $p = 0.810$). Sex experience-induced alterations were not detected in circularity at any of the time points (Appendix C: supplementary figure 1). The DA soma size reduction was dependent on copulation to ejaculation, the most rewarding and reinforcing element of sex behavior (Tenk, Wilson et al. 2009), and not observed if animals were allowed to interact with females or display limited sexual behavior (Appendix C: supplementary figure 2). DA soma size reduction was not dependent on daily mating, as experience during five bi-weekly mating sessions also produced a reduced VTA DA soma size (Appendix C: supplementary figure 3). This reduction in soma size was not due to reduction in TH protein expression, since Western Blot analysis showed that TH levels in the VTA were not significantly reduced by sexual experience (Appendix C: supplementary figure 4). Finally, to explore of VTA DA size reduction results in reduced transport of DA to the NAc as demonstrated following exposure to chronic morphine (Mazei-Robison, Koo et
al. 2011) NAc DA content was determined using HPLC. Sexual experience caused reduced levels of DA in NAc (Appendix C: supplementary figure 5).

**5.3.2 Experiment 2: Sex experience-induced soma size reduction of VTA DA neurons is dependent on MOR activation**

The reduction of VTA DA neuron soma size caused by sexual experience was blocked by systemic administration of the non-selective MOR antagonist NLX. NLX treatment prior to the mating sessions had a significant effect on area ($F_{(1, 22)} = 4.738, p = 0.041$) and trended toward a significant effect on perimeter ($F_{(1, 22)} = 2.892, p = 0.052$). A significant interaction between experience and NLX treatment was found for area ($F_{(1, 22)} = 5.578, p = 0.027$) and perimeter ($F_{(1, 22)} = 8.167, p = 0.009$). Pairwise comparisons showed that sexual experience in Sal treated animals significantly reduced area and perimeter of VTA DA neurons 7 days after the last mating session compared to Sal treated sexually naïve males (Figure 5.1E: area, $p = 0.018$; Figure 5.1F, perimeter, $p = 0.007$). In contrast, sexually experienced NLX treated animals did not differ from NLX treated naïve males (Figure 5.1E: area, $p = 0.483$; Figure 5.1F: perimeter, $p = 0.330$). This effect was specific for sexually experience, as NLX did not affect DA some size in sexually naïve males compared to saline controls. In addition, the soma size of experience saline animals was significantly decreased compared to experienced NLX animals (Figure 5.1E: area, $p = 0.002$; Figure 5.1F: perimeter, $p = 0.002$).
Figure A shows images of Naive and Exp conditions. Figure B presents bar graphs showing the area (µm²) and perimeter (µm) over 3 days for Naive and Exp conditions. Figure C compares area and perimeter measurements over time across different conditions. Figure D illustrates a comparison of area and perimeter between Sal and NLX conditions. Figure E and F provide a visual comparison of area and perimeter for Sal and NLX groups, respectively.
Figure 5.1. EOP-induced soma size changes of DA neurons in the VTA.

Representative images of VTA DA neurons from sexually naïve (A) and experienced (B) animals showing the reduction in soma size 7 days after final mating session. Scale bar indicates 5 µm. Sexual experience (Exp, black bars) caused significant reduction in area (C) and perimeter (D) of VTA DA cells, 1 (Naive, Exp; n = 6) and 7 days (Naive, n = 5; Exp, n = 6), but not 31 days (Naive, n = 6; Exp, n = 8) after final mating, compared to sexually naïve controls (Naïve, white bars). This DA cell plasticity was dependent on EOP action, since NLX (n = 8), but not saline (Sal, n = 7) treatment during mating prevented this reduction in area (E) and perimeter (F) 7 days after final mating session compared to sexually naïve controls (Sal, n = 5; NLX, n = 6). Data represent mean ± SEM. * indicates significant difference compared to sexually naïve controls of the same day or treatment. # indicates significant difference compared to NLX experienced animals.
5.3.3 Experiment 3: Sex experience-induced morphine reward tolerance

The effects of sexual experience on VTA DA soma size by the action of EOP in the VTA are identical to those reported for exogenous opiates (Sklair-Tavron, Shi et al. 1996; Russo, Bolanos et al. 2007). Therefore, it was tested if natural reward-induced VTA DA plasticity affects reward for the opiate morphine. Indeed, sexual experience caused morphine reward tolerance, identical to the effects of chronic opiates (Russo, Bolanos et al. 2007). Sexually experienced males failed to develop a CPP for 0.5 mg/kg morphine dose; whereas, sexually naïve males did form a CPP for this dose indicated by spending a greater amount of time in the morphine-paired chamber compared to the saline-paired chamber during the post-test (Figure 5.2, p = 0.039). Both sexually naïve and experienced groups spent a significantly greater amount of time in the morphine-paired chamber compared to the saline-paired chamber with higher doses of morphine: 5.0 mg/kg (Figure 5.2: Naïve, p = 0.029; Exp, p = 0.012) and 10.0 mg/kg (Figure 5.2: Naïve, p < 0.001; Exp, p = 0.002).

5.3.4 Experiment 4: Facilitation of sexual behavior (systemic naloxone)

The findings thus far demonstrate that EOP acting in the VTA during 5 daily short mating sessions cause plasticity of the VTA DA neurons that is identical to the effects of chronic morphine or heroin (Russo, Bolanos et al. 2007; Mazei-Robison, Koo et al. 2011). We hypothesized that VTA DA soma size reduction is critical for reward learning and specifically for sex experience-induced facilitation of sex behavior. This hypothesis was tested by blocking EOP using NLX during mating and analyzing sex behavior. For experimental design see Figure 5.3A. There was a significant main effect of mating day
Figure 5.2. The effects of sexual experience on morphine reward. Times spent in saline (Sal) or morphine (Mor) paired chambers during the post-test in sexually naïve (Naïve, n = 10-13) or experienced (Exp, n = 9-13) males. Data presented as mean ± SEM. * indicates significant difference compared to Sal-paired chamber within same animals. NS = not significant.
on all parameters of sex behavior (ML: $F_{(2, 55)} = 11.286, p < 0.001$; IL: $F_{(2, 55)} = 8.767, p < 0.001$; EL: $F_{(2, 55)} = 10.368, p < 0.001$) and NLX treatment on latencies to mount ($F_{(1, 55)} = 6.585, p = 0.013$) and intromission ($F_{(1, 55)} = 7.863, p = 0.007$). Pairwise comparisons showed that NLX treatment affected sexual behavior during the first mating session since NLX animals had significantly longer ML ($p = 0.002$) and IL ($p = 0.002$) compared to saline controls on day 1. This NLX effect on initial sex behavior was abated by sexual experience and not observed during subsequent mating sessions. Moreover, systemic NLX prior to each of five mating sessions did not prevent initial facilitation of sexual behavior with experience. Consistent with the reinforcing effects of sexual experience, Sal treated males had decreased latencies to mount (Figure 5.3B, $p = 0.032$) intromission (Figure 5.3C, $p = 0.033$) and ejaculation (Figure 5.3D, $p < 0.001$) during the fifth mating session compared to the first mating session, which indicated facilitation of sexual behavior. Similarly, NLX treatment prior to each mating session did not prevent the experience-induced facilitation of sexual behavior during the 5 consecutive mating days as NLX treated males displayed significantly shorter latencies to mount (Figure 5.3B, $p < 0.001$), intromission (Figure 5.3C, $p < 0.001$) and ejaculation (Figure 5.3D, $p = 0.017$) on the fifth compared to the first day. Moreover, NLX treated males did not differ from saline controls in any of the latencies during the fifth mating session.

In contrast, NLX treatment during sexual experience session did affect the long-term maintenance of facilitated sexual behavior on the test day, which was conducted 7 days after final mating session in the absence of a NLX injection. Sal treated control males displayed the expected experience-induced facilitation of sexual behavior during
the final test day. Specifically, latencies to mount, intromission, and ejaculation did not
differ between the fifth mating session and final test day (Figure 5.3B-D). Whereas, NLX
treated males showed a significant increase in latencies to mount (Figure 5.3B, p =
0.033), intromission (Figure 5.3C, p = 0.036) and ejaculation (Figure 5.3D, p = 0.049) on
the test day compared to the fifth mating session. Also, on the test day NLX animals were
found to be significantly slower than saline treated males as displayed by longer latencies
to mount (Figure 5.3B, p = 0.017) and intromission (Figure 5.3C, p = 0.043). Thus, NLX
treatment blocked the long-term, but not the initial, expression of facilitation of sexual
behavior, thereby confirming a critical role for EOP-induced VTA DA plasticity on long-
term expression of reinforcement of natural reward behavior. These effects of MOR
blockade on loss of long-term reinforcement of sexual behavior were not caused by
exposure to NLX alone, but required NLX treatment with each mating session (Appendix
C: supplementary figure 6) and occurred independent from administration of NLX,
saline, or no injection on the test day (Appendix C: supplementary figure 7). Finally, the
effects of NLX on long-term reinforcement of sexual behavior were not due to decreased
sexual reward during the experience sessions, as NLX treated males formed a preference
for mating identical to saline treated males (Appendix C: supplementary figure 8).

5.3.5 Experiment 5: Facilitation of sexual behavior (intra-VTA)

To confirm EOP act specifically in the VTA to induce long-term facilitation of sexual
behavior, experiment 4 was repeated with intra-VTA infusions of NLX. Results were
identical to systemic administration explained above. There was a significant main effect
of mating day on all parameters of sex behavior (ML: F(2, 33) = 4.494, p = 0.019; IL: F(2,
= 4.042, p = 0.027; EL: F(2, 33) = 5.309, p = 0.010) and intra-VTA NLX treatment on latencies to mount (F(1, 33) = 7.345, p = 0.011) and intromission (F(1, 33) = 6.126, p = 0.019). Intra-VTA NLX did not prevent the initial experience-induced facilitation of sexual behavior during the five days of mating, as both groups demonstrated decreased latencies to mount (Figure 5.3E, p = 0.001), intromission (Figure 5.3F, p < 0.001), and ejaculation (Figure 5.3G, p = 0.001; saline: p = 0.001) on day 5 compared to day 1. NLX treated males did not differ from saline treated males on the fifth day of mating in any of the latencies. Intra-VTA NLX treatment, like systemic administration, caused significantly increased mount (Figure 5.3E, p < 0.001) and intromission latencies (Figure 5.3F, p < 0.001) on the first mating day, which was not observed during subsequent mating sessions.

Similar to systemic NLX, intra-VTA NLX blocked the long-term facilitation of sexual behavior observed in sexually experienced males. Specifically, on the final test day NLX treated males had longer latencies to mount (Figure 5.3E, p = 0.011), intromission (Figure 5.3F, p = 0.010), and ejaculation (Figure 5.3G, p = 0.015) compared to the fifth mating session and compared to the saline males on the final test day (5.3E, p = 0.006; 5.3F, p = 0.008). In contrast, saline treated animals did not differ in latencies to mount and intromission between the final test day and day 5 of mating, thus demonstrating long-term expression of experience-induced facilitation. These effects were specific to delivery of NLX to the VTA, as males with cannulation sites nearby the VTA displayed facilitated sexual behavior with sexual experience – similar to saline controls (Appendix C: supplemental figure 10).
Figure 5.3. The role of EOP in the facilitation of sexual behavior and its long-term maintenance. A, Experimental design for experiments 4 and 5. Sexual behavior parameters for males treated with saline (Sal, white) or NLX (black) with systemic (B-D; Sal, n = 12; NLX, n = 12) or intra-VTA (E-G; Sal, n = 8; NLX, n = 6) administration. Data shown are latency to mount (B, E), intromission (C, F), and ejaculation (D, G) on day 1 and day 5 of five consecutive days of mating. In addition, data are shown for the final test day, 7 days following day 5 in the absence of saline or NLX injection. Data represent mean ± SEM. + indicates significant difference between day 1 and day 5 within treatment. * indicates significant difference between test day and day 5 within treatment. # indicates significant difference between NLX and Sal groups within day. Coronal VTA sections (H, -4.60; I, -5.00; J, -5.25 from bregma) indicating intra-VTA injection sites for all animals in Experiment 5 (Saline; grey; NLX, black; Missed, white). Cannulas were bilateral, but injection sites are represented unilaterally for ease of presentation.
5.3.6 Experiment 6: Sex experience-induced deltaFosB up-regulation

Previously, we have demonstrated that sexual experience-induced deltaFosB in the mesolimbic system. DeltaFosB is a unique transcription factor due to its long-standing stability, which allows it to accumulate with repeated stimulus exposure and then persist in cells in the absence of a stimulus. Moreover, we have shown that sex experience-induced deltaFosB in the NAc is essential for long-term expression of reinforcement of sexual behavior (Pitchers, Frohmader et al. 2010). Here, we tested the hypothesis that EOP-dependent VTA DA plasticity is a critical mediator of NAc deltaFosB expression.

