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Examination of Methamphetamine Reinstatement in Female and Male Rats: A Pre-Clinical Model of Relapse

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EXAMINATION OF METHAMPHETAMINE REINSTATEMENT IN FEMALE AND MALE RATS: A PRE-CLINICAL MODEL OF RELAPSE

by

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EXAMINATION OF METHAMPHETAMINE REINSTATEMENT IN FEMALE AND MALE RATS: A PRE-CLINICAL MODEL OF RELAPSE

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Methamphetamine (meth) dependence is often characterized by persistent and chronic relapse (i.e., return to drug use). There is growing pre-clinical and human evidence suggesting females are at greater risk to relapse. The set of studies presented in this dissertation extended this limited evidence by identifying sex-dependent neural substrates correlated with meth-triggered reinstatement (Experiment 1) and by examining sex-differences in reinstatement triggered by drugs of abuse that are commonly co-abused with meth (Experiment 2). Female and male rats were trained to self-administer meth, received subsequent extinction sessions, and then tested for reinstatement. In Experiment 1, rats were perfused following reinstatement testing and c-Fos activity was examined as a measure of neural activation. Meth triggered reinstatement in both sexes and this effect was more robust in females compared to males. In the females, c-Fos activity was significantly increased following meth-primed reinstatement in the cingulate cortex area 1, lateral orbitofrontal cortex, prelimbic cortex, caudate-putamen, nucleus accumbens core and shell, and central nucleus of the amygdala. In males, there were no significant differences following meth-primed reinstatement. In Experiment 2, nicotine and cocaine were utilized as drug primes to determine if administration of these drugs could

trigger meth-seeking behavior. Nicotine and cocaine reinstated meth-seeking behavior in male and female rats with no difference between the sexes. Females were more sensitive to reinstatement triggered with the original self-administration drug and this effect may not generalize to priming with other drugs of abuse.

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CHAPTER 1

GENERAL INTRODUCTION

Methamphetamine Use and Dependence

Methamphetamine (meth) use and dependence is a serious public health concern. The consequences of meth abuse on an individual are quite grave. Long-term abuse can result in severe dental problems, malnutrition, damage to the cardiovascular system, memory loss, psychotic behavior (including paranoia, visual and auditory hallucinations, and delusions), anxiety, confusion, insomnia, mood disturbances, and violent behavior (National Institute on Drug Abuse, 2013). These health problems can last for months and years following cessation of meth use (Volkow et al., 2001). Despite the well-documented dangers of meth, over 12 million people (4.7% of the population) report using meth at least once (Substance Abuse and Mental Health Services, 2013) and 1.2 million people report using in the past year (Substance Abuse and Mental Health Services, 2013). Hospital emergency departments reported over 102,000 cases in which patients were admitted for meth-related issues, representing 8.2% of all emergency room visits for illicit drugs (Center for Behavioral Health Statistics and Quality, 2013).

In addition to the high cost of meth to the health of the user, the economic burden of meth to society is also substantial. The RAND Corporation estimates the cost to the United States could be as high as $48.3 billion (Nicosia et al., 2009). The cost of meth to society includes premature death, drug treatment, lost worker productivity, crime and criminal justice, health care, production and

environmental hazards, and child endangerment. The dire health consequences, in conjunction with the high economic strain to society, make the search for effective meth cessation strategies a priority (Brackins et al., 2011).

Imagine for a minute a meth user. A 26 year old, single, mother of 2 children; ages 3 and 5. Following a routine traffic stop, several grams of meth are confiscated and our user is arrested. With no prior convictions and a minimal amount of meth seized, she is eligible for drug court. She receives inpatient drug treatment for 90 days. During that time her children are removed from their home and remain wards of the state until her graduation from adult criminal drug court. Our exemplar makes excellent progress in the program. During her abstinence, she recovers her health that has deteriorated from years of meth use, vows to stay clean, and eventually regains primary custody of her two children. Three months later, however, she fails a court-mandated random drug test and is assigned to the county jail, forfeiting her freedom, as well as the custody of her young children. A return to drug use with so much to lose is quite disheartening, and unfortunately, all too common. The majority of addicts return to use within 6 months of treatment (Brackins et al., 2011; Brecht et al., 2004), highlighting the inadequacy of behavioral and pharmacological treatments, as well as demonstrating the impediment that relapse serves to treating meth use disorder.

Reinstatement: A Pre-clinical Model of Relapse

Relapse, or a return to drug use following a prolonged period of drug abstinence, can occur months and even years after drug cessation (Baicy and London 2007; Bamford et al., 2008). A significant body of research has been

dedicated to elucidating factors that may play a role in the re-initiation of drug use after "getting clean". An intense urge or desire to use a drug is often referred to as drug craving. Human studies often utilize subjective reports and physiological responses (e.g., heart rate, skin conductance) to investigate drug craving and the factors that may influence a return to drug use (Breiter et al., 1997; Carter and Tiffany, 1999; de Wit, 1996; Katz and Higgins, 2003; Rosenberg, 2009; Self, 1998; Self and Nestler, 1998; Walsh et al., 2000; Wexler et al., 2001). Clinical studies have shown that cravings can be precipitated by drug-associated cues, by stress, and, the focus of this dissertation, by a priming injection of the drug itself (Blum et al., 2009; Carter and Tiffany, 1999; Chornock et al., 1992; Kaplan et al., 1985; Katz and Higgins, 2003; Preston et al., 1992; Self, 1998; Self and Nestler, 1998; Stockwell et al., 1982; Walsh et al., 2000; Jaffe et al., 1989). In a seminal study, Ludwig et al. (1974) demonstrated this drug-primed effect with alcohol. During abstinence, alcoholics were given ethanol and then craving was measured by subjective rating, as well as the number of times participants pressed a button to obtain more ethanol. Treatment with ethanol increased craving ratings and the number of button presses for ethanol (Ludwig et al., 1974). In the ensuing decades, this priming effect has been replicated in alcohol and extended to other drugs of abuse (Hodgson et al., 1979; Meyer and Mirin, 1979; Jaffe et al., 1989; Stockwell et al., 1982; Preston et al., 1992; Chornock et al., 1992).

In preclinical models, reinstatement is used as a model of relapse. There are several phases to the reinstatement model; the first of which is self-

administration. In intravenous self-administration, an indwelling jugular catheter is surgically implanted into a rat. An access port on the catheter is then connected to a drug-syringe pump. Rats then receive drug infusions contingent on manipulation of an active operandum that activates the drug pump. An inactive operandum is often included to compare response rates. Self-administration is typically defined as more responding on the active compared to the inactive manipulandum. Self-administration is the archetypal paradigm to assess the primary reinforcing effects of a drug. The list of drugs that maintain self-administration responding is extensive, including cocaine (Ciccocioppo et al., 2000; Mello et al., 2014; Gould et al., 2011; Guillem and Peoples, 2010; Montanair et al., 2015, Neisewander et al., 2000; Thomas et al., 2001), heroin (Bossert et al., Montanair et al., 2015; Sedki et al., 2015), alcohol (oral rather than IV self-administration; Ginsburg and Lamb, 2013; Funk et al., 2015; Scuppa et al., 2015; Steensland et al., 2007; Wouda et al., 2011), and pertinent to the studies presented here, methamphetamine (Beardsley et al., 2010; Cornish et al., 2012; Cox et al., 2013; Holtz et al., 2012; Hofford et al., 2014; Reichel et al., 2012; Roth and Carroll, 2004; Rubio et al., 2015; Shepard et al., 2004; Sobieraj et al., 2016). Our lab has refined the meth self-administration procedures and has multiple publications on the subject (Charntikov et al., 2015; Pittenger et al., 2016; Reichel et al., 2008; Reichel et al., 2009).

Following the self-administration component, the second phase of a standard reinstatement paradigm is extinction. Extinction sessions are similar to those of self-administration except the drug that previously was received

following requisite operant responding is no longer available. Removal of the drug reinforcer results in significantly attenuated responding on the previously active manipulandum. Research has revealed that extinction represents new learning, rather than forgetting or destruction of the original learning (Bouton and King, 1983; Pavlov, 1927; Rescorla, 2004). In the reinstatement paradigm, extinction training often lasts long enough for responding on the active lever to diminish to consistently less than 50% of self-administration responding. Previous work in our lab shows this typically occurs between 12 and 15 sessions (Charntikov et al., 2015; Pittenger et al., 2016).

Following extinction, reinstatement testing is the final phase of the reinstatement paradigm. Animals receive the reinstatement trigger and then responding is typically measured in the drug-taking context in the absence of available drug. This approach allows for measurement of drug-seeking behavior rather than drug-taking behavior. Notably, factors that precipitate relapse in human subjects also trigger reinstatement responding in pre-clinical models (Epstein et al., 2006; Katz and Higgins, 2003; Kufahl and Olive, 2011; Shaham et al., 2003). These factors include drug-associated cues, stress, and priming injections of the drug. A study by Hofford et al., (2014) provides an apt example of meth reinstatement with drug-associated cues. Following surgery to implant an indwelling jugular catheter and subsequent recovery, rats were trained to self-administer meth during daily sessions. Requisite lever pressing on the active lever was followed by a 0.1 mg/kg meth infusion and illumination of cue lights for 20-sec. Extinction followed self-administration. Extinction sessions were similar

to self-administration, however responding on the active lever no longer resulted in drug infusion or cue-light illumination. On the day after the last extinction session, cue-induced reinstatement was tested by reintroducing the cue light (contingent on requisite responding), while meth was still not available. This experiment did not have a no-cue control group, so lever pressing in the cue-induced reinstatement session was compared to the last extinction session. Responding was significantly increased in the reinstatement session compared to the last extinction session without the cues available (Hofford et al., 2014). This increase in responding is interpreted as cue-induced reinstatement of meth-seeking.