There was a main effect of sex experience on deltaFosB expression in mPFC subregions (ACA: $F_{(1,27)}= 34.384, p < 0.001$; PL: $F_{(1,27)}= 28.615, p < 0.001$; IL: $F_{(1,27)}= 69.045, p < 0.001$), NAc subregions (shell: $F_{(1,27)}= 28.733, p < 0.001$; core: $F_{(1,27)}= 33.454, p < 0.001$), VTA ($F_{(1,27)}= 73.761, p < 0.001$), CPu ($F_{(1,27)}= 15.283, p < 0.001$) and BLA ($F_{(1,27)}= 6.386, p = 0.019$). No significant differences were detected in sex behavior-associated brain region MPN (Figure 5.4B). These findings are consistent with our previous study (Pitchers, Frohmader et al. 2010). In support of our hypothesis, there was a main effect of NLX treatment during mating sessions on deltaFosB expression in the NAc shell ($F_{(1,27)}= 5.347, p = 0.029$) and core ($F_{(1,27)}= 4.788, p = 0.038$), and a trend towards decreased expression in the BLA ($F_{(1,27)}= 4.189, p = 0.05$). Specifically, in the NAc core and BLA, NLX treatment completely prevented sex experience-induced up-regulation of deltaFosB indicated by levels identical to naïve controls and significantly lower than saline treated experienced males (Figure 5.4A: core, $p < 0.001$; BLA, $p = 0.018$). NLX treatment attenuated deltaFosB up-regulation in the NAc shell, and expression was significantly lower compared to saline experienced males (Figure 5.4A: p
= 0.013), but blockade was partial as deltaFosB expression was significantly increased compared to naïve control groups. NLX treatment did not affect sex experience-induced deltaFosB in the mPFC (ACA (Sal, p < 0.001; NLX, p = 0.01), PL (Sal, p < 0.001; NLX, p = 0.007), IL (Sal, p < 0.001; NLX, p < 0.001)), VTA (Sal, p < 0.001; NLX, p < 0.001), or CPu (Sal, p = 0.004; NLX, p = 0.026) (Figure 5.4B).

Treatment of NLX intra-VTA during mating experience had an identical effect on deltaFosB expression as systemic NLX treatment. In sexually experienced animals, number of deltaFosB-IR cells was significantly decreased in animals treated with NLX compared those treated with saline in the NAc shell (p = 0.007), core (p = 0.048), and BLA (p = 0.023) (Figure 5.4C). Intra-VTA NLX did not affect deltaFosB expression in the mPFC, CPu, or VTA (Figure 5.4D)

5.3.7 Experiment 7: Mating cue-induced pERK expression

Based on the findings above, we hypothesized that EOP activation in VTA during mating experience and subsequent VTA DA soma size reduction are critical for incentive salience of mating reward-associated stimuli or “wanting” of sexual behavior. To test this hypothesis, the effects of blocking MOR during mating on neural activity induced by conditioned cues predictive of sexual reward were examined. Confirming and expanding previous findings (Balfour, Yu et al. 2004), exposure to environmental cues associated with previous sex reward (mating cage) significantly increased pERK expression in sexually experienced males in the mPFC, NAc, and VTA. There were main effects of cue
Figure 5.4. The effects of NLX on sex experience-induced deltaFosB up-regulation.

Numbers of deltaFosB-IR cells in sexually experienced (Exp) or naïve animals that received NLX or saline (Sal) during mating sessions with systemic (A, B) or intra-VTA (C, D) administration. Data are presented as mean (± SEM) fold changes relative to Naïve Sal (A, B) or Exp Sal controls (C, D). * indicates significant difference compared to naïve controls within drug treatment. # indicates significant difference compared to Sal controls within sexually naïve or experience groups. Representative images of deltaFosB-IR cells in the NAc core for Naïve Sal (E), Naïve NLX (F), Exp Sal (G) and Exp NLX (H) following systemic administration. Abbreviation: Sh = NAc shell, Co = NAc core, BLA = basolateral amygdala, ACA = anterior cingulated area, PL = prelimbic cortex, IL = infralimbic cortex, CPu = caudate putamen, VTA = ventral tegmental area, MPN = medial preoptic nucleus, ac = anterior commissure. Scale bar is 100 µm.
exposure in ACA ($F_{(1,12)} = 5.541, p = 0.038$), PL ($F_{(1,12)} = 5.241, p = 0.041$) and NAc core ($F_{(1,12)} = 11.102, p = 0.006$), and NLX treatment in the ACA ($F_{(1,12)} = 15.242, p = 0.002$), PL ($F_{(1,12)} = 7.336, p = 0.019$), and NAc core ($F_{(1,12)} = 6.601, p = 0.025$). There was significant interaction in the ACA ($F_{(1,12)} = 16.060, p = 0.002$), PL ($F_{(1,12)} = 8.235, p = 0.014$), IL ($F_{(1,12)} = 6.965, p = 0.022$), NAc core ($F_{(1,12)} = 10.058, p = 0.008$), and VTA ($F_{(1,12)} = 4.938, p = 0.046$). Specifically, pairwise comparisons in experienced males revealed significant increases in cue-induced pERK in mPFC subregions ACA (Figure 5.5A; $p = 0.007$), PL (Figure 5.5B; $p = 0.02$) and IL (5.5C; $p = 0.03$), NAc shell (5.5D, $p = 0.016$) and core (5.5E, $p = 0.029$), and VTA (5.5F; $p = 0.029$) of saline treated sexually experienced animals (Exp Sal Cue) compared to controls that were not exposed to conditioned cues (Exp Sal). In contrast, exposure to the test cage of sexually naïve animals, which did not associate environmental cues with sexual reward, did not induce pERK in any of the brain areas in either Sal or NLX treated males (Figure 5.5). In support of the hypothesis, NLX treatment during sexual experience significantly attenuated cue-induced pERK expression in mPFC subregions (ACA, 5A, $p = 0.046$; PL, 5B, $p = 0.018$; IL, 5C, $p = 0.028$), NAc core (5E, $p = 0.017$), and VTA (5F, $p = 0.024$) compared to saline treated experienced animals (Sal Cue) and NLX cue males did not differ from control groups not exposed to the cue (Sal or NLX).

5.4 DISCUSSION

The current study demonstrates that EOP acting in the VTA during sexual behavior, a natural reward behavior, caused robust, but transient alterations of VTA DA cells, consisting of reduced some size and reduced DA output to the NAc. This VTA DA
**Figure 5.5. The effects of NLX on mating cue-induced pERK expression.** Numbers of pERK-IR cells in sexually naïve (white) and experienced (Exp; black) animals that were pre-treated with systemic NLX or saline (Sal) during mating sessions and were exposed to the test cage (Cue) or no cues in the ACA (A), PL (B), IL (C), nucleus accumbens shell (D) and core (E), VTA (F), CPu (G), BLA (H), and MPN (I). Data are represent mean ± SEM. * indicates significant difference compared to saline-pretreated no cue-exposed controls (Sal). # indicates significant difference compared to Sal treated cue-exposed controls (Sal Cue). Representative images of pERK-IR cells in the NAc core of sexually experienced males with Sal (J), Sal Cue (K), NLX (L), or NLX cue (M). Abbreviation: ac = anterior commissure. Scale bar is 100 µm.
plasticity appears identical to that induced by chronic opiate exposure (Sklair-Tavron, Shi et al. 1996; Spiga, Serra et al. 2003; Chu, Zuo et al. 2007; Russo, Bolanos et al. 2007; Mazei-Robison, Koo et al. 2011) and caused tolerance to exogenous opiate (morphine) reward. We demonstrated that VTA DA plasticity mediates the long-term, but not short-term, reinforcement of sexual behavior and reward-associated cue-induced neural activity (pERK) in the mPFC, NAc, and VTA. VTA DA plasticity is associated with up-regulation of the transcription factor deltaFosB, a mediator of reinforcement of natural and drug reward, in the NAc and BLA. Thus, these findings are indicative of a role for VTA DA plasticity in the long-term expression of the learned incentive salience of natural reward predictive cues, or reward memory, partially via induction of deltaFosB in areas receiving VTA DA input.

Sexual experience has been well documented to result in facilitation of subsequent sexual behavior, including faster onset to initiation of mating and increased performance (Balfour, Yu et al. 2004; Pitchers, Frohmader et al. 2010; Pitchers, Schmid et al. 2012). This facilitation or reinforcement of sexual behavior is maintained for at least 28 days after mating (Pitchers, Schmid et al. 2012). In addition, it has been shown that sex behavior and conditioned cues predictive of sex reward cause MOR internalization in the VTA and induce neuronal activation throughout the mesolimbic system, including VTA DA neurons, NAc, and BLA (Balfour, Yu et al. 2004; Balfour, Brown et al. 2006). It is currently unknown which EOP is the MOR ligand that acts in the VTA during male sexual behavior. Although both beta endorphin and enkephalin have been implicated in incentive motivation for food reinforcers (Hayward, Pintar et al.
2002), this remains to be established for mating. We have previously shown that beta-endorphin neurons are not activated during mating, nor are there increases in POMC mRNA; suggesting that this beta endorphin may not be the critical EOP (Davis, Fitzgerald et al. 2007). It is well established VTA DA neurons play a critical role in the learning of the incentive salience of reward-associated stimuli (Berridge and Robinson 1998; Kelley and Berridge 2002; Berridge 2007) and are critical for reward prediction (Schultz 2010). The current findings expand on our current knowledge by demonstrating that reward-induced VTA neuroplasticity is critical for these functions, and is dependent on MOR activation by EOP in the VTA. VTA DA plasticity was essential for neural activity in the mPFC, NAc, and VTA following exposure to the sexual reward-predicting environmental cues. Moreover, VTA DA plasticity was critical for the long-term expression of “wanting” or increased initiation and performance of sexual behavior. VTA neuroplasticity caused by sexual experience was not required for hedonic response (“liking”) because sex reward (determined by CPP) and short-term facilitation of sexual motivation and performance (during sex experience or 1 day later) remained intact despite MOR blockade during mating. Instead, the data suggest that VTA DA neuroplasticity mediates the longer term (7 days after last sex experience; Pitchers et al 2012) expression of “wanting” of sex reward and heightened motivated responses to mating cues (Berridge and Robinson 1998).

VTA DA cell activity and plasticity appears to control incentive salience and reward prediction via up-regulation of the transcription factor deltaFosB in the NAc and BLA, since deltaFosB up-regulation in these areas were found to be dependent on EOP
action in VTA during mating. The decrease in sex experience-induced deltaFosB in the NAc after NLX treatment was of particular interest because deltaFosB in the NAc has been shown to be critical for the long-term reinforcing effects of sexual reward (Pitchers, Frohmader et al. 2010). In fact, blocking deltaFosB activity in the NAc through viral vector-mediated expression of a dominant negative binding partner (deltaJunD) attenuated maintenance of facilitated sexual behavior in sexually experienced animals (Pitchers et al 2010), which is similar to the effect produced by blocking EOP action in the VTA in the current study. Furthermore, sex experience-induced deltaFosB up-regulation in the NAc is dependent on D1R activation (Chapter 4). Together, these findings indicate that during sexual behavior, EOP are released in the VTA and bind to MOR, thereby inhibiting GABAergic interneurons and consequently disinhibiting VTA DA neurons resulting in DA release, D1 receptor activation, and deltaFosB induction in the NAc (Balfour, Yu et al. 2004).

Sexually experienced animals demonstrated cross-tolerance to morphine reward, similar to the effects of wheel running in mice - another naturally rewarding behavior – an effect blocked with NLX treatment (Lett, Grant et al. 2001; Lett, Grant et al. 2002) and dependent on VTA DA cell plasticity (current findings). Similar to natural rewards, repeated exposure to the opiates morphine or heroin results in a transient reduction of VTA DA soma size (Sklair-Tavron, Shi et al. 1996; Spiga, Serra et al. 2003; Russo, Bolanos et al. 2007; Mazei-Robison, Koo et al. 2011). Moreover, opiate exposure with short withdrawal periods cause reward tolerance, as implicated by higher doses of drug are required to form reward associations (Shippenberg, Emmett-Oglesby et al. 1988;
Russo, Bolanos et al. 2007), and cause self-administering animals to escalate drug intake (Ahmed, Walker et al. 2000; Walker, Chen et al. 2003; Glass, Kruzich et al. 2004). Hence, EOP and opiates act on common neural substrates to induce reward tolerance during early withdrawal which might reflect a compensatory homeostatic mechanism to counteract stimulation by repeated exposure (Koob and Le Moal 2005). In contrast, during long-term opiate drug abstinence, tolerance is reversed to a sensitivity to the rewarding properties to the drug (Harris and Aston-Jones 2003; Harris and Aston-Jones 2003; Aston-Jones and Harris 2004). Interestingly, sexual experience followed by a sex abstinence period of 7-28 days has been found to cause cross-sensitization for psychostimulant reward (Pitchers, Frohmader et al. 2010), which is dependent on mating-induced deltaFosB expression in the NAc (Chapter 4). Hence, sexual reward experience causes simultaneous opiate reward tolerance and psychostimulant reward sensitization. We postulate that these opposite effects on drug reward may be mediated by the neural plasticity in the different areas of the mesolimbic system: VTA EOP action and DA plasticity mediate the opiate reward tolerance (current study), while NAc deltaFosB expression controls psychostimulant sensitization (Pitchers, Frohmader et al. 2010). Both these events can contribute to escalations of drug take (Ahmed and Koob 1998; Ahmed and Koob 1999; Ahmed, Walker et al. 2000; Ahmed, Kenny et al. 2002; Walker, Chen et al. 2003).

The molecular mechanisms by which EOP influence the VTA DA neurons during natural reward behavior remain unknown. The IRS2-Akt-mTOR pathway is a major mediator of decreased soma size in the VTA caused by repeated morphine (Russo,
Repeated morphine administration induced changes in the size of DA neurons in the VTA can be prevented by intra-VTA infusions of BDNF (Sklair-Tavron, Shi et al. 1996). Brain-derived neurotrophic factor (BDNF) activates this pathway through TrkB signaling (Russo, Bolanos et al. 2007), a receptor kinase with high affinity for BDNF and part of the IRS2-Akt pathway (Seroogy and Gall 1993; Numan and Seroogy 1999), and expressed on DA and GABA neurons in the VTA. Down-regulation of the various components of the IRS2-Akt pathway using viral vector gene transfer technology mimics the effects of chronic opiate exposure and reduces DA neuron soma size and effects of the drug can be rescued by restoring this signaling pathway (Russo, Bolanos et al. 2007). Overexpression of component of mTORC2 prevents morphine-induced VTA DA soma reduction (Mazei-Robison, Koo et al. 2011). Therefore, previous work investigating the effects of chronic opiates on VTA DA soma size shows that morphine-induced down-regulation of the IRS2-Akt-mTOR pathway is both sufficient and necessary for this effect (Russo, Bolanos et al. 2007; Mazei-Robison, Koo et al. 2011). Thus, it is tempting to speculate that the effects of sexual experience on VTA DA neuroplasticity are mediated by BDNF and the IRS2-Akt-mTOR pathway.