Stress-induced reinstatement of meth-seeking can be achieved by physiological or pharmacological stressor. A study by Beardsley et al. (2010) trained rats to self-administer meth, extinguished the responding and then utilized stress-induced reinstatement with a physiological stressor. During the 15 min immediately prior to a reinstatement session (meth unavailable), rats received intermittent foot-shock administered at 0.63 mA for 0.5 sec with an average inter-shock interval of 40 s. Active lever pressing during the subsequent reinstatement session was then compared to the last extinction session. Following the foot-shock, rats significantly increased meth-seeking behavior, successfully demonstrating stress-induced reinstatement with a physiological stressor (Beardsley et al., 2010).

Stress-induced reinstatement with a pharmacological stressor was nicely demonstrated by Shepard et al. (2004). Rats were again trained in a self-

administration procedure, followed by extinction training. Prior to reinstatement tests (meth unavailable), rats were given either saline, 1.25, or 2.5 mg/kg of yohimbine in a within-subjects design. Yohimbine is a α-2 adrenoceptor antagonist that functions as an anxiogenic in humans (Bremner et al., 1996a; Charney et al., 1983; Holmberg and Gershon, 1961) and non-human subjects (Davis et al., 1979; Bremner et al., 1996b). Active lever responding following yohimbine injection was compared to levels following saline administration. Both the 1.25 and 2.5 mg/kg doses successfully increased lever presses, demonstrating stress-induced reinstatement with a pharmacological stressor (Shepard et al., 2004).

Consistent with a drug prime precipitating drug relapse in humans, priming injections result in robust reinstatement in pre-clinical models. Our lab has an extensive record utilizing this particular reinstatement model. For example, Pittenger et al. (2016) clearly demonstrates meth primed reinstatement of meth-seeking behavior. In that study, rats were implanted with indwelling jugular catheters and trained to self-administer 0.05 mg/kg meth or saline with timeout cues. Rats in the meth group responded significantly more than rats in the saline group. Rats then received extinction without meth or saline infusions available and the timeout cues intact. Rats in the meth group attenuated active lever pressing to levels that were equivalent to the saline group. After the last extinction session, all rats received a meth (0.3 mg/kg SC) injection 15 min prior to the start of a reinstatement session with timeout cues but without meth or saline infusions available. These procedures allowed for meth-induced

reinstatement of meth-seeking behavior to be determined based on the comparison between active lever responding in the meth vs saline group; recall responding was equivalent at the end of extinction. A meth-trigger produced robust reinstatement of meth-seeking. The studies presented herein utilize this drug-trigger reinstatement paradigm.

Sex Differences in Methamphetamine Dependence

Numerous differences between males and females in meth addiction have been documented. These discrepancies range from differences in human patterns of meth use to differences in meth intake in pre-clinical models. In humans, differences between males and females arise as early as the reported motivation for the initiation of meth use. Brecht et al. (2004) interviewed 350 individuals who were treated for meth use disorder in a treatment center regarding their meth behavior. The motivation for initial meth use varied between the sexes; women start using to control weight and increase energy, whereas more men report being motivated by the desire to work more hours (Brecht et al., 2004). Brecht et al. (2004), as well as several other studies (Dluzen and Liu, 2008; Hser et al., 2005; Lin et al., 2004; Westermeyer and Boedicker, 2000; Wu et al., 2007), also found that women initiate use at a younger age than men. Once meth use is initiated, women also tend to transition to regular use more quickly than men (1.6 years for females vs 2.56 years for males; Brecht et al., 2004; Rawson et al., 2005).

While women appear to be more vulnerable to meth use, they are also more likely to identify and acknowledge their own meth dependence and seek

treatment (Dluzen & Liu, 2008; Kim & Fendrich, 2002). Additionally, women may be more responsive to meth treatment (Hser et al., 2005). Hser and colleagues (2005) used the Addiction Severity Index (ASI; McLellan et al., 1992) to assess problem severity in alcohol use, drug use, employment, family and social relationships, legal, psychological and medical status among 1073 meth-abusing patients from 32 treatment centers. Data was collected before and 9 months after treatment admission. Hser and colleagues (2005) found that treatment successfully reduced severity in all 7 problem areas in both sexes. Women had greater changes pre- to post-treatment in ASI scores on family/social relationships and medical status (sexes comparable on the other measures). Notably, these greater reductions reflected higher pre-treatment scores in the females and not lower post-treatment scores. Following treatment, women still had higher severity scores in drug use, employment, psychological status, and medical status than their male counterparts, the sexes were comparable on the other 3 measures. Even with treatment having a greater effect in females, the enduring elevated severity scores may mean that the women were still at greater risk for relapse (Hser et al., 2005).

Despite this converging evidence that women may be more susceptible to meth dependence, animal models rarely use female subjects. This leaves a critical need for empirical research on meth-taking/seeking in preclinical models employing females. The limited animal research that has investigated sex differences in meth drug models is consistent with work in humans. That is, research shows females may be more vulnerable to meth-abuse disorder. The

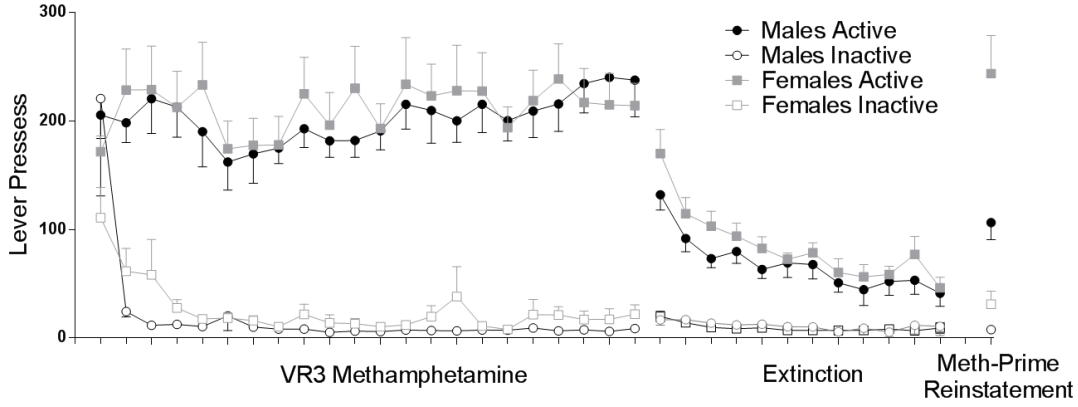
meth locomotor activity literature is concordant with this notion. Locomotor activity following drug administration has long been employed to measure both the activating effects, as well as sensitization to drugs of abuse. While meth administration, both acute and chronic, increases locomotor activity in both sexes, female rats are more sensitive to this activating effect of meth (Mattei and Carlini, 1996; Milesi-Halle et al., 2007; Schindler et al., 2002). Schindler et al. (2002) provided support for this notion in 2 separate experiments. In the first experiment, rats received 10 habituation days during which rats were administered saline prior to placement in a locomotor chamber (a 16 x 16 infrared photocell array measured activity). A range of meth doses (saline, 0.1, 0.3, 1.0, 3.0 mg/kg) was then tested. Rats received administration of assigned dose prior to placement into the locomotor chamber. Rats did not differ in locomotor activity following the saline injection, but did differ following administration of the higher doses of meth (0.3, 1.0, and 3.0 mg/kg) with females showing more meth activation than the males. In the second study, rats were repeatedly administered 0.3 mg/kg meth on 4 test days prior to placement in the locomotor chamber. Each test day was separated by 2 intervening days. Consistent with the finding with acute meth, females continued to show more locomotion following repeated administration (Schindler et al., 2002).

Meth sex differences are also found when a self-administration model is utilized. Roth and Carroll (2004) found that a greater percentage of female rats self-administered a low dose of meth (0.02 mg/kg/inf) during 6 h self-administration sessions (fixed-ratio 1 schedule: FR1) and females acquired meth

self-administration quicker than their male counterparts. They also found that when rats were switched to a progressive ratio (PR) schedule, female rats responded more across a range of meth doses (0.01, 0.02, 0.04, 0.08 mg/kg/inf) during 6 h self-administration sessions (Roth and Carroll, 2004). These sex differences in meth self-administration are not universal and appear to be dependent on the parameters used in the study (i.e., meth dose, session duration, reinforcement schedule, etc.). For example, Reichel et al. (2012) found that sex differences were apparent in meth-intake (females more than males) and escalation of intake (females faster than males) when self-administration sessions were 6 h, but did not find differences between the sexes with 1-h self-administration sessions (Reichel et al., 2012).

Given these varied findings, we conducted a preliminary study to investigate possible sex differences using the self-administration procedures that are utilized in our lab. Male and female rats (n=24) were trained to self-administer meth (0.05 mg/kg/infusion) by pressing an active lever on a variable ratio (VR) 3 schedule in our lab's standard protocol (for details see General Methods).

Figure 1.



Results of preliminary study investigating sex differences using our procedures. Female (grey) and male (black) responding during methamphetamine self-administration, extinction, and meth-primed reinstatement is displayed.

Males and females displayed robust self-administration and discrimination between the two levers (Figure 1; VR3 Methamphetamine), and there was not a significant difference in meth intake between the sexes. This outcome was not surprising as our procedures use 2-h sessions, rather than a more extended access duration.

Following self-administration, we wished to further explore potential sex differences using our procedures by utilizing the meth-primed reinstatement model detailed earlier. To do so, rats entered an extinction phase, where meth was no longer available. Responding on the active lever decreased across sessions in both sexes (Figure 1; Extinction). Although there was a tendency for male responding to be lower in the early extinction sessions, there was not a statistically significant sex difference and the lower levels in males did not persist later in extinction. Differences in resistance to extinction following self-

administration have been noted in previous sex differences work (Cox et al., 2013). In a study by Cox et al. (2013), females, on average, required more extinction sessions to meet an extinction criteria of <25% baseline responding; baseline was calculated from the end of meth self-administration. This finding is not ubiquitous. Others have not reported a sex difference in extinction (Reichel et al., 2012; Holtz et al., 2012).