In conclusion, the current study demonstrates VTA neuroplasticity is caused by the natural rewarding behavior of sexual behavior. Specifically, EOP act in the VTA to reduce DA soma size, which in turn results in a hypodopaminergic system, and alters mesolimbic system functioning in response to cues that are predictive of sexual reward. Furthermore, VTA neuroplasticity is critical for the incentive motivation (“wanting”) and
memory of incentive salience, but not for the hedonic impact (“liking”) of sexual behavior. Finally, VTA neuroplasticity caused by natural reward behavior followed by a short period of reward abstinence have a direct influence on opiate reward and may therefore affect the vulnerability to development of drug addiction.
5.5 REFERENCES


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CHAPTER 6:

Natural reward experience alters AMPA and NMDA receptor distribution and function in the nucleus accumbens¹

6.1 INTRODUCTION

The mesolimbic system consists of interconnected brain areas, including the ventral tegmental area, medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) (Morgane, Galler et al. 2005) and mediates naturally rewarding behaviors including feeding (Avena, Bocarsly et al. 2008; Avena 2009; Avena, Rada et al. 2009; Vucetic and Reyes 2010), drinking (Yoshida, Yokoo et al. 1992), maternal behavior (Numan 2007), social bonding (Young, Lim et al. 2001; Young and Wang 2004) and sexual behavior (Bradley and Meisel 2001; Bradley, Boulware et al. 2005; Wallace, Vialou et al. 2008; Frohmader, Pitchers et al. 2010; Pitchers, Balfour et al. 2010). Behavioral studies have demonstrated that male rat sexual behavior is rewarding and reinforcing as male rats form a conditioned place preference for copulation (Agmo and Berenfeld 1990; Agmo and Gomez 1993; Tenk, Wilson et al. 2009), develop faster running speeds in T-mazes (Kagan 1955), straight-arm runway (Lopez and Ettenberg 2002) or hurdle climbing (Sheffield, Wulff et al. 1951), and perform operant tasks to gain access to sexually receptive females (Everitt, Fray et al. 1987; Everitt and Stacey 1987). Moreover, sexual experience causes facilitation of subsequent sexual behavior, including increased sexual motivation and performance (Pitchers, Frohmader et al. 2010), and influences expression of conditioned place preference for mating (Tenk, Wilson et al. 2009). These behavioral changes suggest the occurrence of natural reward-related learning and memory, which is hypothesized to be mediated by alterations in the mesolimbic system induced by mating behavior experience (Pitchers, Balfour et al. 2010).

In support of this hypothesis, we have previously shown that sexual experience caused an up-regulation of deltafosB in the NAc, which in turn, was critical for the
facilitation of initiation and performance of sexual behavior following sexual experience (Pitchers, Frohmader et al. 2010). In addition, sexual experience caused an increase in dendritic arborization and number of spines in the NAc (Pitchers, Balfour et al. 2010). Similar changes in transcription and morphology are induced by psychostimulants (McClung and Nestler 2003; Robinson and Kolb 2004; Dietz, Dietz et al. 2009). Sexual experience has been shown to alter responsiveness to drugs of abuse, including sensitization to psychostimulant-induced locomotor activity (cross-sensitization) and enhanced psychostimulant reward (Frohmader, Pitchers et al. 2010; Pitchers, Balfour et al. 2010; Frohmader, Lehman et al. 2011). It has been demonstrated that a withdrawal or abstinence period following drug exposure is critical for an increased craving for the drug, termed incubation of craving (Grimm, Hope et al. 2001). Likewise, a mating abstinence period following sexual experience is critical for sexual experience-induced enhancement of psychostimulant reward and increased dendritic branching and spines in the NAc (Pitchers, Balfour et al. 2010). Hence, sexual experience and subsequent abstinence from this natural reward cause long-term alterations in the mesolimbic system that are similar to those induced by psychostimulants, and affect behavioral responses to both natural and drug reward.

Psychostimulants have been reported to induce numerous additional alterations in the NAc, several of which are dependent on a drug abstinence period. These alterations include changes in glutamate receptor trafficking and function (Thomas, Kalivas et al. 2008; Wolf 2010). Repeated cocaine followed by a long abstinence period (3-7 weeks), causes an increase in surface expression of α-amino-3-hydroxy-5-methylisoxazole-4-
propionate (AMPA) receptor subunits and N-methyl-D-aspartate (NMDA) receptor subunits, whereas no changes were observed after a short abstinence period (1 day). Moreover, different subunits of AMPA and NMDA receptors are differentially affected by drug exposure and subsequent abstinence periods, as surface expression of GluA2-lacking AMPARs is up-regulated after prolonged abstinence periods (5-7 weeks) (Conrad, Tseng et al. 2008; McCutcheon, Wang et al. 2011). Electrophysiological studies have shown that repeated cocaine causes alterations in AMPA/NMDA ratio in PFC-responding NAc shell neurons and changes in intrinsic excitability of NAc neurons that may be dependent on a drug abstinence period and NAc subregion (Zhang, Hu et al. 1998; Zhang, Cooper et al. 2002; Hu, Basu et al. 2004; Hu, Ford et al. 2005; Ishikawa, Mu et al. 2009; Kourrich and Thomas 2009; Moussawi, Pacchioni et al. 2009; Mu, Moyer et al. 2010). Such drug-induced neural plasticity contribute to changes in psychostimulant behavioral sensitization (Pierce, Bell et al. 1996; Wolf 1998; Vanderschuren and Kalivas 2000; Wolf and Ferrario 2010) and drug seeking (Cornish and Kalivas 2000; Suto, Tanabe et al. 2004; Conrad, Tseng et al. 2008).

It is currently unknown whether similar adaptations of glutamate receptor trafficking and function occur following natural reward experience and subsequent abstinence from natural reward. Therefore, the goal of the current study was to determine changes in AMPA or NMDA receptor trafficking in the NAc and alterations in synaptic strength of PFC-responding NAc shell neurons following sexual experience. In addition, these measures were determined at 3 different times following the final mating session to
investigate the effects of an abstinence period from natural reward on plasticity in the NAc.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Adult male Sprague Dawley rats (experiment 1 and 2: 200-225 grams; 10-12 weeks; experiments 3, 150-175 grams; 8-10 weeks upon arrival) were obtained from Charles River Laboratories (Senneville, QC, Canada). Animals were housed in Plexiglas cages with a tunnel tube in a temperature-regulated room maintained on a 12/12 hr light dark cycle with food and water available ad libitum except during behavioral testing. Stimulus females (210-220 grams) for mating sessions received a subcutaneous implant containing 5% estradiol benzoate and 95% cholesterol following bilateral ovariectomy. Sexual receptivity was induced by administration of 500 µg progesterone in 0.1 mL sesame oil approximately 4 hours before testing. All procedures were approved by the Animal Care and Use Committees of the University of Western Ontario and conformed to Canadian Council on Animal Care guidelines involving vertebrate animals in research.

6.2.2 Sexual behavior

All male rats were sexually naïve prior to the onset of the experiments. Mating sessions occurred during the early dark phase (between 2-6 hours after onset of the dark period) under dim red illumination. During each mating session male rats were allowed to copulate to ejaculation or until 1 hour, and parameters for sexual behavior were recorded, including: mount latency (ML; time from introduction of female until first mount),
intromission latency (IL; time from introduction of female until first mount with vaginal penetration), and ejaculation latency (EL; time from first intromission to ejaculation) (Agmo 1997). Sexually naïve controls were handled, housed in the same rooms as the mating animals and thus exposed to the same levels of noise, general disturbance and distant smells of estrous females, but were not allowed to interact or mate with receptive females.

6.2.3 Experiment 1: Sex facilitation

6.2.3A Experimental design

Sprague Dawley male rats mated in their home cages for 5 consecutive, daily mating sessions. Animals were divided in 2 experimental groups and were tested for sexual behavior either 1 week or 1 month following after last mating session (Test Day; n = 7 per group). Groups were matched on parameters of sexual behavior and no significant differences were detected between groups for any sexual behavior measures during any mating session while gaining sexual experience.

6.2.3B Data analysis: Sex facilitation

Within group comparisons were made assessing Day 1 and 5 of sexual experience to determine facilitation of sexual behavior with sexual experience, between Day 1 and Test day, and between Day 5 and Test day (either 1 week or 1 month after Day 5) for mount, intromission and ejaculation latencies using Wilcoxon Signed Rank test with significance level of 5%.
6.2.4 Experiment 2: Ionotropic glutamate receptor subunit expression and redistribution

6.2.4A Experimental design

To examine ionotropic receptor redistribution, a paradigm similar to experiment 1 was utilized. Sexually naive male Sprague Dawley rats were divided into sexually experienced and naïve groups. For the sexually experienced groups, sexual experience was gained through 5 consecutive, daily mating sessions (as described above). The sexually experienced males were then divided into 3 experimental groups (matched for sexual behavior parameters) for tissue collection, 1 day (1D), 1 week (1W), or 1 month (1M) following last mating session. Brains from sexually naïve controls (handled as described above) were collected at identical time points after final handling. The groups included: sexually experienced (AMPAR: 1D, n = 9; 1W, n = 12; 1M, n = 12; NMDAR: 1D, n = 9, 1W or 1M, n = 6) or sexually naïve (AMPAR: 1D, n = 9; 1W, n = 12; 1M, n = 12; NMDAR: 1D, n = 9, 1W or 1M, n = 6).

6.2.4B Surface receptor cross-linking

Animals were euthanized with sodium pentobarbital (270 mg/kg; i.p.) followed by decapitation. Following decapitation, each brain was rapidly removed and immediately placed into ice-cold saline. Bilateral NAc was dissected using a rat brain matrix (ASI Instruments, Warren, MI, USA) and scalpel blade according to NAc boundaries defined by Paxinos & Watson (1998). Next, NAc tissue was chopped into 400 x 400 μm cubes using a McIlwain tissue chopper (Vibratome, St. Louis, MO, USA). Methodology for AMPA and NMDA receptor subunit cross-linking were based on Boudreau & Wolf
Immediately following chopping, brain tissue was rapidly transferred to an Eppendorf tube containing 1 mL of ice-cold aCSF spiked with the protein cross-linking reagent bis(sulfosuccinimidyl)suberate (BS₃, 2mM; Pierce Biotechnology, Rockford, IL, USA) and incubated for 30 minutes on a rocker at 4°C. BS₃ does not cross cell membranes, enabling it to selectively cross-link surface-expressed proteins with sulfide bonds, thus forming high molecular weight aggregates, while intracellular proteins remain unmodified. This reaction enables surface and intracellular pools of protein to be distinguished based on MW using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot analysis. The cross-linking reaction was quenched by the addition of 100 µL of 1M glycine for 10 min at 4°C. Tissue was pelleted by centrifugation at 14 000 rpm for 2 minutes at 4°C and the supernatant was discarded. The pellets were resuspended in 400 µL of ice-cold lysis buffer [25mM Hepes (pH 7.4), 500 mM NaCl, 2 mM EDTA, 1mM DTT, 1mM PMSF, 20 mM NaF, 0.1% Nonidet P-40 (0.1%), protease inhibitor cocktail (Ministop, Roche Diagnostics GmbH, Mannheim, Germany) and 1x phosphatase inhibitor cocktail (Phosstop, Roche Diagnostics GmbH,)]. Samples were sonicated for 5 seconds to disrupt tissue, then centrifuged at 14 000 rpm for 2 min at 4°C and immediately placed back into ice block. Supernatant was transferred to a new Eppendorf tube, from which 30 µL of sample were put on ice and remaining supernatant was stored at -80°C for Western Blot analysis. The 30 µL sample was used to determine protein concentration of cross-linked lysates using a BCA assay (ThermoFisher Scientific Inc., Waltham, MA, USA) and NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific Inc., Waltham, MA, USA).
6.2.4C Western Blot analysis

Protein samples (20 μg) were loaded and electrophoresed on 4-15% gradient Tris-HCl gels (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) using a Mini Trans-Blot Cell system (Bio-Rad Laboratories Ltd.) and Tris-Glycine-SDS running buffer [25mM Tris, 192 mM Glycine, 0.1% SDS (pH8.3)]. Precision Plus protein All Blue standards (Bio-Rad Laboratories Ltd.) were used as molecular weight markers. Following separation, proteins were transferred to Millipore Immobilon-FL polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA) using the Trans-Blot Cell wet Blotting system (Bio-Rad) for immunoblotting. The protein transfer was run in transfer buffer (20% methanol and 0.037% SDS in Tris-Glycine [25 mM Tris, 192 mM Glycine (pH 8.3)] at 82 V for 1 hr at room temperature (RT). Transfer buffer for non cross-linked samples had a lower percent of methanol and no SDS (10% methanol in Tris-Glycine). All samples were run in at least duplicate, balanced across groups, and across individual gels.