Following extinction, rats received meth-primed reinstatement testing. For reinstatement, rats were injected intraperitoneally (IP) with 0.3 mg/kg of meth and placed in the conditioning chamber. Female rats responded on the active lever (reinstated) significantly more than males (Figure 1; Meth-Primed Reinstatement). This finding is concordant with similar studies that also found greater reinstatement induced by a meth-priming injection in females compared to males (Cox et al., 2013; Holtz et al., 2012; Reichel et al., 2012). Replication of this finding using our procedures allowed for the detailed exploration of sex differences in meth-primed reinstatement that are presented in this dissertation. The reinstatement protocol for the set of dissertation experiments reported herein matched this preliminary work.

Dissertation Aims

Human and preclinical research indicates that females are more vulnerable to meth addiction and relapse (Brecht et al., 2004; Carroll and Anker, 2010; Cox et al., 2013; Dluzen and Liu, 2008; Holtz et al., 2012; Hser et al., 2005; Lin et al., 2004; Mattei and Carlini, 1996; Rawson et al., 2005; Reichel et al., 2012; Schindler et al., 2002; Westermeyer and Boedicker, 2000; Wu et al.,

2007). However, the neurobiological and behavioral factors that contribute to this increased vulnerability remains understudied. The goal of the present research was to begin to fill a paucity of preclinical studies investigating sex differences in meth-taking. To do so, we completed 2 separate studies. In Experiment 1, we used immunohistochemistry to identify sex-dependent neural substrates correlated with meth-triggered reinstatement; thereby identifying brain regions that may be targeted for functional analysis in future meth-triggered reinstatement studies. In Experiment 2, we sought to programmatically investigate if the sex difference found with meth-triggered reinstatement extends to meth reinstatement triggered by other drugs of abuse that have high comorbidity with meth abuse disorder (i.e., nicotine and cocaine; Brecht et al., 2004). Relapse, or a return to meth use following abstinence, is a hallmark of meth addiction. The increased vulnerability to reinstatement found in females is of particular interest and further investigation of the behavioral and neural factors that contribute to sex difference is warranted.

CHAPTER 2

GENERAL METHODS

Subjects

Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN, USA) at approximately 9 weeks of age (total n=165). Rats were housed individually in clear polycarbonate cages (35.5 × 32 × 18 cm; length × width × depth) with TEK-Fresh® cellulose bedding. The colony room was temperature- and humidity-controlled and maintained a 6:00 AM light/6:00 PM dark cycle. Rats were allowed to acclimate to the colony room for 3 days. At that time, 90% free-feeding weights were calculated and maintained for the duration of the experiment. Rats received *ad libitum* access to water in the home cages. All experimental procedures were conducted during light phase of the cycle. Protocols were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee.

Apparatus

Behavioral testing was conducted in conditioning chambers purchased from Med Associates (ENV-008CT; Georgia, VT, USA). Each chamber measured 30.5 × 24.1 × 21 cm and was enclosed in a sound-attenuating cubicle. A variable-speed syringe pump (PMH-100VS; Med-Associates) was located outside each cubicle. Tygon® tubing was threaded from the pump syringe, through a leash, into the chamber to be attached to the catheter port that exited below the scapula of the rat. A recessed receptacle (5.2×5.2×3.8 cm) was centered on 1 sidewall of

each chamber. A dipper arm, when raised, provided access to 0.1 ml of 26% (w/v) sucrose in this recessed receptacle. A retractable lever was located on each side of the receptacle. A white cue-light (2.54 cm diameter; 28V, 100-mA) was mounted 7 cm above each lever. A house-light (two white 28V, 100-mA lamps) was located in the cubicle, 10 cm above the Perspex chamber ceiling.

Drugs

(+)-Methamphetamine hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline. Meth was infused IV at a volume of 35.74 µl over 1 sec at 0.05 mg/kg/infusion for Experiment 1 and 0.05 mg/infusion for a 250 g rat in Experiment 2 (see Experiment 2 Self-administration Methods for details). For reinstatement, meth was injected IP at 1 ml/kg. Meth doses are reported in salt form. (–)-Nicotine hydrogen tartrate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Nicotine was dissolved in 0.9% sterile saline and adjusted to a pH of 7.0 ± 0.2 using a dilute NaOH solution. Nicotine doses are reported as the base form. Injections of nicotine were subcutaneous (SC) at 1 ml/kg. (–)-Cocaine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in 0.9% sterile saline, and injected IP at 1 ml/kg (salt form).

GENERAL PROCEDURES

Preliminary lever training

Following acclimation to the colony room and food restriction to maintain 90% of free-feeding weight, rats were trained to lever press, on both levers, in our

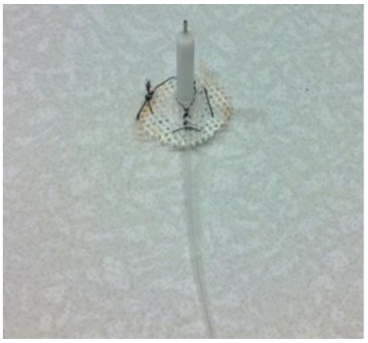
lab's standard procedure (Charntikov et al., 2015; Charntikov et al., 2013; Pittenger et al., 2016). The start of each session was signaled by illumination of the house light and insertion of a randomly selected lever (right or left). A lever press or a lapse of 15 sec resulted in 4-sec access to sucrose, retraction of the lever, and commencement of a timeout (average=60 sec; range=30 to 89 sec). Following the timeout, a randomly selected lever was again inserted with the condition that the same lever could not be presented more than twice in a row. This protocol was repeated for 60 sucrose deliveries. Daily sessions range from 65 to 80 min depending on individual performance. Training continued until a lever press was made on at least 80% of the lever insertions for two consecutive days. All rats met criterion between sessions 3 to 5. This autoshaping protocol ensured rats were pressing at relatively robust levels.

Catheter Surgery and Recovery

Indwelling jugular catheters were implanted using our standard protocol (previously described in Charntikov et al., 2015; Charntikov et al., 2013; Pittenger et al., 2016). Rats were anesthetized with a 2:1 ketamine HCl (100 mg/kg; MWI, Boise, ID) plus xylazine HCl (20 mg/kg; MWI, Boise, ID) cocktail (intramuscular; IM). The neck and back were then shaved and cleaned with isopropyl alcohol and betadine. Mineral oil was applied to the rat's eyes and their nails were clipped to prevent eye dryness and scratching of the wound, respectively. Three incisions were then made, two in the back and one in the right side of the neck. The catheter (pictured in Graphic 1) was then placed under the skin with the base

mount setting flat on the back, the cannula access port sticking through the skin, and the catheter tubing threaded under the skin to the neck incision.

Graphic 1.



Catheter implanted during indwelling jugular surgery

The right jugular vein was then isolated and partially cut in order to gently guide the end of the catheter tubing into the jugular vein. Once completed, the tubing was fixed inside the vein by suturing the vein, tubing, and surrounding tissue together. Rats were then thoroughly cleaned with hydrogen peroxide and the incisions were sutured closed. Following surgery, rats were administered buprenorphine (0.1 mg/kg, SC; MWI, Boise, ID) for pain management and atipamezole (0.5 mg/kg, IM; MWI, Boise, ID) to terminate anesthesia. Buprenorphine was again administered 24 h post-surgery. Rats were allowed to recover for 7 days. During recovery, they remained in their home cages and catheters were flushed daily with a cocktail of 0.2-ml baytril (5.0 mg/ml; MWI, Boise, ID) to treat and prevent infections and heparin (30 Units/ml; MWI, Boise, ID) to prevent catheter non-patency as a result of blood clotting and blocking fluid flow. Catheter patency was checked on the last day of recovery by IV infusion of 0.05-ml xylazine (20 mg/ml). Rats that displayed motor ataxia within

20 sec were considered patent (Charntikov et al., 2015; Charntikov et al., 2013; Reichel et al., 2008; Pittenger et al., 2016). Patency was again checked upon the completion of the self-administration phase; rats were excluded from the study if catheters were not patent.

Post-surgery Lever Training

Following recovery, rats were place on a variable ratio 3 (VR3) schedule of sucrose reinforcement. Under the VR3 schedule, on average every 3rd lever press (range 1 to 5) was followed by 4-sec access to sucrose. Levers were again inserted individually with the condition that the same lever was not inserted more than 2 times in a row. These procedures ensured robust responding with both levers having a similar reinforcement history (Charntikov et al., 2015; Charntikov et al., 2013; Pittenger et al., 2016). This training lasted for 3 daily 1-h sessions conducted on consecutive days. On session 3, all rats earned more than 71% (range of 43-55) of the 60 possible sucrose deliveries.

Self-administration

Rats then began the self-administration phase of the experiment. Drug (meth or saline) was available on a VR3 schedule of reinforcement. During training, two levers were present, active and inactive. Rats were randomly assigned which of the two levers served as the active (meth or saline infusion) vs inactive lever. Sessions were 120 min and conducted daily, 7 days per week. Before a rat was attached to the leash/tubing at the start of each session, the catheter was flushed with 0.2-ml heparin (30 Units/ml) in sterile saline. The session commenced with insertion of both levers and priming of the catheter with

meth or saline [ca. 31 µl (90% of internal catheter volume)]. Requisite VR3 responding on the active lever initiated an infusion of meth or saline, retraction of both levers, and illumination of the house light for a 20-sec timeout. Following the timeout, both levers were extended and the house light was terminated. Responding on the inactive lever was recorded but had no programed outcome. After each session, the catheter was flushed with a cocktail of 0.2 ml-baytril (5.0 mg/ml) and heparin (30 Units/ml) in sterile saline.

Extinction

Extinction sessions commenced 24 h after the last self-administration session. Extinction sessions were identical to self-administration sessions except drug was no longer infused. Requisite VR3 responding on the active lever still produced the same cues and the timeout. Responding on the inactive lever was recorded but held no programed consequence. Sessions were 120 min and conducted daily, 7 days per week.

Reinstatement

Twenty-four hours after the last extinction session, rats began reinstatement testing. Drug-primes were administered prior to the reinstatement sessions. Reinstatement sessions were identical to extinction sessions (i.e., drug not available). Requisite VR3 responding on the active lever still produced the same cues and the timeout. Responding on the inactive lever was recorded but held no programed consequence. See Experiment 1 Methods and Experiment 2 Methods for detailed accounts of reinstatement testing, as they differed between experiments.

CHAPTER 3

EXPERIMENT 1

IDENTIFICATION OF SEX-DEPENDENT NEURAL SUBSTRATES CORRELATED WITH METH-TRIGGERED REINSTATEMENT

Introduction

Neurobiology of Methamphetamine-Primed Reinstatement

Our preliminary study demonstrated behavioral sex differences in meth-primed reinstatement. Consistent with previous studies, females reinstate lever pressing significantly more than their male counterparts (Cox et al., 2013; Holtz et al., 2012; Reichel et al., 2012). While these behavioral differences have been reliably found, little is known regarding the neurobiology of these differences. The aim of Experiment 1 was to identify potential neural correlates that show differential activation between the sexes following meth-primed reinstatement. The goal of this study was to be the initial experiment in a programmatic line of study investigating the neurobiology of meth-primed reinstatement and associated sex differences. To this end, 20 brain regions were examined for differential expression of c-Fos following reinstatement testing. c-Fos is an early immediate gene that has long been used as a marker for neuronal activation (Curran and Morgan, 1985; Curran and Morgan, 1995; Kovacs, 1998; Greenberg and Ziff, 1984). c-Fos is critical in the formation of a transcription factor known as activator protein-1. c-Fos is commonly utilized as a marker for 2 major

reasons; there are low levels of c-Fos transcription under basal conditions and it is readily activated in response to various stimuli throughout the brain (Kovacs, 1998). The c-Fos immunohistochemical approach has the advantage that it allows for examination of a number of brain regions. While determining the functionality of identified regions using ablation, chemogenetic, and optogenetic techniques is a long-term goal of the programmatic line of research referenced above, the first step is identification of regions of interest.

The brain regions examined in this study were the cingulate cortex area 1 and 2 (Cg1; Cg2), prelimbic cortex (PrL), infralimbic cortex (IL), lateral orbital cortex (LO), dorsal medial caudate-putamen (dmCPu), dorsal lateral caudate-putamen (dlCPu), ventral medial caudate-putamen (vmCPu), ventral lateral caudate-putamen, (vlCPu), nucleus accumbens core (NAcC), nucleus accumbens shell (NAcSh), hippocampus proper (CA1; CA2; and CA3) and ventral subiculum (VS), amygdala [central (CEA); basolateral (BLA)], lateral hypothalamus (LH), ventral tegmental area (VTA), and substantia nigra (SNR). Given the importance of relapse in drug abuse disorder, a number of studies have examined the neurobiology of reinstatement. The 20 brain areas examined in this study were carefully selected based on prior research implicating these regions in the reinstatement process (detailed below), however, there are lacunae in our understanding on the specificity to meth and drug-primed reinstatement. Much of what we know has been inferred from general reinstatement circuitry, although several recent studies that begin to fill this gap are noted.

Given the importance of the prefrontal cortex in decision making and executive function, several regions of the cortex were examined. The cingulate cortex (Cg) was of interest as it has been implicated in both drug- and cue-induced reinstatement (Breiter et al., 1997; Childress et al., 1999; Ciccocioppo et al., 2001; Neisewander et al., 2000; Thomas and Everitt, 2001; Wexler et al., 2001). Following self-administration and extinction, Neisewander et al. (2000) examined c-Fos expression after cocaine- or cue-induced reinstatement in a pre-clinical model. Male rats were trained to self-administer cocaine or saline. Extinction training then occurred, followed by reinstatement testing (controls did not receive cues or a cocaine injection) and brain extraction. Reinstatement behavior (both cue- and cocaine-induced) was detected in the rats that had received self-administration with cocaine. In the Cg, c-Fos expression was higher in the groups that received the reinstatement triggers compared to controls (Neisewander et al., 2000). These results have been replicated in cocaine reinstatement (Kufahl et al., 2009a; 2009b) and extended to meth-primed reinstatement (Recinto et al., 2012). However, females were not examined in these studies. When females were examined in a cue-induced cocaine seeking paradigm, the Gg did indeed show increases in c-Fos expression (Zhou et al., 2014). This increase in activation in females and possible sex differences has yet to be determined in a meth-primed model.

The medial prefrontal areas, including the PrL and IL, are abundantly interconnected with other regions that mediate the reinforcing effects of psychostimulants including the VTA, striatum, and amygdala (Aoki and Pickel,

1989; Murase et al., 1993; Sesack and Pickel, 1992; Taber and Fibiger, 1995). These areas have been implicated in aspects of addictive behavior in work by Weissenborn et al. (1997) that utilized ablation to show these medial prefrontal cortical regions mediate the ability of drug-associated cues to induce cocaine-seeking behavior. This outcome has been supported by additional work showing the medial prefrontal cortex is critical to the expression of reinstatement behavior induced by cues and drug prime (Fuchs et al., 2005; McFarland and Kavivas, 2001; McLaughlin and See, 2003). Parsegian and See (2014) demonstrated that during cue- and meth-primed reinstatement the dorsal medial prefrontal cortex (defined by their methods as containing regions of the Cg1 and PrL) had increased efflux of dopamine and glutamate, implicating transmission of these neurotransmitters in these regions in the meth reinstatement process.

Additionally, Kufahl et al. (2009) showed that the PrL and IL had increased c-Fos activation following reinstatement of cocaine seeking behavior by cues previously paired with drug. This effect was replicated in a meth reinstatement procedure (Recinto et al., 2012). Recinto et al. (2012) found increased c-Fos activation following meth-primed reinstatement in the medial prefrontal cortex, however no distinction was made in this latter study between the CG, PrL, and IL and this effect was only examine in male rats. The lack of differentiation between regions is notable, as Rocha and Kalivas (2010) found differential involvement of the PrL and IL in meth reinstatement induced by cues or meth-prime. That is, inactivation of the PrL eliminated both cue- and meth-induced reinstatement behavior, but inactivation of the IL only inhibited cue-

induced reinstatement; suggesting dissociable control of behavior in the regions (Rocha and Kalivas, 2010). Further, some work actually shows the IL is a primary site of the inhibition of behavior produced by extinction. Reinstatement of cocaine seeking triggered by a cocaine prime was blocked by increased neuronal activity induced by a direct glutamate agonist microinjection into the IL and reinstatement was enhanced by inhibition with GABA agonists in the IL (Peters et al., 2007; Peters et al., 2008). Examining these areas in a meth-primed paradigm was warranted.

The LO was also examined. The LO shares significant neuroanatomic connections with the mesolimbic reward pathway and is crucial in processing reward salience (Schoenbaum et al., 2006). In humans, the LO is hypermetabolic in cocaine abusers reporting intense craving (Bonson et al., 2002; Volkow et al., 1991, Volkow et al., 1999). In rats, ablation of the LO results in the inability to use outcome expectancies to guide behavior (Gallagher et al., 1999; Izquierdo et al., 2004). Kufahl et al. (2009) also showed that in male rats, the LO had a higher number of c-Fos-positive nuclei following cocaine reinstatement induced by cues. The study presented herein examined if and how activation of these cortical areas was differential in males and females following meth-primed reinstatement.

The caudate-putamen, part of the dorsal striatum and basal ganglia macrocircuit, serves as a critical structure in the neurobiology of drug addiction (Volkow et al., 2006). In humans addicts, a cocaine prime activates the caudate-putamen (Breiter et al., 1997) and previously paired drug-cues elicit dopamine increases in the caudate-putamen with the magnitude of this increase positively

correlated with self-reports of craving (Garavan et al., 2000; Volkow et al., 2006,). Neutral stimuli repeatedly paired with a rewarding drug will acquire the ability to increase dopamine in the caudate-putamen, facilitating drug-seeking behavior in pre-clinical models (Ito et al., 2002; Vanderschuren et al., 2005). Additionally, pre-clinical work with male rats has shown that cocaine-priming injections enhance c-Fos expression in the caudate-putamen (Neisewander et al., 2000). The role of the caudate-putamen in mediating the habitual nature of compulsive drug seeking (Robbins and Everitt, 1999; Tiffany, 1990) makes it a prime target for investigation of potential sex differences in drug-primed reinstatement. Previous work has found a significant effect of sex in c-Fos activation of the caudate-putamen in one particular reinstatement model (Zhou et al., 2014). Females showed significantly more positively labeled c-Fos in the dmCPu following cue-induced reinstatement of cocaine seeking, but no other caudate-putamen region. In one of the few studies that has examined neuronal activation during meth reinstatement, Rubio et al. (2015) found significant c-Fos activation in the dorsal medial and dorsal lateral caudate-putamen following reinstatement induced by re-exposure to a meth-taking context (i.e., one form of cue-induced reinstatement). This meth-reinstatement effect was only examined in male rats (Rubio et al., 2015). Given the size of the caudate-putamen and findings that different subregions may have different functions (Charntikov et al., 2012; Fuchs et al., 2006; Zhou et al., 2014), sex differences in c-Fos activation following meth-primed reinstatement were examined in 4 different regions of interest; dlCPu. dmCPu, vlCPu, and vmCPu.