Next, membranes were incubated in a 2:3 solution of Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) and Tris-Buffered Saline (TBS; 50 mM Tris and 150 mM NaCl (pH 8.0)) for 1 hr on a shaker tray at RT. Membranes were then individually incubated for 16 hr on a shaker at 4°C with either rabbit polyclonal anti-GluA1 (~106 kDa; 1:1K; Millipore, Cat # AB1504) and GluA2 (~100 kDa; 1:4K; Millipore, Cat # AB1768), or mouse monoclonal anti-NR1 (~130 kDa; 1:2K; Upstate (Millipore), Cat # 05-432). These primary antibodies have been previously used and validated (Gao and Wolf 2008; Nelson, Milovanovic et al. 2009), and produced a single band at the
appropriate molecular weight that was prevented by preabsorption of the antibody with peptide when used on non cross-linked tissue. All antibodies were diluted in a 2:3 mix of Odyssey Blocking Buffer with TBS-T (TBS + 0.05% Tween-20 (pH8.0). Following three 10-min washes in TBS-T, the membranes were incubated in secondary antibodies diluted in a 2:3 mix of Odyssey Blocking Buffer and TBS-T for 1 hr at RT. The secondary antibodies included Alexa-680 conjugated goat anti-rabbit (1:5K; Invitrogen, Carlsbad, CA, USA) or IR Dye800 CW-conjugated goat anti-mouse (1:10K; LI-COR Biosciences). Fluorescent immunoreactivity was visualized and images captured using an Odyssey 2.1 scanner (LI-COR Biosciences).

6.2.4D Quantification and statistical analysis

For each protein sample, fluorescence intensity levels for each band (High MW, > 250 kDa for surface expression, and bands at the specific MW listed above for intracellular expression) were determined using the Odyssey software and averages were calculated for each animal. Surface (S), intracellular (I), ratio (S/I, measure of receptor subunit distribution) and total (S+I, measure of total receptor subunit expression) band density values were normalized to the mean values of corresponding sexually naïve control groups. All Western Blot data was analyzed between sexually experienced and sexually naïve controls at the same time point using unpaired t-tests with a significance level of 0.05.
6.2.5 Experiment 3: Electrophysiology

6.2.5A Experimental design

The same experimental design was used as described above for experiments 1 and 2. Sexually experienced males were divided into 3 experimental groups based on the time of tissue collection 1 day, 1 week, or 1 month following last mating session (1 day, n = 7; 1 week, n = 9; 1 month, n = 10). Rats were anaesthetized with sodium pentobarbital (270 mg/kg; i.p) followed by transcardial perfusion with ice-cold oxygenated (95% O₂, 5% CO₂) preparation solution containing [250 mM Sucrose, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 4 mM MgCl₂, 0.1 mM CaCl₂, 26 mM NaHCO₃, 10 mM glucose, 3 mM myoinositol, 2 mM sodium pyruvate, and 0.5 mM ascorbate]. The brain was excised and placed in ice-cold oxygenated preparation solution. Sagittal brain slices, 400 μm thick, were obtained from each animal using a vibratome (Microm, Walldorf, Germany). A total of 4 slices per brain were transferred to a holding chamber with artificial cerebrospinal fluid (aCSF) [125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 1.3 mM MgCl₂, 2.5 mM CaCl₂, 26.2 mM NaHCO₃ and 10 mM glucose], heated to 32°C for 30 min, and then allowed to recover to room temperature for at least 1 hr before a single slice was placed in a recording chamber. This chamber was superfused with oxygenated aCSF at 22°C. There were no differences between groups for any sexual behavioral parameters. Brains were collected from sexually naïve controls at each of the 3 time points after final handling (n = 3-4 each).

Shell neurons were examined in medial NAc slices that did not contain dorsal striatum. Stainless steel bipolar microelectrodes were placed in the prelimbic cortex-NAc
border for presynaptic stimulation of cortical afferent fibers. Afferents were stimulated with paired pulses (50ms ISI) at 0.1 Hz and neurons were voltage-clamped at -80 mV. AMPA/NMDA ratios were determined by taking the average of EPSCs at +40 mV in the absence or presence of the NMDAR antagonist AP5 (50 µM; whole cell currents 30x control and 30x in the presence of AP5). NMDA response was calculated by subtracting AP5 recordings from control. The peak of AMPAR EPSC was divided by the peak of the NMDAR EPSC to yield an AMPA/NMDA ratio. In order to determine paired pulse ratios, measurements were taken at -80 mV with presynaptic stimulation (30x).

6.2.5B Data analysis: Electrophysiology

Data from the sexually naïve controls were combined to form one control group (n=10 neurons in 10 animals) since no statistical differences were detected between time points within controls. Sexually experienced groups (1 day, n=7; 1 week, n=9; 1 month, n=10 neurons; typically one neuron per animal) were compared to the sexually naïve control group using a one-way ANOVA (factors: abstinence period) followed by a Fisher LSD for post hoc comparisons with a significance level of 5%.

6.3 RESULTS

6.3.1 Experiment 1: Sex facilitation

The objective of experiment 1 was to determine a time course for facilitation of sexual behavior following sexual experience. Previously, facilitation of sexual behavior has been detected up to 1 week after final mating session (Pitchers, Frohmader et al. 2010), but it has not been previously investigated if sex experience-induced facilitation of sexual
behavior is maintained after a protracted abstinence period. Here, two groups of male rats were tested for long-term expression of facilitation of sexual behavior, either 1 week or 1 month following last mating session. First, both groups showed facilitation of sexual behavior during five daily mating sessions as was demonstrated by a significantly shorter latency to mount (Figure 6.1A; 1 week, \( p = 0.028 \); 1 month, \( p = 0.019 \)), intromission (Figure 6.1B; 1 month, \( p = 0.016 \); trend at 1 week, \( p = 0.078 \)) and ejaculation (Figure 6.1C; 1 week, \( p = 0.016 \); 1 month, \( p = 0.008 \)) on day 5 compared to day 1 of sexual experience. Furthermore, facilitation of sexual behavior was maintained both 1 week and 1 month after last mating session, as latencies to mating parameters were shorter on Test day (1 week or 1 month after day 5 of sexual experience) compared to mating day 1 (Figure 6.1A: mount latency, 1 month, \( p = 0.016 \); Figure 6.1B: intromission latency, 1 month, \( p = 0.046 \); Figure 6.1C: ejaculation latency, 1 week, \( p = 0.016 \); 1 month, \( p = 0.008 \)) and no significant differences existed between mating day 5 and Test day for any behavioral parameter at any time (except for mount latency at 1 month; \( p = 0.016 \)). Hence, experience-induced facilitation of sexual behavior was maintained during a 1 month period of mating abstinence.

### 6.3.2 Experiment 2: Ionotropic glutamate receptor subunit expression/redistribution

The objective of experiment 2 was to determine the expression of ionotropic glutamate receptor subunits in sexually experienced and naïve males following different mating abstinence periods. AMPAR and NMDAR subunit surface and intracellular pools were quantified using a membrane-impermeant cross-linking reagent (BS\(^3\)), which selectively modified surface-expressed protein enabling distinction from unmodified intracellular
Figure 6.1. Facilitation of sexual behavior and its long-term maintenance. Latencies to mount (A), intromission (B), and ejaculation (C) for two experimental groups during 5 daily mating sessions and a final Test either 1 week (white, dashed) or 1 month (black, continuous) following last mating session. Data are expressed as group means (± SEM). * indicates significant difference from Day 1; # indicates significant difference from Day 5. Proteins using SDS-PAGE and Western Blot analysis. Representative blots illustrating surface (High MW, > 250 kDa) and intracellular bands are presented in Figure 6.2M.
One day following sexual experience, expression of NR1 subunit in surface (S), intracellular (I) and total (S+I) were significantly increased (S, Figure 6.2A, p = 0.025; I, Figure 6.2D, p = 0.035; S+I, Figure 6.2J, p = 0.023) in sexually experienced animals compared to sexually naïve controls. One week following last mating, there was a significant decrease in surface to intracellular ratio expression of NR1 (S/I ratio; Figure 2G; p = 0.024) without significant changes in surface or intracellular expression, compared to sexually naïve animals. Expression of GluA2 significantly increased one week following last mating, in intracellular and total expression, without changes in surface expression (I, Figure 6.2E, p = 0.026; S+I, Figure 6.2K, p = 0.014) in sexually experienced animals compared to naïve. Whereas, one month following last mating, GluA2 was significantly increased in S/I ratio (Figure 6.2H; p = 0.046) accompanied with a statistical trend towards an increase in surface expression (Figure 6.2B; p = 0.055) in sexually experienced animals compared to naïve controls. Changes in GluA1 were not detected at 1 day or 1 week following last mating, but after 1 month, GluA1 surface and S/I ratio expression appeared to be increased in sexually experienced animals compared to naïve controls, albeit only statistical trends were detected (S, Figure 6.2C, p = 0.098; S/I, Figure 6.2I, p = 0.083). Hence, in summary, initially at 1 day of abstinence, a total increased expression of NR1 was detected, followed by increased intracellular expression of GluA2 after 1 week of abstinence and increased surface expression of GluA2 and GluA1 after 1 month of abstinence (the latter as a statistical trend).
Figure 6.2. Glutamate receptor subunit expression and distribution in the NAc.

Quantitative analysis of expression for NR1 (A, D, G, J), GluA2 (B, E, H, K) and GluA1 (C, F, J, L) for surface (A, B, C), intracellular (D, E, F), surface to intracellular ratios (G, H, I) and total protein levels (J, K, L) in sexually experienced and naïve males at 1 day, 1 week and 1 month following last mating or handling session. Data are expressed as group means (± SEM). * indicates significant difference from sexually naïve males at same time point. Statistical trends are indicated by p-values. Images of representative blots demonstrating surface (>250 kDa) and intracellular bands for NR1 (∼130 kDa), GluA2 (∼100 kDa) and GluA1 (∼106 kDa) (M). Representative images demonstrating increased surface expression of NR1 (N) and intracellular expression of GluA2 (O) at 1D and 1W respectively.
6.3.3 Experiment 3: Electrophysiology

The objective of experiment 3 was to determine whether sexual experience alters synaptic strength in PFC-responding NAc shell neurons. Synaptic currents were recorded in medium spiny neurons in the NAc shell of sexually experienced and naïve males following stimulation of fibers deriving from the PFC. AMPA/NMDA ratio was significantly reduced in sexually experienced animals 1 day (p = 0.005), 1 week (p = 0.016), and 1 month (p = 0.005) after final mating session compared to the sexually naïve control group (Figure 6.3A, B, C). In order to determine if there was a change in presynaptic transmitter release probability, paired-pulse ratios were investigated. The magnitude of the facilitation of transmitter release that occurred in response to paired-pulse stimulation did not differ between sexually experienced and naïve animals at any time interval (Figure 6.3D).

6.4 DISCUSSION

The current study demonstrated that sexual experience causes changes in ionotropic glutamate receptor distribution and function in the NAc of male rats and that some of these changes varied with the length of the abstinence period from sexual behavior. Compared to sexually naïve males, sexually experienced males showed a short-term increase in total NR1 subunit expression due to increases in both surface and intracellular receptor pools. GluA2 expression was increased intracellularly and at the surface after 1 week and 1 month of reward abstinence, respectively. Finally, GluA1 expression was moderately increased at the surface, only after a 1 month sex abstinence period. Furthermore, sexually experienced animals had an immediate and long-lasting decreased
Figure 6.3. AMPA/NMDA ratios for sexually experienced and naïve males at 1 day, 1 week or 1 month after last mating or handling session. A) Average AMPA/NMDA ratios in NAc shell neurons of sexually naïve (N) and sexually experienced animals, either 1 day (1D), 1 week (1W) or 1 month (1M) after final handling or mating sessions. Data are expressed as group means (± SEM). * indicates significant difference from sexually naïve males. B) Ratio of the peak amplitudes of AMPA and NMDA current traces for each recorded cell. C) Representative AMPA and NMDA traces recorded in medium spiny neurons in the NAc shell of naïve and sexually experienced (1 month after final mating session) animals. Vertical scale bar indicates 20 mV; horizontal scale bar indicates 20 ms. D) Average paired pulse ratios in NAc shell neurons of sexually naïve (N) and sexually experienced animals, either 1 day (1D), 1 week (1W) or 1 month (1M) after final handling or mating sessions. Data are expressed as group means (± SEM). Representative paired pulse current traces of naïve and sexual experienced (1 month after final mating session) animals. Vertical scale bar indicates 50 pA; horizontal scale bar indicates 25 ms.
AMPA/NMDA ratio recorded from PFC-responding NAc shell neurons compared to naïve controls. Our results indicate that glutamate receptor trafficking depends on the length of an abstinence period from sexual behavior, whereas alterations in synaptic strength at synapses formed by prefrontal cortical afferents to the NAc shell do not. These main findings are similar to reports following repeated cocaine in terms of AMPAR subunit increased surface expression after long drug abstinence and an immediate decrease in AMPA/NMDA ratio, but differ in regards to short-term NMDAR subunit expression, and a persistent decrease in AMPA/NMDA ratio.

Sexual behavior is highly rewarding and reinforcing. Thus, as a male rat gains sexual experience, it displays increased sexual motivation and performance, which are demonstrated by shorter latencies to initiate mating and display ejaculation, and increased copulation efficiency (Frohmader, Pitchers et al. 2010; Pitchers, Frohmader et al. 2010). Here, it was confirmed that this facilitation of sexual behavior was present at 1 week following mating, and it was determined that facilitated sexual behavior was maintained for up to 1 month of mating abstinence. The temporal profile of sex experience-induced facilitation of sex behavior corresponds with our previous study showing cross-sensitization to the locomotor-inducing effects of amphetamine (Amph) when tested at the same mating abstinence periods (Pitchers, Balfour et al. 2010). Previous studies have implicated the NAc as a mediator of the maintenance of facilitated sexual behavior. Both NAc core and shell neurons are activated by mating or cues-associated with mating (Balfour, Yu et al. 2004), and sexual experience results in increased dendritic branching and spines in the NAc core and shell a week after mating (Pitchers, Balfour et al. 2010).
In addition, decreasing the activity of the transcription factor deltaFosB in the NAc via viral vector-mediated gene transfer attenuates experience-induced facilitation of sexual behavior (Pitchers, Frohmader et al. 2010; Vialou, Robison et al. 2010). These findings suggest that the NAc is specifically associated with reinforcement of sexual behavior, and is potentially critical for the cross-sensitized drug effects of sexual experience (Pitchers, Frohmader et al. 2010).

The current study demonstrated that sexual experience caused changes in AMPAR trafficking that were dependent on the length of the sex abstinence period: an increase in intracellular expression of GluA2 expression at 1 week, and increased GluA1 and GluA2 surface expression after 1 month after last mating. Changes in AMPAR trafficking following cocaine exposure, were also dependent on the length of the drug abstinence period (Ferrario, Li et al. 2010). In general, cell surface and synaptosomal GluA1 and GluA2/3 levels are increased after a week of abstinence from cocaine and are maintained at elevated levels for up to 3 weeks after last cocaine injection (Boudreau and Wolf 2005; Boudreau, Reimers et al. 2007; Kourrich, Rothwell et al. 2007; Boudreau, Ferrario et al. 2009; Ghasemzadeh, Mueller et al. 2009). After a more prolonged period of drug abstinence (5 weeks), GluA1 surface expression remained elevated and there was a small decrease in GluA2 surface/intracellular ratio, and hence GluA2-lacking AMPARs were up-regulated (Conrad, Tseng et al. 2008; Mameli, Halbout et al. 2009). This increased expression of GluA2-lacking receptors at the surface was detected after prolonged withdrawal (5-7 weeks) from extended-access cocaine self-administration, but not following prolonged withdrawal from non-contingent cocaine (McCutcheon, Wang et
Moreover, blockade of GluA2-lacking AMPAR prevented drug-seeking (Conrad, Tseng et al. 2008; Famous, Kumaresan et al. 2008). The changes in AMPA receptor trafficking following sexual experience and abstinence vary slightly from those induced by cocaine. Sexual experience caused an increase in surface expression and surface/intracellular ratio of GluA1 and GuA2 after a month abstinence period and an increase in total GluA2 expression (due to an increased intracellular pool) after 1 week. In terms of increased GluA1 surface expression, our data are similar to the changes induced by cocaine after 3 and 5 weeks, but differ in terms of GluA2 which underwent a small decrease after 5 or more weeks without cocaine (Conrad, Tseng et al. 2008). We therefore hypothesize that AMPA receptor up-regulation in NAc may be critical for the long-term effects of sexual experience on subsequent reward behavior and increased Amph reward (Pitchers, Balfour et al. 2010) following the prolonged abstinence periods tested in the current study. In contrast, alterations in AMPA receptor expression or trafficking are not critical for the short term effects of sexual experience on reward behavior. In support, numerous reports have demonstrated that altered AMPAR transmission is not necessary for drug-induced behavioral sensitization to psychostimulants (for review (Wolf 2010)). Sensitization and cross-sensitization have been observed after 1 day of reward abstinence without changes in AMPAR up-regulation (Boudreau and Wolf 2005; Pitchers, Balfour et al. 2010). Moreover, behavioral sensitization has been demonstrated with repeated exposure to the psychostimulant Amph, which was not associated with any changes in AMPAR transmission (Boudreau and Wolf 2005; Bachtell and Self 2008; Ferrario, Li et al. 2010).
The role of NMDARs has been much less studied than AMPARs in regards to behavior sensitization and receptor trafficking. A non-selective NMDAR antagonist (MK-801) prevented the development of locomotor sensitization with cocaine or Amph, but failed to block expression of this sensitization (Karler, Calder et al. 1989; Schumann and Yaka 2009). Also, longer (3 weeks) but not short (1 day) abstinence periods from repeated cocaine increased the expression of NMDAR subunits (NR1, NR2A, NR2B) in the NAc (Ghasemzadeh, Mueller et al. 2009; Schumann and Yaka 2009). Likewise, the role of NMDAR in sexual behavior has been minimally examined through systemic or intra-medial preoptic area administration of MK-801, which impaired sex behavior in naïve and experienced male rats (Fleming and Kucera 1991; Powell, Dominguez et al. 2003), (Dominguez, Balfour et al. 2007). Here, sexual experience caused an increase in total NR1 expression at 1 day due to increased levels in both the surface and intracellular receptor pools, in addition to decreased surface/intracellular ratio after a 1 week abstinence period. Hence, we hypothesize that an initial increase in NMDAR transmission may be critical for the short-term effects of sexual experience on subsequent reward behavior.

Moreover, the short-term increase in NR1 subunit following sexual experience may be indicative of sex experience-induced silent synapse formation. Huang et al (2009) demonstrated that repeated cocaine generated silent synapses in the NAc shell, in which the post-synaptic recruitment of new NMDAR was crucial (Huang, Lin et al. 2009). Silent glutamatergic synapses express functional NMDAR-mediated currents in absence of AMPAR mediated currents (Liao, Zhang et al. 1999; Pickard, Noel et al. 2000; Groc,
It has been shown that previous cocaine exposure can generate silent synapses throughout the brain and that these newly generated synapses provide a substrate for subsequent experience (Marie, Morishita et al. 2005; Huang, Lin et al. 2009). NMDAR are composed of at least one NR1 subunit in combination with one or more NR2 subunits (A-D). NR1 is required for the formation of functional channels; hence changes in NR1 expression may provide an index for changes in the number or functional NMDA receptors. Repeated cocaine drives the insertion of NR1 and NR2B-containing NMDARs and generates silent synapses in the NAc shell. These NR2B-containing silent synapses were inhibited during cocaine administration which attenuated subsequent locomotor sensitization with cocaine (Brown, Lee et al. 2011). The number of silent synapses decreased after several days of cocaine abstinence whereas locomotor sensitization persisted, suggesting premature synapses may engage in the formation of new plastic circuits that can be altered (potentially by reward abstinence) to mediate these persistent behaviors. Thus, we hypothesize that up-regulation of NR1 total expression shortly following sexual experience may be due to the increase number of NMDAR in newly formed silent synapses. Furthermore, sexual experience followed by protracted abstinence may result in decreased silent synapses, explaining both the decreased NR1 surface/intracellular ratio and the increase in AMPAR subunits with prolonged abstinence periods, as synapses are unsilenced.

The current findings that sexual experience and reward abstinence periods caused alterations in glutamate receptor trafficking and expression suggested alterations in the synaptic strength at excitatory synapses in the NAc. Therefore, an electrophysiology
procedure based on Thomas et al (2001) was utilized to study glutamate receptor function in NAc shell neurons following the same abstinence periods shown to result in sensitized sex and drug behavior, and glutamate receptor redistribution. Similar to previous studies following cocaine exposure, plasticity was determined in NAc shell synapses that were responsive to PFC input. PFC is well established to play a key role in compulsive behavior and drug abuse (Feil, Sheppard et al. 2010; Goldstein and Volkow 2011). Likewise, the PFC is critical for development or expression of compulsive sexual behavior, as PFC lesions cause maladaptive seeking of sexual behavior in male rats (Davis, Loos et al. 2010). The current data showed that sexual experience caused an immediate and long-lasting decrease in AMPA/NMDA ratio in the PFC-responding NAc shell neurons.

Previously, it was hypothesized that the primary difference between natural and drug reward is that changes in AMPA/NMDA ratio following natural reward are temporary and will dissipate with time whereas drug-induced changes will persist (Chen, Bowers et al. 2008). This hypothesis was based on findings that cocaine, but not food or sucrose induced a long-lasting increase AMPA/NMDA ratio in the VTA (Chen, Bowers et al. 2008). In contrast, the current study demonstrates that sexual experience did indeed cause a long-lasting change in AMPA/NMDA ratio in the NAc. However, the sex-induced the NAc shell plasticity differs from that following cocaine exposure, which have been shown to be associated with a bidirectional change in AMPAR mediated excitability. Following cocaine exposure and one day of drug abstinence, a reduction in AMPA/NMDA ratios (Thomas, Beurrier et al. 2001) and depression in firing capacity
(Kourrich and Thomas 2009) was found, similar to the results obtained for short term effects of sexual experience. However, after a 14 days abstinence period from cocaine, NAc shell neurons have increased AMPA/NMDA ratios, suggesting that abstinence from cocaine enhanced synaptic strength in the NAc (Kourrich, Rothwell et al. 2007). Hence, cocaine exposure results in a bidirectional change in synaptic plasticity with reduced AMPA-mediated responses shortly after drug exposure, but increased AMPAR-mediated excitability with protracted drug abstinence. In contrast, protracted abstinence from sexual experience resulted in decreased AMPAR-mediated excitability in NAc shell, even though sensitization of locomotor activity and reward associated with Amph are observed during those abstinence periods (Pitchers, Balfour et al. 2010). However, other reports have shown decreased intrinsic membrane excitability in both the core and shell at short and long abstinence period following cocaine exposure (Zhang, Hu et al. 1998; Zhang, Cooper et al. 2002; Hu, Basu et al. 2004; Hu, Ford et al. 2005; Ishikawa, Mu et al. 2009; Moussawi, Pacchioni et al. 2009; Mu, Moyer et al. 2010), showing a lack of bidirectional change, similar to the effects of sexual experience. Moreover, the current results show that sexual experience does not cause changes in presynaptic glutamate release probability based on lack of differences between groups for paired pulse ratio. Kalivas and colleagues have demonstrated that repeated cocaine induces changes in mediators of extracellular non-synaptic glutamate pools in the NAc, whether such alterations occur with sexual experience remain to be determined (Kalivas 2009).

It is likely that glutamatergic inputs to the NAc from other brain areas than the PFC play a functional role in the effects of sexual experience on subsequent reward
behavior. The NAc receives glutamatergic input from the basolateral amygdala (BLA) and the ventral subiculum of the hippocampus (Belujon and Grace 2011). The BLA plays a role in natural reward-seeking behavior (Ishikawa, Ambroggi et al. 2008; Stuber, Sparta et al. 2011), is critical for the association of environmental stimuli with sexual reward (van Furth, Wolterink et al. 1995) and for instrumental behavior to initiate mating (Everitt, Cador et al. 1989). Hence, it is tempting to speculate that sex experience induced alterations in synaptic strength at BLA-responding synapses as well.

The decreased AMPA/NMDA ratio at PFC synapses in the NAc shell is consistent with the increased expression of NR1 shortly after sexual experience, but inconsistent with increased GluA2 and GluA1 after prolonged abstinence periods. One explanation is that receptor trafficking occurred at synapses receiving glutamatergic input arising from other brain areas besides the PFC. Also, the inclusion of NAc core, in addition to the NAc shell, in the protein samples excludes the possibility to make accurate correlations between the protein expression and synaptic strength data. Nonetheless, the current findings clearly demonstrate that natural reward behavior and subsequent reward abstinence cause synaptic plasticity in the NAc with similarities and differences from those induced by psychostimulants.

In conclusion, sexual experience and subsequent reward abstinence in male rats was shown to cause immediate and long-lasting facilitation in sexual behavior, similar to the effects of sexual experience on sensitization to the locomotor inducing effects of Amph (Pitchers, Balfour et al. 2010), and was associated with an immediate and long-
lasting decrease in AMPA/NMDA ratios in synapses in NAc shell. Moreover, sexual experience caused a fast up-regulation of NMDAR and slowly developing redistribution of AMPAR (both GluA1 and GluA2) to the surface of NAc neurons. Hence, similar to the effects of psychostimulants, natural reward behavior can cause long-lasting alterations in glutamate receptor expression - increased expression of both GluA1 and GluA2 subunits, trafficking, and excitability. However, protracted abstinence from sexual experience, unlike that following cocaine, resulted in persisted decrease in AMPA/NMDA ratio in the NAc shell. A number of testable hypotheses for potential underlying contributing factors for this discrepancy were proposed, including alterations of BLA-responding synapses, following natural reward experience. These data once again show both similarities and differences between the long-term consequences of natural and drug reward exposure and may contribute to a better understanding of how drugs may act on this natural reward pathway.
6.5 REFERENCES


CHAPTER 7: GENERAL DISCUSSION
7.1 SUMMARY OF RESULTS, CONCLUSIONS AND FUTURE DIRECTIONS

7.1.1 Sexual experience and subsequent reward abstinence causes neuroplasticity and sensitized drug responses

The findings in Chapter 2 established that natural reward experience caused long-lasting neuroplasticity in the nucleus accumbens (NAc) and that some sex experience-induced changes were dependent on sex abstinence period. Specifically, a sensitized locomotor response was detected as early as one day and up to 28 days after sex experience. Whereas, enhanced drug reward and increased dendritic complexity and spines in the NAc shell and core were not detected until a period void of sex reward (Figure 7.1). These data clearly demonstrated the convergence of natural and drug reward on the mesolimbic circuit, and that natural reward experience followed by an abstinence period may make an individual more susceptible to addiction (Pitchers, Balfour et al. 2010).

This initial research chapter produced an interesting avenue of future study: what are the underlying cellular and molecular mediators of sex experience-induced neuroplasticity?

7.1.2 DeltaFosB in the NAc is critical for the neural alterations caused by sexual experience

Studies in Chapters 3 and 4 demonstrated that the transcription factor deltaFosB is up-regulated, notably in the NAc, by sexual experience via D1 dopamine (DA) receptor (D1R) activation. Moreover, hindering deltaFosB activity in the NAc either attenuated or completely abolished a number of outcomes of sexual experience, including initial and long-term facilitation of sexual behavior (Pitchers, Frohmader et al. 2010), enhanced
amphetamine reward, and increased dendritic spines in the NAc. Overall our data demonstrated that deltaFosB mediates long-term plasticity produced by natural reward experience, which suggests that deltaFosB is important for reward reinforcement and memory (Figure 7.1).