The nucleus accumbens was of particular interest as a key structure in the mesolimbic pathway. The mesolimbic pathway serves as the brain's primary reward circuitry (Hyman et al., 2006; Kalivas and Volkow, 2005; Robison and Nestler, 2011). The reinforcing effects for drugs of abuse, as well as natural rewards (e.g., food and sex) are largely mediated by dopaminergic neurotransmission projecting from the VTA to the nucleus accumbens, caudate-putamen, amygdala, hippocampus, and prefrontal cortex (Hyman et al., 2006; Kiyatkin and Stein, 1996; Koob and Simon, 2009; Phillips et al., 2003; Robison and Nestler, 2011).

In addition to dopaminergic transmission, glutamatergic neurotransmission in the mesolimbic pathway is also a key component in the expression of drug-seeking behavior (for a review see Kalivas and Volkow, 2005). For example, Parsegian and See (2014) found increased glutamate levels during both cue- and meth-triggered reinstatement of meth-seeking behavior. Di Ciano and Everitt (2001) also showed that NMDA receptor antagonists microinjected into the NAcC and NAcSh decreased cocaine-seeking and cocaine-taking respectively, demonstrating a dissociation of the two areas. Differential conditioned dopamine release in the core and shell further supports dissociation in these regions of the nucleus accumbens. Ito and colleagues (2000) showed that while dopamine increased in both areas during cocaine self-administration, only in the NAcC was dopamine elevated by non-contingent presentations of a cocaine paired cue; indicating that the NAcC may be particularly important in mediating responding to drug-associated stimuli. Parkinson et al. (2000b) also

determined that the core plays a more prominent role in reward-related learning compared to the shell by demonstrating that lesions of the core, but not shell, impaired acquisition of a Pavlovian conditioning in an autoshaping procedure.

These nucleus accumbens subregion specific lesion effects were also found using a meth reinstatement procedure. Rocha and Kalivas (2010) found that inactivation of the NAcC reduced cue- and meth-primed reinstatement, while inactivation of the NAcSh reduced neither reinstatement type. Further, Kufahl et al. (2009) established that c-Fos activation was increased by cue-induced reinstatement of cocaine seeking in the NAcC and NAcSh. However, this effect was more robust in the core compared to the shell (i.e., differences were only detected in the shell by an uncorrected t-test comparing cue group to no-cue control group). Curiously, this c-Fos increase following cue-induced reinstatement in the shell and core of the nucleus accumbens has not been universally found. On the contrary, Zhou et al. (2014) found that c-Fos expression was actually deceased in the core and shell in male and female rats following cue-induced reinstatement of cocaine seeking. Additionally, the main effect of sex was significant in the shell, but not in the core, with females showing more activation than the males (Zhou et al., 2014). Further examination of these sex differences and determining if these findings replicate in a different reinstatement model (i.e., drug-primed) with a different drug (i.e., meth) was one of the goals of this study.

The hippocampus is a brain structure vital to memory and learning functions (Squire, 1992). As memory and learning are central components in the

reinstatement of drug-seeking following extinction, the hippocampus was carefully examined in Experiment 1. The ventral subiculum, as well as 3 separate loci in the hippocampus proper (CA1, CA2, and CA3) were investigated. The ventral subiculum of the hippocampus was examined based on work identifying it as an important interface between the hippocampus proper and brain reward circuitry including the nucleus accumbens and prefrontal cortex (for a review see Cooper et al., 2006). Taepavarapruk and Phillips (2003) provided support for the VS's role in reinstatement by demonstrating that electrical stimulation of the VS reinstated pressing on a D-amphetamine paired lever and induced an increase of dopamine efflux in the nucleus accumbens measured by *in vivo* microdialysis. The finding that VS stimulation increased both drug-seeking behavior and accumbal dopamine levels has also been demonstrated with cocaine (Vorel et al., 2001). Accordantly, inhibition of the VS via temporary inactivation by local bilateral microinjections of lidocaine decreased cocaine-induced reinstatement compared to saline microinjections (Sun and Rebec, 2003). These decreases following VS inactivation were specific to drug-seeking behavior; lidocaine injections had no effect on cocaine self-administration (Sun and Rebec, 2003). Zhou et al. (2014) found that increases in c-Fos expression in the VS correlated with drug seeking in male and female rats, replicating previous work using only male rats (Kufahl et al., 2009). Zhou et al. (2014) utilized cocaine as the drug in question and reinstatement was induced by cues. Under these parameters, females showed more c-Fos immunoreactivity in the VS than the males (Zhou et al., 2014).

Regions of the hippocampus proper were included as previous work has shown differential activation of these 3 regions. All 3 regions have been shown to be more active during cue-induced reinstatement (Kufahl et al., 2009; Neisewander et al., 2000; Zhou et al., 2014). However, females when tested showed more neuronal activation in the CA1 and CA3 regions (Zhou et al., 2014). While the role of the hippocampus in certain reinstatement models has been established, possible differential activation between the sexes in a meth-primed reinstatement model need to be determined.

The amygdala is a key component of the limbic system mediating motivated behavior. Human studies have found cues previously paired with drug induce changes in amygdala metabolic activation and these increases are correlated with craving in addicts (Bonson et al., 2002; Childress et al., 1999; Grant et al., 1996; Kilts et al., 2001). In preclinical studies, inactivation of the BLA via dopamine antagonism or ablation can reduce reward-seeking behavior (Di Ciano and Everitt 2004; Fuchs et al., 2005; McLaughlin and See, 2003; See et al., 2001; Whitelaw et al., 1996). The CEA also plays a role in the addiction process with research showing that inactivation of this area reduces the activating effects of psychostimulants, and may play a role in certain types of reward-related learning (Gallagher et al., 1990; Parkinson et al., 2000a; Robledo et al., 1996). Kufahl et al. (2009) found that there may be differential activation between the two areas during reinstatement induced by previously paired cues. c-Fos activation in the BLA was significantly increased in the group that received cue-induced reinstatement, however, this increase was not found the CEA (Kufahl et

al., 2009). Neisewander et al. (2000) also found exposure to a drug-paired cue enhanced c-Fos expression in the BLA, but not the CEA. Notably, cocaine administration increased c-Fos in the CEA but not the BLA. However this increase was observed in rats that received cocaine self-administration and saline self-administration (Neisewander et al., 2000). That is, the cocaine increased c-Fos in the CEA expression when it was functioning as a drug-trigger in reinstatement of cocaine-seeking behavior and upon acute administration independent of prior drug learning. Further examination of possible differences in neuronal activation between a drug-prime and acute drug injection, as well as identification of regions differentially altered between the sexes, was completed by Experiment 1.

As mentioned above, dopaminergic projections from the VTA to the nucleus accumbens, caudate-putamen, amygdala, hippocampus, and prefrontal cortex serves as the brain's primary reward circuitry and the reinforcing properties of drugs of abuse are mediated through this system (Hyman et al., 2006; Kalivas and Volkow, 2005; Robison and Nestler, 2011). As expected, the VTA has been implicated in drug reinstatement and shows increased activation following a cocaine prime in cocaine-dependent subjects (Breiter et al., 1997). Morphine, known to activate mesolimbic dopamine neurons, directly microinjected into the VTA produces drug-primed reinstatement in cocaine or heroin self-administration trained rats (Stewart, 1984). Accordingly, transient VTA inhibition by direct microinjection reduces cocaine-induced reinstatement of cocaine-seeking behavior (McFarland and Kalivas, 2001). Examination of

neuronal activation of the VTA revealed that it did show increased c-Fos expression during cocaine reinstatement (Neisewander et al., 2000). Additionally, there appear to be sex differences in c-Fos immunoreactivity in the VTA. Zhou et al. (2014) found that females had higher levels of c-Fos activation in this area. However, this effect was found in the group that received cue-induced reinstatement of drug-seeking and a control group that did not receive cue-induced reinstatement, suggesting basal differences (Zhou et al., 2014). Determining basal neuronal activation in male and female rats following our basic protocol (i.e., saline self-administration and saline reinstatement trigger) was one goal of this study.

The LH is a central structure the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis functions as the primary stress system; mediating a litany of bodily processes in response to a physical or psychological stressor (de Kloet et al., 2005). Additionally, this circuitry shows significant dysregulation following drug intake (for reviews see Becker-Krail and McClung 2016; Fosnocht and Brand, 2016). As discussed earlier, stress readily reinstates meth-seeking behavior (Beardsley et al., 2010; Shepard et al., 2004). While the procedures used in this set of studies did not explicitly use a pharmacological or physical stressor, stress may have still played a role. The restraint and administration of a subcutaneous injection that is necessary in the drug priming procedure, may have served as a stressor in itself. Also, the drug seeking induced by a meth-trigger without the subsequent outcome of meth infusion may be a stressful event (recall that meth is unavailable in the reinstatement session). When neuronal

activation in the LH was investigated in cocaine seeking induced by a cocaine paired cue, females tended to show more c-Fos positive cells (Zhou et al., 2014). Although this effect did not reach significance, this tendency may have been due to the limited n of 7 per group (Zhou et al., 2014). With this in mind, the LH was included as a locus of interest.