The behavioral and morphological effects of sexual experience have been shown to require both deltaFosB activity in the NAc and a sex reward abstinence period (Figure 7.1). It was hypothesized in Chapter 4 that deltaFosB exerts its transcriptional control of numerous genes during this abstinence period to mediate the long-term effects of reward experience. During this abstinence period, deltaFosB activates or represses transcription in a gene-specific manner. Nestler and colleagues have identified several genes that are affected by deltaFosB following repeated drug exposure (Bibb, Chen et al. 2001; Kumar, Choi et al. 2005; Renthal, Carle et al. 2008; Russo, Wilkinson et al. 2009; Maze, Covington et al. 2010; Vialou, Robison et al. 2010). However, downstream genes targeted by sex experience-induced deltaFosB during this sex reward abstinence period need to be further explored. Furthermore, the mechanism by which deltaFosB actually alters target gene expression remains unclear. In Chapter 3, c-Fos was identified as a deltaFosB target indicated when sex-induced c-Fos being decreased by sexual experience. The C-Fos gene contains an AP-1 like site in its promoter region (Morgan and Curran 1989). Like sex experience, c-Fos is greatly induced by acute psychostimulant exposure, but is only weakly induced after repeated exposure (Hope, Kosofsky et al. 1992; Persico, Schindler et al. 1993; Renthal, Carle et al. 2008). After chronic amphetamine, deltaFosB was found to be bound to the c-fos promoter, where it
recruited the histone deacetylase HDAC1 (Renthal, Carle et al. 2008). A similar mechanism may be mediating the sex experience-induced decrease in subsequent sex-induced c-Fos induction. Future studies will continue to investigate potential downstream targets of deltaFosB, and the co-repressors and co-activators recruited by deltaFosB to regulate transcription following sexual experience.

The identification of deltaFosB recruitment of HDAC1 (Renthal, Carle et al. 2008) sparks another interesting line of future research involving epigenetic mechanisms by which sexual experience can induce stable changes to gene expression. An epigenetic mechanism enables environmental signals to be transduced into alterations in chromatin structure to regulate the access transcriptional machinery has to DNA, thus, ability to activate or repress gene expression (Berger, Kouzarides et al. 2009). As a result, specific experiences can alter specific gene transcription through enzymatic modifications of DNA and histones (Berger, Kouzarides et al. 2009). In addition, these modifications are potentially stable making epigenetic regulation a great candidate for mediating transcriptional changes and consequently, neural plasticity to underlie the long-term effects of reward experience. Nestler and colleagues have conducted a great deal of work implicating altered histone acetylation and deacetylation, and DNA methylation in drug addiction (Kumar, Choi et al. 2005; Renthal, Carle et al. 2008; Maze, Covington et al. 2010; Lester, Tronick et al. 2011; Maze and Nestler 2011; Robison and Nestler 2011). We hypothesize that natural reward experience caused long-term neuroplasticity via transcriptional and epigenetic mechanisms. Epigenetic alterations at global and gene
promoter levels following natural reward experience will be investigated in future experiments.

7.1.3 Sexual experience causes neuroplasticity in the ventral tegmental area as well

In contrast to Chapters 2, 3, 4 and 6 which focus on neuroplasticity in the NAc, Chapter 5 investigated sex experience-induced neuroplasticity in the ventral tegmental area (VTA). The VTA contains DA neurons that project to the NAc and other reward-related areas. Similar to exogenous opiates, endogenous opioids (EOP) released during sex behavior caused a reduction in VTA DA soma size, which was critical for reinforcement of sexual reward, tolerance to morphine reward, and deltaFosB up-regulation. These data suggested that by blocking EOP during mating and as a result leaving VTA DA morphology unchanged, it blocked the “wanting” and memory for sexual behavior and reward. Indeed, exposure to a cue associated with mating and predictive of sexual reward did not elicit neuronal activity in NAc and basolateral amygdala (BLA) when the effects of sexual experience on VTA DA plasticity were blocked. Together, these results suggested that sexual experience caused neuroplasticity in the VTA, which was critical for sex reward memory (Figure 7.1).

We have largely investigated the behavioral implications of the sex experience-induced decrease in VTA DA soma size, yet the molecular pathway responsible for this morphological change remains unexplored following natural reward. Nestler and colleagues have chronic morphine or heroin self-administration to cause a similar reduction in VTA DA soma size as we find with sexual experience (Sklair-Tavron, Shi et
Brain-derived neurotropic factor (BDNF) is the most prevalent growth factor in the central nervous system and its signaling pathway influence neuronal survival, growth, differentiation, and structure (Autry and Monteggia 2012). BDNF signals through 3 major pathways: IRS2-Akt, phospholipase Cγ, and Raf-ERK. Previous studies have implicated BDNF-IRS2-Akt-mTOR signaling in mediating opiate-induced neuroadaptations in the VTA (Russo, Bolanos et al. 2007; Mazei-Robison, Koo et al. 2011). As described in Chapter 5, sexual experience resulted in similar changes as chronic opiate exposure in terms of VTA DA morphology, tolerance to morphine reward, and decrease DA in the NAc. Our data suggest that EOP released during mating are affecting the same VTA DA neurons via the same signaling pathway. Future studies will seek to identify what molecules are affected by sexual experience and whether manipulations of specific molecules apart of the IRS2-Akt-mTOR pathway will also stop or replicate the effects of sexual experience. By conducting this investigation, we will be able to determine whether natural reward experience affects VTA DA soma size and neuronal function through the same signaling pathway as opiates.

To this point, our investigation into VTA neuroplasticity has been restricted to the population of DA neurons - largely ignoring the nearby and intermingled population of GABAergic neurons in the VTA. As described in Chapter 5, EOP released during mating act on MOR to inhibit GABAergic interneurons in the VTA (Johnson and North 1992; Balfour, Yu et al. 2004). These VTA GABAergic neurons provide inhibitory input to the adjacent DAergic neurons and are themselves activated by reward-related stimuli.
(Steffensen, Lee et al. 2001; Balfour, Yu et al. 2004). It would be interesting to investigate if we can replicate the effects of sexual experience on decreasing VTA DA soma size by administering GABA antagonists or block the effects of sexual experience with GABA agonists administered concomitantly with mating. Furthermore, these VTA GABAergic neurons have been implicated in mediating the transition from a drug-naïve state to a state of drug addiction (Bechara, Harrington et al. 1992; Nader and van der Kooy 1997; Laviolette, Gallegos et al. 2004; Laviolette and van der Kooy 2004). Specifically, a subset of GABAergic neurons in the VTA express GABA_A receptors which switch from inhibitory to excitatory signaling depending on drug history. In a drug-naïve state, GABA_A receptors are inhibitory and opiates produce their acute rewarding effects through a DA-independent mechanism via descending projections to the brainstem (Semba and Fibiger 1992; Steininger, Rye et al. 1992; Nader and van der Kooy 1997; Olmstead, Munn et al. 1998; Dockstader, Rubinstein et al. 2001). In contrast, once an animal is drug-experienced, the motivational effects of opiates are DA-dependent and mediated by the mesolimbic system (Nader and van der Kooy 1997; Dockstader, Rubinstein et al. 2001; Laviolette, Nader et al. 2002). During mating, DA is released into the NAc once the female is first noticed and remains elevated until conclusion (Pfaus, Damsma et al. 1990; Damsma, Pfaus et al. 1992). However, we find DA (either D1R or D2R) antagonists infused into the NAc during mating did not affect sexual reward as indicated by undisturbed facilitation of sexual behavior with sexual experience, potentially because sexual reward is being mediated in a DA independent manner. In fact, male rats with NAc lesions demonstrated normal copulatory behavior (Paredes and Agmo 2004) and DA antagonists did not affect sex CPP (Agmo and Berenfeld 1990). It might
be interesting to test whether sexual reward in a sexually naïve male is mediated via a DA-independent mechanism, since our data suggest DA is required for ‘wanting’ of sexual reward in a sex experienced animal.

### 7.1.4 Sexual experience alters glutamate receptor expression, distribution, and function in the NAc.

In Chapter 6, experimental focus turned from DA input to the NAc to its equally important glutamatergic input from the medial prefrontal cortex (mPFC), BLA, and hippocampus (Belujon and Grace 2011). Sexual experience caused an immediate increase in total NR1 subunit expression followed by increased surface expression of GluA2 after 1 month sex abstinence period in the NAc. Moreover, sex experience caused an immediate and long-lasting reduction in AMPA/NMDA ratio of synaptic currents in NAc shell neurons following stimulation of cortical afferents. These findings did not exactly match those caused by psychostimulants, yet similarities were apparent. These experiments showed that the glutamatergic system in the NAc was dynamic following sexual experience and may play a role in the long-term effects on natural and drug reward (Figure 7.1).

Although our initial glutamatergic studies focused on PFC inputs, there is reason to suspect glutamatergic inputs from the BLA to the NAc are more important in natural reward behaviors. BLA projections to the NAc are thought to modulate cue-induced motivated behaviors (Cador, Robbins et al. 1989; Setlow, Holland et al. 2002; Di Ciano and Everitt 2004; Ambroggi, Ishikawa et al. 2008; Shiflett and Balleine 2010). In fact,
inhibiting BLA-to-NAc fibers during cue-reward pairing caused an attenuated response to reward cue and subsequent intake of natural rewards (Stuber, Sparta et al. 2011).

Moreover, BLA neural responses to reward cues precede those of NAc neurons and cue-evoked activation of NAc neurons is dependent on BLA inputs (Ambroggi, Ishikawa et al. 2008). As a result, future studies will investigate the neural activity and plasticity in the BLA following sexual experience and further investigate the importance BLA projections to the NAc in reward seeking. In addition, our data showing sexual experience produced a sensitized drug reward suggests that there still may be important change in projections from the mPFC to the NAc since these inputs are thought to modulate compulsive reward-seeking behavior (McFarland, Lapish et al. 2003; Kalivas and Volkow 2005). Clearly, there is great potential for future investigations into the role of glutamate - originating from the mPFC or BLA - in sex experience-induced neuroplasticity in the NAc.

Although the set of studies included in my thesis provide a critical and in-depth investigation into the underlying cellular and molecular mechanisms mediating neuroplasticity induced by sexual experience and subsequent reward abstinence, many questions remain. Future studies will build on some of the major findings of this thesis in order to advance the breadth of knowledge on mechanisms mediating natural reward experience-induced neuroplasticity. Together, the studies included in this thesis and proposed future studies will be critical for further understanding reward and reinforcement in general, and how the reward system ‘malfunctions’ following drug reward leading to drug addiction.
Figure 7.1. Thesis summary figure. Schematic illustrating the number of neural and behavioral alterations that occur following sexual experience and subsequent reward abstinence.
7.2 REFERENCES


APPENDIX A
**Supplemental Information for Chapter 2**

**Supplemental Figure 1.** Sexually experienced animals in experiment 2 showed a greater locomotor response to the sex-paired environment after the first day of mating. Data are presented as mean ± SEM of total number of crossovers over 15 minutes prior to introduction of a female. Data for experienced animals on Day 4 are missing due to computer failure. * indicates statistical significant difference between experience and naïve groups (p < 0.05).

**Supplemental Figure 2.** Locomotor response during the first 30 minutes of the test of sexually experienced and naïve animals to SAL or AMPH injection one day (A), one week (B) or one month (C) following the pre-test mating sessions. No differences in responses to AMPH were found between experienced and naïve animals, in contrast with the sensitized response during the subsequent 60 minutes (Figure 2.2). Mean ± SEM of total number of crossovers during the first 30 minute time interval after injection.* indicates statistically significant difference between AMPH and SAL (p < 0.05).
Supplemental Figure 3. Animals in experiment 1 that did not mate to ejaculation do not show a sensitized locomotor response. Shown are locomotor responses of sexually experienced (Exp), naïve (Naïve) animals, and animals that only mounted and/or intromitted (M/I; n = 6) during mating sessions, to saline or AMPH one week after final mating session. Mean ± SEM of total number of crossovers over 90 minutes. These data are also found in Figure 2.2 except for M/I group. * indicates statistically significant difference between AMPH and saline. # indicates statistically significant difference between experienced and naïve groups (p < 0.05).

Supplemental Experiment 1

Methods: The purpose of this experiment was to test if social and sexual interactions that do not include ejaculation result in behavioral sensitization to AMPH. Sexually naïve male rats (Sprague Dawley) were divided into 4 groups and received the following treatment during five consecutive days in mating test cages: 1. Mate with receptive female to 1 ejaculation (n = 7); 2. Mate with receptive female to 8 intromissions (n = 8); 3. Exposure to scents of receptive female placed on top of the cage (n = 7); 4. Control (handled, housed in the same room, but no direct exposure to females; n = 8). Six days
after last treatment, animals received three days of saline injections and were placed in
the locomotor activity chamber (Med Associates Inc., St Albans, VT, USA) for 60
minutes. Locomotor activity on the third day served as the baseline saline response
measure. On the following day (10 days following last treatment day), all animals
received 0.5 mg/kg AMPH (s.c.), were placed in the locomotor activity chambers and
total distance traveled (in centimeters) in a period of 60 minutes was measured. All drug
and behavior treatments and activity analysis were performed during the first half of the
dark phase.

**Results:** Locomotor response to AMPH was significantly increased (sensitized) in males
allowed to mate to ejaculation, confirming our previous findings (supplementary figure
4). In contrast, display of intromissions or access to female scents did not cause sensitized
responses to AMPH. In conclusion, ejaculations are essential for the development of
locomotor sensitization to AMPH induced by sexual experience. These findings indicate
that alterations of the mesolimbic system induced by mating are dependent on display of
ejaculation; thus, not caused by social or sexual interactions that do not include
copulation to ejaculation.
**Supplemental Figure 4.** Locomotor response of animals exposed to the scent of receptive females (Scent), restricted mating to only mounts and intromissions (M/I), mated to ejaculation (Exp), or controls (Naïve), to saline (sal) or amphetamine (amph). Mean ± SEM of total distance traveled (cm) over 60 minutes. * indicates statistically significant difference between Exp amph and M/I amph groups. # indicates statistically significant difference between Exp amph and Naïve amph groups (p < 0.05).
APPENDIX B
Supplemental Information for Chapter 3

**Supplemental Table 1.** Behavioral paradigm used in experiment 1 to test the effects of sexual experience, and consequential deltaFosB induction, on the induction of the immediate-early gene c-fos by sexual behavior. Animals were intracardially perfused either 1 hour (NS, ES) or 18-24 hours after final mating session (NNS, ENS).

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Final Day</th>
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<tr>
<td>Naïve No Sex</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Naïve sex (NS)</td>
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<td>-</td>
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<td>perfusion</td>
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<tr>
<td>Experienced no sex (ENS)</td>
<td>sex</td>
<td>sex</td>
<td>sex</td>
<td>sex</td>
<td>sex</td>
<td>perfusion</td>
</tr>
<tr>
<td>Experienced sex (ES)</td>
<td>sex</td>
<td>sex</td>
<td>sex</td>
<td>sex</td>
<td>sex;</td>
<td>perfusion</td>
</tr>
</tbody>
</table>

**Supplemental Table 2.** Sexual behavior data for animals in experiment 1 (mean ± sem). Animals were sacrificed either 1 hour (NS, ES) or 18-24 hours (ENS) after first (NS) or fifth (ENS, ES) mating session (n = 6/group). *indicates significant difference from first mating session. No significant differences were detected between groups for any behavioral measures within the appropriate mating session. Sexual behavior measures: ML, mount latency; IL, intromission latency; EL, ejaculation latency; M, number of mounts; IM, number of intromissions.
<table>
<thead>
<tr>
<th>Group</th>
<th>Sexual Behavior Measure</th>
<th>Mating Session 1</th>
<th>Mating Session 2</th>
<th>Mating Session 3</th>
<th>Mating Session 4</th>
<th>Mating Session 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experienced No Sex</td>
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<td>207 +/- 11</td>
<td>70 +/- 27*</td>
<td>108 +/- 41</td>
<td>23 +/- 9*</td>
<td>19 +/- 8*</td>
</tr>
<tr>
<td></td>
<td>IL</td>
<td>207 +/- 11</td>
<td>82 +/- 32*</td>
<td>118 +/- 43</td>
<td>23 +/- 9*</td>
<td>19 +/- 8*</td>
</tr>
<tr>
<td></td>
<td>EL</td>
<td>1105 +/- 356</td>
<td>369 +/- 62*</td>
<td>789 +/- 399</td>
<td>355 +/- 74*</td>
<td>298 +/- 117*</td>
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<td>13 +/- 4</td>
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<td>IM</td>
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<td>25 +/- 9</td>
<td>19 +/- 1</td>
<td>10 +/- 1</td>
</tr>
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<td>Experienced Sex</td>
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<td>389 +/- 119</td>
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<td>162 +/- 126</td>
<td>43 +/- 17*</td>
<td>73 +/- 33*</td>
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<td></td>
<td>IL</td>
<td>392 +/- 117</td>
<td>118 +/- 56*</td>
<td>165 +/- 129</td>
<td>50 +/- 18*</td>
<td>101 +/- 46*</td>
</tr>
<tr>
<td></td>
<td>EL</td>
<td>1008 +/- 242</td>
<td>769 +/- 348*</td>
<td>746 +/- 336</td>
<td>442 +/- 151*</td>
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<td>16 +/- 8</td>
<td>9 +/- 3</td>
<td>4 +/- 2</td>
</tr>
<tr>
<td></td>
<td>IM</td>
<td>13 +/- 1</td>
<td>16 +/- 3</td>
<td>18 +/- 4</td>
<td>18 +/- 7</td>
<td>9 +/- 2</td>
</tr>
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<td>Naïve Sex</td>
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<td>-</td>
<td>-</td>
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<td></td>
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<td>-</td>
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<tr>
<td></td>
<td>IM</td>
<td>11 +/- 2</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>
Supplemental Figure 1. Schematic drawings illustrating the area of analysis (indicated by the boxes) of deltaFosB-IR in the caudate putamen (CP; B), and nucleus accumbens core and shell at rostral (A), middle (B) and caudal (C) levels. Abbreviations: C, NAc core; S, NAc shell; ac, anterior commissure; LV, lateral ventricle; cc, corpus callosum.
Supplemental Figure 2. Schematic drawings illustrating the area of analysis (indicated by the boxes) of deltaFosB-IR in the ventral tegmental area at rostral (A), middle (B), caudal (C) and tail levels (D). Abbreviations: ML, medial lemniscus; FR, fasciculus retroflexus; pc, posterior commissure; AQ, cerebral aqueduct; SC, superior colliculus; PAG, periaqueductal grey; PH, posterior hypothalamus; MGv, medial geniculate ventral part; cpd, cerebral peduncle; RN, red nucleus; IPN, interpeduncular nucleus; MM, medial mammary nucleus.
Supplemental Figure 3. Schematic drawings illustrating the areas of analysis (indicated by the boxes) of deltaFosB-IR in the medial prefrontal cortex (A) and medial preoptic nucleus (B). Abbreviations: cc, corpus callosum; ac, anterior commissure; ACA, anterior cingulate area; PF, prelimbic area; IL, infralimbic; MPN, medial preoptic nucleus; MPO, medial preoptic area; LV, lateral ventricle; AQ, cerebral aqueduct; CP, caudate putamen; fx, fornix; BST, bed nucleus of the stria terminalis; GPe, globus pallidus external segment; och, optic chiasm; ec, external capsule.
Supplemental Figure 4. Representative NAc section showing DAB-GFP staining for injection site verification. Abbreviations: AC, anterior commissure; LV, lateral ventricle.
Supplemental Information for Chapter 5

Supplemental Experiment 1

Methods: Methods described in section 5.2.3.

Results: Data described in 5.3.1.

Supplementary Figure 1. Sexual experience does not alter circularity of VTA DA neurons (1D: Naive, Exp, n = 6; 7D: Naive, n = 5, Exp, n = 6; 31D: Naive, n = 6, Exp, n = 8). Data represent mean ± SEM.

Supplemental Experiment 2

Methods: To determine which elements of sexual behavior are essential for the sex experience-induced decrease in VTA DA soma size, animals were exposed to different elements of sexual behavior and then analyzed for reduced VTA DA soma size. During 5 consecutive days, male rats either mated to one ejaculation as described in experiment 1 (n = 4; including group average of 17 intromissions per mating session), exposed to odors from an estrous female placed on top of their cages (n = 4), mated including mounts and intromissions, but no ejaculation (n = 4; females were removed after display of 6 intromissions), or handled as described in experiment 1 (n = 4; sexually naïve controls). All animals were perfused with paraformaldehyde (as described in experiment 1) 7 days
after the last day of mating. Sectioning and immunohistochemistry for DA soma size were conducted as described above in experiment 1. Data were analyzed by one-way ANOVA (factor: mating) followed by post hoc analysis using Holm-Sidak method.

**Results:** Mating caused a significant decrease in perimeter ($F_{(3,12)} = 3.56; p = 0.048$) and a trend toward a decrease for area ($F_{(3,12)} = 3.56; p = 0.060$). Pairwise comparisons with naïve controls revealed a significant reduction in soma size was only detected in males that mated to ejaculation (supplemental figure 2: A, area, $p = 0.033$; B, perimeter, $p = 0.007$). In contrast, exposure to an estrous female, or display of mounts and intromissions did not result in a significant reduction of VTA DA soma size (Female: area, $p = 0.164$, perimeter, $p = 0.133$; Intro, area, $p = 0.251$, perimeter, $p = 0.162$).

**Supplemental Figure 2.** VTA DA cell area (A) and perimeter (B) in animals that mated to ejaculation (Ejac), exposed to female odors (Estrous), displayed mounts and intromissions (Intro), or remained sexually naïve (Naïve) ($n = 4$/group). Data represent mean ± SEM. * indicates significant difference compared to sexually naïve controls.
Supplemental Experiment 3

Methods: To determine whether mating had to occur daily for the sex experience-induced VTA DA soma size reduction, a different mating paradigm was tested consisting of bi-weekly mating sessions, but consisted of the same number of total mating sessions (5). Animals were euthanized 1 day after final mating session. Sectioning and immunohistochemistry for VTA DA soma size were conducted as described in experiment 1. Area and perimeter data were compared between sexually naïve (n = 8) and sexually experienced (n = 8) groups using t-tests.

Results: Reduced VTA DA soma size in terms of area and perimeter were detected in sexually experienced animals compared to naïve control following a mating paradigm consisting of bi-weekly mating sessions (supplemental figure 3: area, p = 0.004; perimeter, p < 0.001). Therefore, the reduction in VTA DA some size is an outcome of sexual experience independent of the frequency of mating.

Supplemental Figure 3. Fold change in VTA DA soma area and perimeter in sexually naïve (white) and experienced (black) animals. Data represent mean ± SEM. * indicates significant difference compared sexually naive controls.
Supplemental Experiment 4

Methods: To determine whether the reduced VTA DA soma size caused by sexual experience was associated with decreased TH expression in the VTA, Western Blot analysis was used to measure TH levels in sexually experienced (n = 5) and naïve (n = 5) males.

Tissue Collection and Protein Isolation

Male rats received sexual experience or were handled (naïve) as described above. One day after last mating or handling session, animals were deeply anesthetized using sodium pentobarbital (270 mg/kg; i.p.). Brains were rapidly removed, frozen on dry ice, and stored at -80°C until processing. The VTA was microdissected and homogenized mechanically in RIPA buffer [50 mM Tris-HCl; 150 mM NaCl; 1% Nonidet P 40; 0.1% sodium dodecyl sulphate (SDS); 0.5% sodium deoxycholate] supplemented with a protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA). The homogenized mixture was centrifuged at 12 000 rpm for 20 min at 4°C and supernatant collected. Protein concentrations were determined using a BCA assay (ThermoFisher Scientific, Waltham, MA) and a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific).

Western Blot Analysis

Protein samples (10 μg) were boiled at 96°C for 4 minutes using an Accublock Digital Dry Bath (Labnet International, Edison, NJ, USA) and loaded on a 10% polyacrylamide gel and separated under reducing conditions using a Mini Trans-Blot Cell system (Bio-Rad) and Tris-Glycine-SDS running buffer [25 mM Tris, 192 mM Glycine, 0.1% SDS]
Precision Plus protein All Blue standards (Bio-Rad) were used as molecular weight markers. Following separation, proteins were transferred to Millipore Immobilon-FL polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA) using the Trans-Blot Cell wet blotting system (Bio-Rad) for immunoblotting. The protein transfer form gel to membrane was in transfer buffer (20% methanol and 0.037% SDS in Tris-Glycine [25 mM Tris, 192 mM Glycine (pH 8.3)]) (Bio-Rad) at 82 V for 1 hr at RT. All samples were run in triplicate, and balanced across groups and individual gels.

Next, membranes were incubated in a 2:3 solution of Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) and Tris-Buffered Saline (TBS; 50 mM Tris and 150 mM NaCl (pH 8.0)) for 1 hr on a shaker tray at RT. Membranes were then individually incubated for 16 hr on a shaker at 4°C with mouse polyclonal anti-TH (1:200K; AB152; Millipore, Temecula, CA) and mouse anti-GAPDH (1:20K, MAB374; Millipore, Temecula, CA, USA) and for 1 hr in IR Dye 800 CW-conjugated goat anti-mouse (1:10K; L-COR Biosciences). All antibodies were diluted in a 2:3 mix of Odyssey Blocking Buffer with TBS-T (TBS + 0.05% Tween-20 (pH 8.0))

Quantification and statistical analysis

Bands of fluorescent immunoreactivity were visualized and images captured using an Odyssey 2.1 scanner (LI-COR Biosciences). For each protein sample, fluorescence intensity levels for each band (supplemental figure 4B: TH, 62 kDa; GAPDH 35-37 kDa) were determined using the Odyssey software. Ratios of TH/GAPDH were determined for each protein sample and averages calculated for each animal. Ratios for each animal were
expressed as a fold change over the mean of the naïve control group to calculate group mean. Sexually experienced and sexually naïve groups were compared using unpaired t-tests with a significance level of 0.05.

**Results:** Sexual experience did not alter TH protein expression in the VTA (supplemental figure 4A; p = 0.37). Hence, the reduction in VTA DA soma size caused by sexual experience is not correlated with loss of TH protein expression or immunoreactivity.

**Supplemental Figure 4.** A, VTA TH/GAPDH expressed as fold change over the mean of sexually naïve control group. B, Representative bands for TH and GAPDH in sexually experienced and naïve males. Data represent mean ± SEM.

**Supplemental Experiment 5**

**Methods:** To determine whether the reduced VTA DA soma size caused by sexual experience altered basal DA levels in the NAc, High Performance Liquid Chromatography (HPLC) and electrochemical detection were used to measure NAc DA levels in sexually experienced (n = 7) and naïve (n = 7) males.
**Tissue Collection and Protein Isolation**

Animals were deeply anesthetized using sodium pentobarbital (270mg/kg; i.p.) 7 days after final mating session. Following decapitation, each brain was rapidly removed and immediately placed into ice-cold saline. Bilateral NAc was dissected using a rat brain matrix (ASI Instruments, Warren, MI, USA) and scalpel blade according to NAc boundaries defined by Paxinos & Watson (1998). NAc samples were immediately frozen on dry ice and stored in -80°C until processing. NAc samples were thawed, weighed and homogenized in a Tissuemiser (ThermoFisher Scientific) on ice in 400 µL of internal standard solution [600pg/µL dihydroxybenzylamine (Sigma-Aldrich, St. Louis, MO, USA), 1.6 mM ethylenediaminetetraacetate, 8mM sodium metabisulfate and 0.05M perchloric acid]. The homogenate was centrifuged at 14 000 rpm for 5 min at 4°C. The supernatant was collected and filtered through a 0.22 µm syringe before analysis.