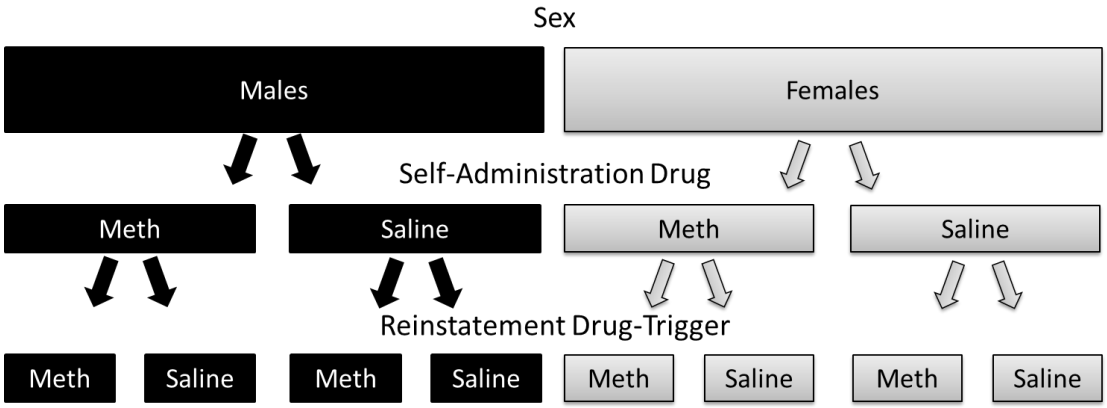
Finally, the SN was also examined for c-Fos activation following meth-primed reinstatement. The SN is a key component of the basal ganglia and key source of dopaminergic projections to the striatum (Nicola et al., 2000). This circuit is often referred to as the nigrostriatal system and, similar to the mesolimbic system, plays an important role in drug addiction (Jasinska et al., 2014; Ikemoto et al., 2015; Wise, 2009). The substantia nigra, specifically, has been implicated by work that shows that activation of dopaminergic neurons in this region is highly rewarding (Ilango et al., 2014; Rossi et al., 2013). In fact, rats responded at similar rates to deliver photostimulation of dopamine neurons in the SN and the VTA (Ilango et al., 2014). When neuronal activation was examined in cue- and cocaine-induced cocaine-seeking reinstatement procedures, there was significant increase in the SN (Kufahl et al., 2009; Neisewander et al., 2000). Determining if this finding is replicated with our meth procedures and examining possible sex differences was one goal of Experiment 1.

As detailed above, while significant progress has been made in elucidating the neurobiological circuitry that underlies drug relapse, there remains a significant paucity in our understanding. With females rarely utilized, the gap is particularly glaring with regard to identification of brain areas that may differ

between the sexes. Experiment 1 began to fill this gap by identifying sex-dependent neural substrates correlated with meth-triggered reinstatement of meth-seeking behavior.

Design

Graphic 2.

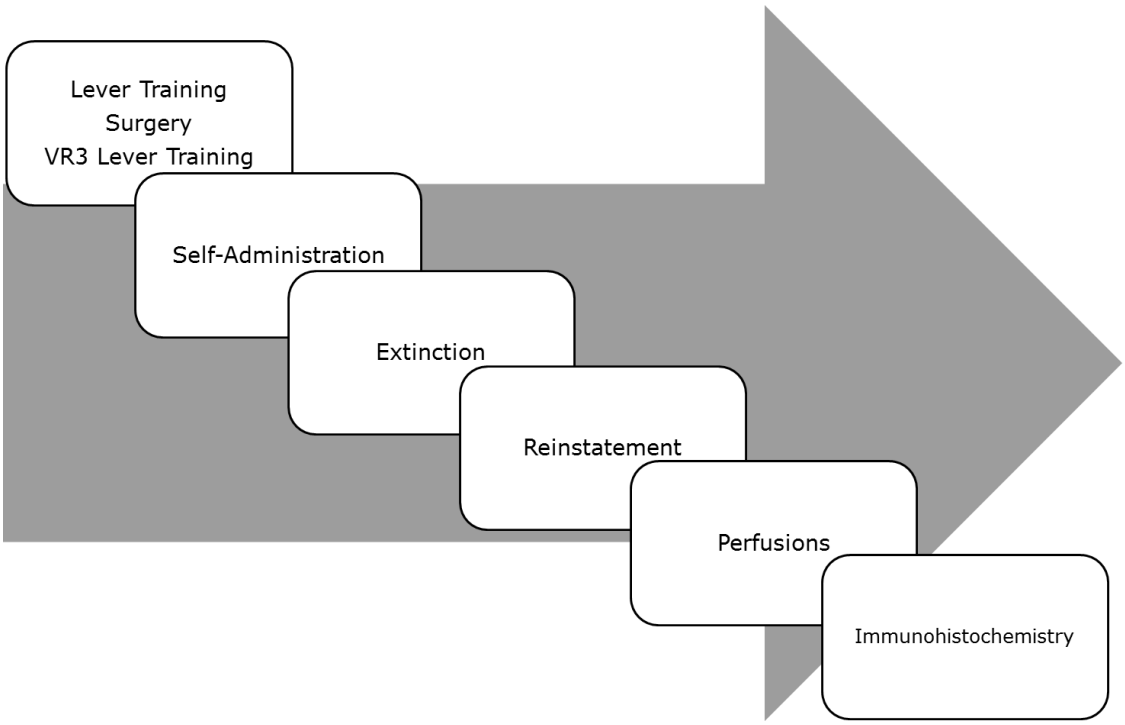


Experiment 1 utilized a 2 x 2 x 2 factorial design with sex (male or female), self-administration drug (saline or meth), and drug-trigger (saline or meth) as between-subjects factors (Graphic 2). This design resulted in 4 separate groups for each sex (SalineSA/SalineT, SalineSA/MethT, MethSA/SalineT, MethSA/MethT) allowing for careful investigation of brain regions correlated with meth-triggered reinstatement. The first part of each name indicates the drug each group self-administered (saline or meth) and the second part of each name indicates the drug that "triggered" reinstatement (Saline or Meth; n=14 per group; see Graphic 2). Each group provided us with vital information regarding the activation of brain regions associated with reinstatement. The SalineSA/SalineT group provided baseline c-Fos activation for both sexes in the

absence of meth self-administration or an acute meth injection. The MethSA/SalineT groups allowed for examination of c-Fos activation that is correlated with an extinction (i.e., drug abstinent) period following self-administration of meth. The SalineSA/MethT groups was used to detect neural substrates correlated with acute meth injection while controlling for exposure to chambers, handling, etc. Finally, the MethSA/MethT groups allowed us to identify sex-dependent neural substrates correlated with meth-triggered reinstatement following meth self-administration. Given the number of animals included in this project (n=112) and the time consuming nature of self-administration experiments, 4 replications were required (rep 1: n=25, rep 2: n=29, rep 3: n=29, rep 4: n=29) to complete this experiment. Each replication contained all 8 groups.

Procedures

Graphic 3.



Preliminaries and Surgery

Following acclimation to the colony room and food restriction to maintain 90% of free-feeding weight, rats received preliminary lever training (see GENERAL PROCEDURES for details). Indwelling jugular catheters were then implanted using our standard protocol (see GENERAL PROCEDURES) and rats were allowed to recover for 7 days. Following recovery, rats were placed on a variable ratio 3 (VR3) schedule of sucrose reinforcement in standard post-surgery lever training (see GENERAL PROCEDURES).

Self-administration

Following these preliminaries, male and female rats were separated into 2 self-administration conditions: MethSA or SalineSA. Rats in the MethSA conditions began self-administration of meth during daily 2 h sessions. Rats in

the SalineSA condition began daily 2 h sessions identical to those received by the MethSA condition except saline was available in lieu of meth. Self-administration continued for 21 days (see GENERAL PROCEDURES for details).

Extinction

In Experiment 1, extinction sessions commenced 24 h after the last self-administration sessions. Extinction sessions were identical to self-administration sessions except meth and saline were no longer infused. Requisite VR3 responding on the active lever still produced the same cues and the timeout. Extinction was conducted daily for 12 consecutive sessions.

Reinstatement

At the end of extinction, the MethSA and SalineSA conditions were split further into 2 different reinstatement-trigger groups: SalineT or MethT. Rats were pseudo-randomly assigned with the caveat that the groups did not differ statistically in responding at the end of extinction. This created 4 groups of males and 4 groups of females: SalineSA/SalineT, SalineSA/MethT, MethSA/SalineT, MethSA/MethT. Rats in the MethT groups were administered a 0.3 mg/kg injection of meth (IP) 15 min before a 70-min reinstatement session, whereas the SalineT groups received a saline injection (IP) 15 min before their 70-min reinstatement session. The reinstatement session was identical to extinction sessions (i.e., no available infusions) except for the truncated time; decreased from 120 to 70 min. The session length was shortened due to preliminary data revealing that lever pressing peaked during the first 10 min of reinstatement sessions. c-Fos is primarily expressed approximately 60-90 min after neuronal

activation (Kovacs, 1998). Limiting the reinstatement session to 70 min allowed for sufficient time to gather the crucial behavioral data, as well as identify brain regions correlated with the meth trigger and the reinstatement behavior.

Perfusion and Brain Extraction

Immediately following the reinstatement session, rats were deeply anesthetized by injection with Fatal-Plus (25 mg/kg; MWI, Boise, ID). Rats were then transcardially perfused with 200 ml of ice cold 0.9% saline followed by 200 ml of ice cold 4% paraformaldehyde. Brains were rapidly removed and post-fixed in 4% paraformaldehyde for 24 h at 4°C. Brains were then cryoprotected in 30% sucrose for 72 h at 4° C. Following cryoprotection, brains were frozen on dry ice and stored at -80° C until all 4 replications of the experiment were completed.

Histology

Table 1.

Batch 1			Batch 2			Batch 3		
Replication	Subject	Group	Replication	Subject	Group	Replication	Subject	Group
1	64	Male MethSA/MethT	1	70	Male MethSA/SalT	1	63	Female SalSA/MethT
1	69	Female MethSA/MethT	1	72	Female MethSA/SalT	1	65	Female SalSA/SalT
2	104	Female MethSA/SalT	2	103	Male SalSA/MethT	1	68	Male SalSA/SalT
2	107	Male MethSA/SalT	2	109	Female SalSA/MethT	1	89	Male SalSA/MethT
3	139	Male SalSA/MethT	3	142	Female SalSA/SalT	2	102	Female SalSA/SalT
3	146	Female SalSA/MethT	3	147	Male SalSA/SalT	2	105	Male SalSA/SalT
4	179	Male SalSA/SalT	4	175	Male MethSA/MethT	2	111	Female MethSA/MethT
4	182	Female SalSA/SalT	4	176	Female MethSA/MethT	2	122	Male MethSA/MethT
						3	145	Male MethSA/MethT
						3	148	Female MethSA/MethT
						4	181	Male MethSA/SalT
						4	205	Female MethSA/SalT

Batch 4			Batch 5			Batch 6		
Replication	Subject	Group	Replication	Subject	Group	Replication	Subject	Group
1	67	Male MethSA/MethT	1	73	Male MethSA/MethT	1	85	Male MethSA/MethT
1	71	Female MethSA/MethT	1	86	Male MethSA/SalT	1	91	Male MethSA/SalT
2	108	Female MethSA/SalT	1	87	Female MethSA/SalT	2	115	Female MethSA/SalT
2	114	Male MethSA/SalT	1	93	Female MethSA/SalT	2	126	Male SalSA/MethT
3	141	Male SalSA/MethT	2	110	Female SalSA/MethT	3	153	Female SalSA/SalT
3	144	Female MethSA/SalT	2	117	Male SalSA/SalT	3	154	Female MethSA/MethT
3	152	Female SalSA/MethT	2	119	Male SalSA/MethT	3	156	Female MethSA/MethT
3	157	Male MethSA/SalT	2	134	Female SalSA/MethT	3	162	Female SalSA/SalT
4	177	Male SalSA/MethT	3	151	Female SalSA/SalT	4	183	Male SalSA/MethT
4	180	Female SalSA/MethT	3	171	Male SalSA/SalT	4	184	Female SalSA/MethT
4	185	Male SalSA/SalT	4	193	Male MethSA/MethT	4	189	Male SalSA/SalT
4	186	Female SalSA/SalT	4	199	Female MethSA/MethT	4	195	Male MethSA/SalT

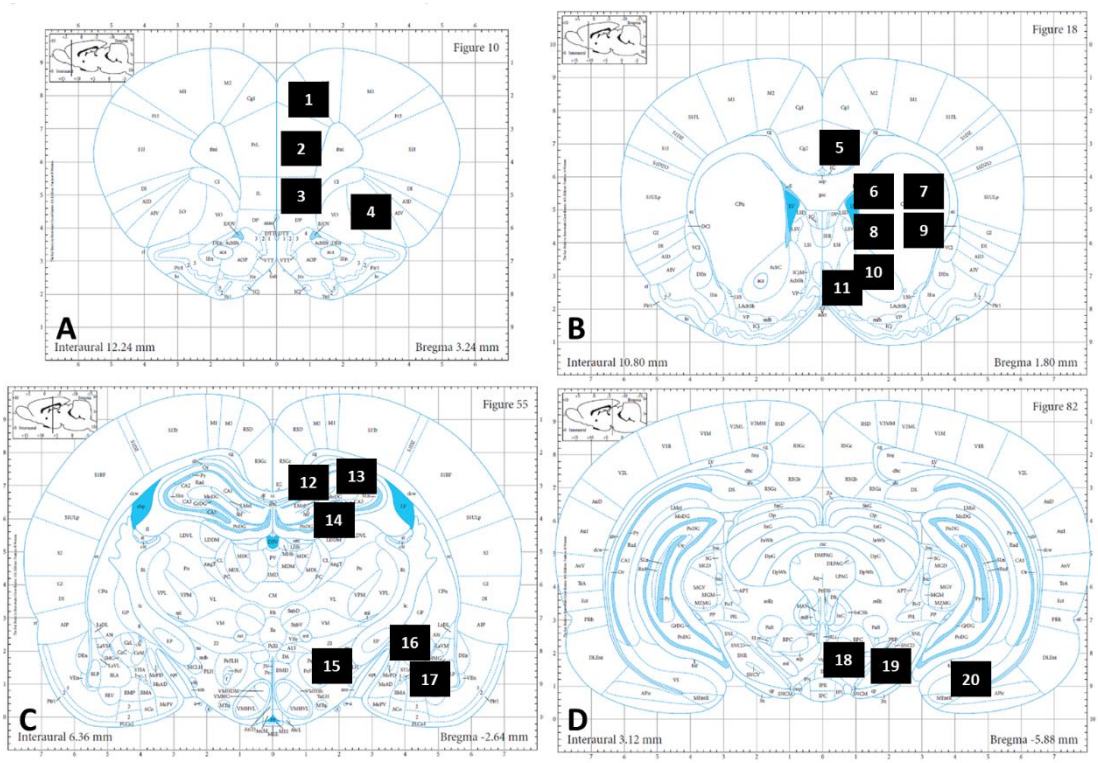
Batch 7			Batch 8			Batch 9		
Replication	Subject	Group	Replication	Subject	Group	Replication	Subject	Group
1	76	Female MethSA/MethT	1	92	Male SalSA/MethT	1	81	Female SalSA/MethT
1	77	Male SalSA/SalT	2	128	Male SalSA/SalT	1	90	Female MethSA/MethT
1	84	Female SalSA/SalT	2	135	Male MethSA/MethT	1	95	Female SalSA/MethT
1	96	Male MethSA/SalT	3	155	Female MethSA/SalT	2	118	Female MethSA/MethT
2	113	Female MethSA/MethT	3	160	Female SalSA/MethT	2	123	Female SalSA/SalT
2	116	Female MethSA/SalT	3	161	Male SalSA/MethT	2	127	Female SalSA/SalT
2	131	Male MethSA/MethT	3	169	Male MethSA/SalT	2	129	Male MethSA/SalT
2	133	Male MethSA/MethT	3	172	Female MethSA/MethT	2	132	Female MethSA/SalT
3	150	Female MethSA/SalT	4	188	Female SalSA/SalT	3	159	Male MethSA/MethT
3	165	Male MethSA/SalT	4	196	Female SalSA/SalT	4	191	Male SalSA/MethT
4	187	Male SalSA/MethT	4	197	Male SalSA/SalT	4	204	Male MethSA/SalT
4	192	Female SalSA/MethT	4	201	Female SalSA/MethT	4	206	Male SalSA/SalT

Batch 10		
Replication	Subject	Group
1	94	Male SalSA/SalT
2	121	Female MethSA/MethT
2	136	Male MethSA/SalT
3	158	Female MethSA/SalT
3	166	Female MethSA/SalT
3	167	Male MethSA/MethT
3	168	Female SalSA/MethT
3	173	male MethSA/MethT
4	202	Male SalSA/MethT
4	203	Female SalSA/SalT
4	207	Male SalSA/MethT
4	208	Male SalSA/SalT

Following the 4th and final replication, brains were pseudo-randomly assigned, controlling for group type, to 1 of 10 batches (see Table 1). Psuedo-random assignment was to control for slightly variations in the staining process

across batches (Rhodes et al., 2005). Brain slices were cut at 40 µm on a freezing microtome. Brain regions were identified according to the atlas of Paxinos and Watson (2007). Coronal sections were collected at 3.24 mm bregma to assess the Cg1, PrL, IL, and LO (see Graphic 4). Coronal slices at 1.80 mm bregma were used to examine the Cg2, dmCPu, dlCPu, vmCPu, vlCPu, NAcC, and NAcSh (Graphic 4). Regions of the hippocampus (CA1; CA2; and CA3), amygdala (CEA; BLA) and LH were identified on sections collected at -2.64 mm bregma (Graphic 4). Finally, sections at -5.88 mm bregma contained the VTA, SNR, and VS (Graphic 4). Each area of interest was examined in a single hemisphere from 3 separate tissue sections per rat (cf. Zhao and Li, 2010).

Graphic 4.



Brain regions identified by the atlas of Paxinos and Watson (2007) and examined for c-Fos expression. **A**: Bregma 3.24; **1**-Cg1, **2**-PrL, **3**-IL, **4**-LO. **B**: Bregma 1.80; **5**-Cg2, **6**-dmCPu, **7**-dlCPu, **8**-vmCPu, **9**-vlCPu, **10**-NAcC, **11**-NAcSh. **C**: Bregma -2.64; **12**-CA1, **13**-CA2, **14**-CA3, **15**-LH, **16**-CEA, **17**-BLA. **D**: Bregma -5.88; **18**-VTA, **19**-SNR, **20**-VS.