**High Performance Liquid Chromatography**

NAc tissues were assayed using HPLC with electrochemical detection for DA. Processed tissue samples were separated on an ESA HPLC column (HR-80 33.2, 3 mm particle size, 80 mm length; ESA Biosciences, Chelmsford, MA, USA) with a mobile phase consisting of 75 mM NaH₂PO₄, 0.2 mM EDTA, 2.0 mM OSA (1-octanesulfonic acid sodium salt monohydrate, Fluka Cat#74882), and 15% methanol (pH 3.0). Flow rate through the column was set to 0.7 mL/min. DA was quantified using a coulometric detector (Coulochem III, ESA Biosciences) equipped with a high-sensitivity analytical cell containing dual coulometric working electrodes (ESA model #5014B). The detector settings were as follows: detector 1 - 75 mV and detector 2 +100 mV. Output from
detector 2 was used for DA quantification. Chromatograms were integrated using an internal standard calibration on a HP 3396 integrator.

Quantification and statistical analysis

The amount of DA and 3,4-Dihydroxyphenylacetic acid (DOPAC; DA metabolite) were expressed ng/mg. Amount for each animal was normalized to sexually naïve controls and thus expressed as a fold change over the mean of naïve controls. Data were analyzed between sexually experienced and sexually naïve controls using unpaired t-tests with a significance level of 0.05.

Results: Reduced VTA DA soma size was detected up to 7 days after final mating session. To investigate the effect of this reduced soma size on the function of these DA neurons, DA levels in the NAc were measured. Levels of DA were significantly decreased (p = 0.049), whereas levels of DOPAC trended towards a decrease (p = 0.09) in sexually experienced animals compared to naïve controls (supplemental figure 5). Therefore, reduced VTA DA soma size is associated with decreased DA in the NAc.
Supplemental Figure 5. Levels of DA and DOPAC of sexually experienced animals expressed as fold change over the mean of sexually naïve control group. Data represent mean ± SEM. * indicates significant difference compared to sexually naïve controls.

Supplemental Experiment 6

Methods: To determine whether it was NLX treatment when paired with sexual experience and not repeated to NLX alone that causes impairment of sexual behavior 7 days after last treatment, sexually naïve animals received either NLX (10 mg/kg; s.c.) or saline injections on 5 consecutive days prior to a mating test 7 days after final injection. On this final test day, animals did not receive any injection. Sexual behavior was observed and recorded. Parameters of mating were compared between groups using unpaired t-tests. For all statistical tests a probability value less than 5% was considered to be statistically significant.

Results: No significant differences were detected for any mating parameter between saline and NLX pre-treated groups (supplemental figure 6: A, ML, p = 0.753; B, IL p =
These results indicate that NLX alone is not sufficient to alter subsequent sexual behavior. Rather, NLX paired with sexual behavior is required for the loss of experience-induced facilitation of sex behavior.

Supplemental Figure 6. Latencies to mount (A), intromission (B) and ejaculation (C) for animals that received pretreatment with either NLX or Saline for 5 consecutive days, 7 days before mating. Data represent mean ± SEM.

Supplemental Experiment 7

Methods: In experiment 4, it was demonstrated that NLX action during sexual behavior blocked the sex experience-induced facilitation of sexual behavior when tested 7 days after last mating session on Test day. On Test day, animals did not receive an injection. In order to show that altered sexual behavior on Test day was not due to the absence of NLX, we administered either NLX or saline on the Test day to animals that received NLX with mating while they gained sexual experience (supplemental figure 7A: experimental design). Specifically, all animals received NLX injection (10 mg/kg, s.c.) 30 minutes prior mating to one ejaculation during 5 consecutive days as described in experiment 1. On test day (7 days later), animals received an injection of NLX (10
mg/kg, n = 7) or saline (n = 6) 30 minutes before introduction of a receptive female. Sexual behavior was observed and recorded.

Parameters of mating were compared to determine whether NLX affected either sex experience-induced facilitation of mating (day 1 vs day 5) or maintenance of this facilitation (day 5 vs test) using a two-way ANOVA (factors: treatment (saline versus NLX) and day (day 1, day 5 or test)) and Holm-Sidak method for post hoc comparisons. For all statistical tests a probability value less than 5% was considered to be statistically significant.

Results: There was a significant main effect of mating day on latencies to mount ($F_{(2,27)} = 30.031; p = 0.038$) and intromission ($F_{(2,27)} = 10.686; p = 0.048$). Similar to experiment 4, NLX treatment during mating did not affect the facilitation of sexual behavior during the 5 sexual experience sessions. Both NLX and saline groups (both received NLX during mating) demonstrated facilitated sexual behavior on day 5 compared to day 1 showed significantly shorter latencies to first mount (supplemental figure 7B: Sal, $p = 0.033$; NLX, $p = 0.014$) and intromission (supplemental figure 7C: Sal, $p = 0.034$; NLX, $p = 0.026$). Both groups had longer latencies to mount (supplemental figure 7B: Saline, $p = 0.018$; NLX, $p = 0.029$) and intromission (supplemental figure 7C: Saline, $p = 0.019$; NLX, $p = 0.020$) on test day compared to the fifth day of sex experience. Therefore, the administration of NLX or saline on the test day immediately prior to mating did not influence the effect of NLX treatment during sexual experience sessions and attenuation
of long-term facilitation of sexual behavior was identical to that shown in animals that did not receive any injection on Test day (experiment 4).

**Supplemental Figure** 7. A, Experimental design for supplemental experiments 7. Latencies to mount (B) and intromission (C) on day 1 and day 5 for two groups that both received systemic NLX during each of five consecutive days of mating. In addition, data are shown for the final test day, 7 days following day 5, when animals were administered either Saline (dark grey) or NLX (black). Data represent mean ± SEM. * indicates significant difference between day 1 and day 5 within treatment. # indicates significant difference between Test day and day 5 within treatment.
Supplemental Experiment 8

Methods: We hypothesize that NLX treatment during the acquisition of sexual experience disrupts the long-term expression of sex experience-induced facilitation of sexual behavior. To test this further, the effects of NLX treatment during mating was tested on subsequent sexual behavior during a final Test day, which was conducted only 1 day after last mating (supplemental figure 8A: experimental design). The design was similar to that described in experiment 4 and supplemental experiment 7.

Results: There was a significant main effect of mating day on mount (F(2,20) = 19.780; p < 0.001) and intromission latencies (F(2,20) = 19.041; p < 0.01). Similar to experiment 4 and supplement experiment 7, NLX treated males showed facilitation of sexual behavior during the 5 sexual experience sessions indicated by significantly shorter latencies to mount (supplemental figure 8B: Saline, p = 0.002; NLX, p = 0.018) and intromission (supplemental figure 8C: Saline, p = 0.006; NLX, p = 0.009) on day 5 compared to day 1. More importantly, NLX treatment during mating did not significantly affect the sex experience-induced facilitation of sex behavior when tested 1 day after sex experienced in saline (n = 5) or NLX (n = 4) injected males.
Supplemental Figure 8. A, Experimental design for supplemental experiment 7.

Latencies to mount (B) and intromission (C) on day 1 and day 5 for two groups that both received systemic NLX during each of five consecutive days of mating. In addition, data are shown for the final test day, one day following day 5, when animals were administered either Saline (dark grey) or NLX (black). Data represent mean ± SEM. * indicates significant difference between day 1 and day 5 within treatment.

Supplemental Experiment 9

Methods: One possibility for NLX’s attenuating effects on the display of maintenance of facilitated sexual behavior is that NLX blocks the rewarding effects of sexual behavior. To test this possibility, CPP was conducted for sexual behavior immediately following NLX or saline injection. The utilized CPP paradigm consisting of a pre-test, conditioning
days and post-test, and apparatus was identical to that outlined in experiment 5 and based on Tenk et al. (2009). Sex behavior was paired with the initially non-preferred chamber. In a counterbalanced manner, each animal received an injection of NLX (n = 12) or saline (n = 11) 30 minutes prior to receiving access to a receptive female. The average duration of mating session was approximately 13 minutes. Approximately, 1 minute following ejaculation, the animal was placed in the paired chamber. On the other conditioning day, animals received an injection of either NLX or saline (whichever they received before mating) and were placed into the unpaired chamber 30 minutes later. In order to determine chamber preference 3 measures were used: (i) Preference score, defined as percent of total time spent in the pair chamber (P) divided by the sum of time spent in the pair and unpaired (U) chambers (P/(P+U)); (ii) Difference score, defined as the time spent in the paired chamber minus time spent in the unpaired chamber (P-U); and (iii) TIP score, defined as the time spent in the paired chamber. For statistical analysis, paired t-tests were used to compare preference, difference scores, and TIP scores during the pre-test and post-test to determine whether CPP was formed for sexual behavior. A p-value of less than 0.05 was considered significant.

**Results:** NLX administered immediately before mating did not alter formation of CPP for mating, hence did not alter sexual reward. Both saline and NLX treated groups formed a significant CPP for sexual behavior as indicated by significantly higher preference (supplemental figure 9A: Saline, p = 0.016; NLX, p < 0.001), difference (supplemental figure 9B: Saline, p = 0.016; NLX, p < 0.001), and TIP (supplemental figure 9C: Saline, p = 0.038; NLX, p = 0.002) scores during the post-test compared to the
pre-test. Therefore, NLX does not exert its detrimental effect on maintenance of facilitated sexual behavior by blocking the reward associated with sexual behavior.

**Supplemental Figure 9.** NLX does not alter sexual reward. Preference (A), Difference (B), and TIP (C) scores during the pre-test (white) and post-test (black) for animals receiving either NLX or Saline prior to mating. Data represent mean ± SEM. * indicates significant difference compared to pre-test.

**Supplemental Experiment 10**

**Methods:** Methods described in section 5.3.6.

**Results:** Similar to saline control males, the missed injection NLX group displayed facilitated sexual behavior with sexual experience (supplemental figure 10). Moreover, a significant difference in latencies to mount and intromission on the test day between NLX and missed injection animals was detected using a Mann-Whitney U in terms of mount and intromission latencies on the test day (ML, IL: p = 0.024; NLX data in Figure 5.2.). However, only one of the three animals mated on day 1 hindering statistical analysis between groups on day 1 and within the group between day 1 and day 5.
Supplementary Figure 10. Sexual behavior for animals that had cannulas directed adjacent to the VTA (miss, Figure 5.2H-J). Latencies to mount (A), intromission (B), and ejaculation (C) on any mating day (Day 1, n = 1; Day 5, n = 3; Test, n = 2) were not different from saline controls (Experiment 4). Number of animals varied due to not all animals mating each day. Data represent mean ± SEM.
Appendix D
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 E
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 F

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CURRICULUM VITA

Name: Kyle K. Pitchers

Educational Background:

PhD in Neuroscience
University of Western Ontario, London, ON, Canada
September, 2007 – present

Bachelor of Science: Honors Specialization in Life Sciences
Queen’s University Kingston, ON, Canada
September 2003 – May 2007

Teaching Experience:

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The University of Western Ontario, London, ON, Canada
September 2009 – December 2010
Lab Coordinator, Systemic Human Anatomy 3309

Scholarships:

2009-2012: Alexander Graham Bell Canada Graduate Scholarship (NSERC, CGS-D3)
2009-2010: Ontario Graduate Scholarship (declined)
2008-2009: NSERC PGS-M
2008-2009: Ontario Graduate Scholarship (declined)
2007-2012: Western Graduate Research Scholarship
2007: NSERC Undergraduate Student Research Award
2004 & 2004: Principal’s Scholarship

Honours and Awards:

2012: Young Investigator Award for Society for Behavioral Neuroendocrinology
2010: Drs. Madge and Charles Macklin Fellowship for Teaching and Research
2009: Travel Award for Society for Behavioral Neuroendocrinology
2008 – 2011: University Students’ Council Teaching Honour Roll Member
2008: Graduate Student Teaching Award
2007: Ben Kropp Prize for Highest Standing in Anatomy

Publications:


Seminar Presentations:

Sex, Drugs and Neuroplasticity. Presentation was demonstrated for the Anatomy and Cell Biology Departmental Seminar Series, 2010.

Sex, Drugs and Neuroplasticity. Presentation was demonstrated at the Anatomy and Cell Biology Departmental Research Day, 2009.

Abstracts:

Kyle K. Pitchers and Lique M. Coolen (2012). D1 dopamine receptors in the nucleus accumbens are critical for enhanced amphetamine reward and nucleus accumbens deltafosb accumulation caused by sexual experience in male rats. Society for Behavioral Neuroendocrinology Abstracts, Madison, WI, USA [International Conference].

Kyle K. Pitchers and Lique M. Coolen (2012). D1 dopamine receptors in the nucleus accumbens are critical for enhanced amphetamine reward and nucleus accumbens deltafosb accumulation caused by sexual experience in male rats. Winter Conference on Brain Research, Snowbird, UT, USA [International Conference].


Study of Sex Differences Abstracts, Ann Arbor, MI, USA [International Conference].

Kyle K. Pitchers, Caroline M. Coppens, Jonathon Fuller, Sandy Van, and Lique M. Coolen (2009). Opioid-induced neuroplasticity of dopaminergic neurons in the ventral tegmental area mediates reinforcement of natural reward. Society for Neuroscience Abstracts, Chicago, IL, USA [International Conference].


Services:

2008-2010: Student Representative for Graduate Affairs Committee (elected), Anatomy & Cell Biology, University of Western Ontario.

2008-2010: Student Chair (elected), Anatomy & Cell Biology, University of Western Ontario.

2008-2010: Member of Advisory Committee for Associate Dean of Graduate Studies (appointed), University of Western Ontario.