c-Fos Immunohistochemistry

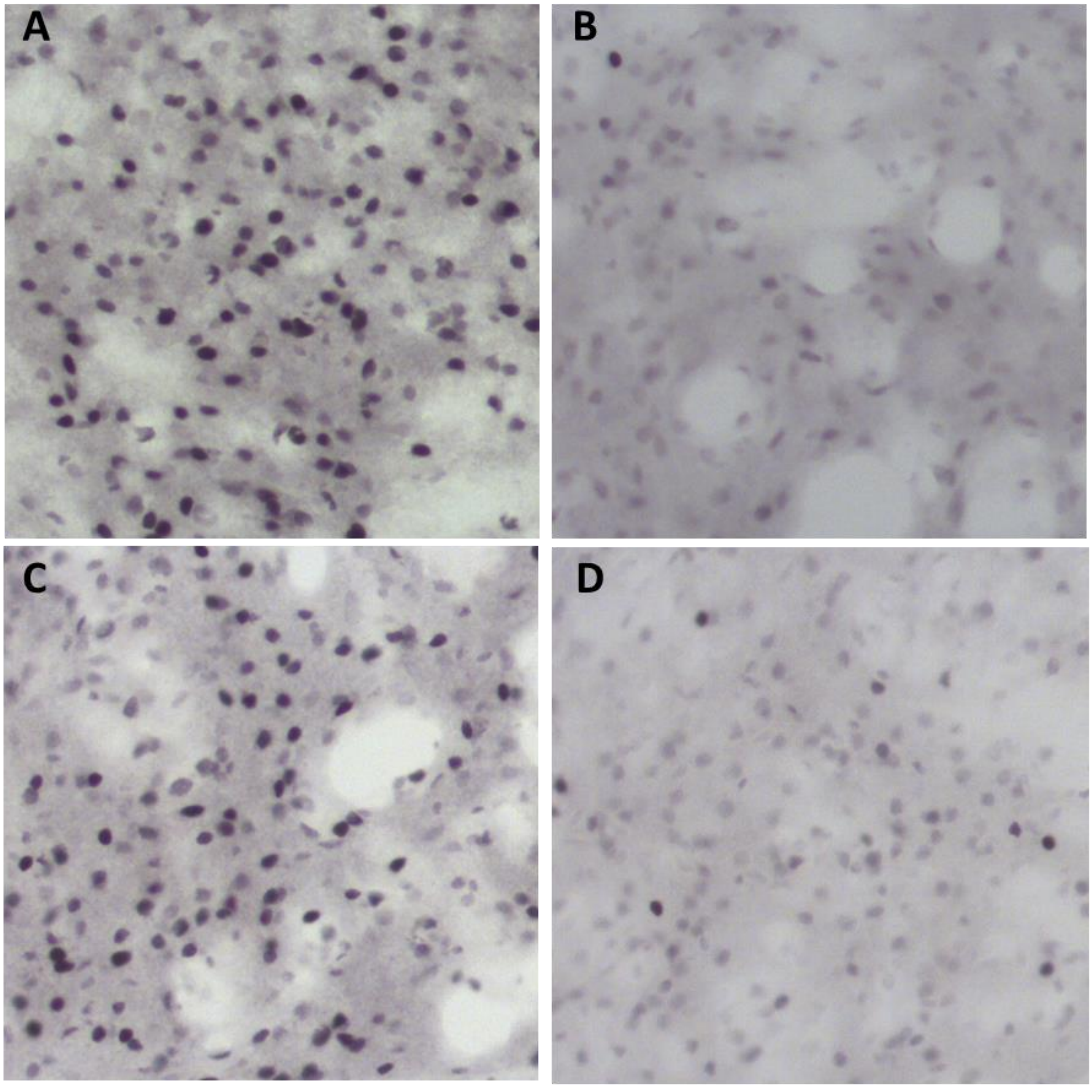
Following sectioning, brain slices were stored for no more than 48 h in a 0.02 M phosphate buffered saline (PBS): 0.1% sodium azide solution. For c-Fos immunohistochemistry, brain sections incubated on ice for 1 h in blocking solution [10% normal goat serum (NGS): 0.3% Triton X-100: 0.02 M PBS]. Sections were then washed 3 times in wash buffer (0.3% Triton X-100: 0.05% NGS: 0.02 M PBS) for 10 min per wash. Washing was proceeded by incubation in 1.5% hydrogen peroxide: 50% methanol for 30 min on ice. This was followed by another round of 3 washes with wash buffer (10 min per wash). Sections were then incubated with c-Fos primary antibody (Santa Cruz Biotechnology, Dallas, TX, USA; 1:3000 dilution) in 0.3% Triton X-100:1% NGS: 1% blocking reagent:0.02 M PBS for 48 h at 4°C. Following incubation with the primary antibody, sections were washed 3 times for 10 min per wash with wash buffer. Then sections were incubated with biotinylated goat anti-rabbit secondary antibody (Vector Labs, Burlingame, CA, USA; 1:200 dilution) in 1% NGS:0.02 M PBS for 2 h on ice. This was followed by 3 washes (10 min per wash) with 0.02 M PBS. Sections were then incubated with horseradish peroxide avidin-biotin complex (Vectastain Elite ABC Kit, Vector Labs, Burlingame, CA, USA: 1:200 dilution) in 0.02 M sodium azide- free PBS. This was followed by 3 washes with 0.05 Tris-HCl. Sections were then incubated for 5 min in diaminobenzidine-based peroxide substrate (DAB Substrate Kit, Vector Labs, Burlingame, CA, USA) to aid in protein visualization. Brain slices were then mounted on gelatin-coated

slides and allowed to air-dry at room temperature. They were then dehydrated in ascending alcohol concentrations, cleared in xylene and cover slipped with mounting medium (Permount, Fisher Scientific, Suwanee GA, USA).

c-Fos Imaging and Quantification

A digital image (20x objective lens magnification; 490 μm^2) was captured from each region of interest from anatomically matched sections (1 image from each tissue section x 3 tissue sections per area for each rat) using a light microscope (Olympus CX41RF, Tokyo, Japan) fitted with a digital camera (Infinity Lite, Ottawa, ON, Canada). For each image, c-Fos immunoreactivity was automatically identified and counted using NIH ImageJ software (Abramoff et al., 2004; Charntikov et al., 2012). Sample photomicrographs of c-Fos expression are provided in Graphic 5. Imaging and immunoreactivity quantification using ImageJ were performed blind to the treatment status of the sections.

Graphic 5.



Photomicrographs of c-Fos expression in the CEA. **A**: Subject 199; Female MethSA/MethT. **B**: Subject 205; Female MethSA/SalineT. **C**: Subject 184: Female SalineSA/MethT. **D**: Subject 182: Female SalineSA/SalineT

Dependent Measures

Lever-pressing was the primary dependent measure during the behavioral phases of the experiment. To show inactive lever responding relative to active lever responding during self-administration, a discrimination index was

calculated using the following formula: Discrimination Index = [Active Lever Presses/ (Inactive Lever Presses + Active Lever Presses)]. A Discrimination Index value of 0.5 indicates equal responding on the active and inactive lever (i.e., no discrimination between levers); a value >0.5 indicates more pressing on the active lever. Lever pressing on the inactive lever was near zero following early acquisition and remained for the rest of the experiment (data not displayed). Positively identified c-Fos cells in each brain region was the primary dependent measure used for neuronal activation. For each rat, the number of positively labeled nuclei was averaged between the 3 tissue sections in each region and used as a unit of measurement (cf. Charntikov et al., 2012; Zhao and Li, 2010 and Shram et al., 2007).

Statistical Analyses

Active lever responding in acquisition and extinction were analyzed by separate 3-way mixed measures analysis of variance (ANOVA; Type III Sum of Squares) with Sex (Female vs Male) and Group (MethSA vs SalineSA) as between-subjects factors and Session as a within-subjects factor. This same ANOVA was also utilized to examine the discrimination index. Active lever responding in reinstatement was analyzed by a 3-way ANOVA with Sex (Female vs Male) and Self-Administration Drug (Meth vs Saline) and Reinstatement Drug (Meth vs Saline as between-subjects factors). Three-way ANOVAs with Sex, Self-Administration Drug, and Reinstatement Drug as between-subjects factors were also used to analyze regional c-Fos activation following reinstatement testing. Post-hoc analyses were conducted on significant interactions and on planned

comparisons in reinstatement behavior and c-Fos activation. The complete list of *a priori* comparisons can be found in Table 2. To adjust for multiple comparisons, Tukey HSDs were utilized for post-hoc analysis of behavioral data. Statistical significance was declared at $p<0.05$.

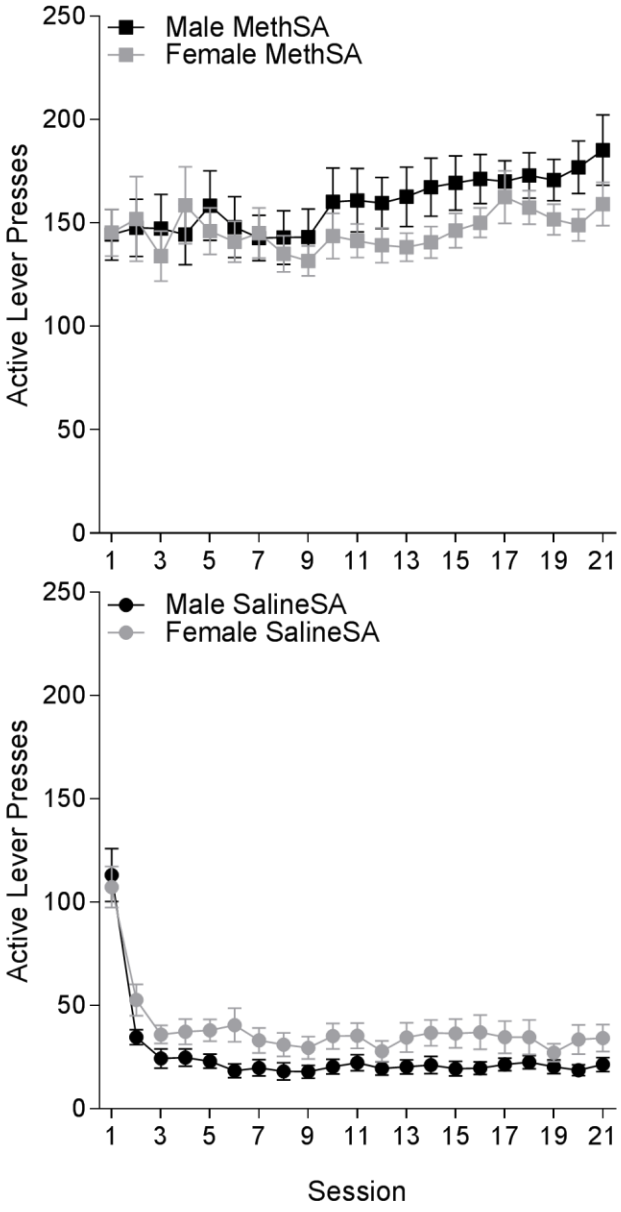
Table 2.

Planned Comparisons	
Within Females	
1	MethSA/MethT vs MethSA/SalineT
2	MethSA/MethT vs SalineSA/MethT
3	MethSA/MethT vs SalineSA/SalineT
4	MethSA/Saline T vs SalineSA/MethT
5	MethSA/Saline T vs SalineSA/SalineT
6	SalineSA/Meth T vs SalineSA/SalineT
Within Males	
7	MethSA/MethT vs MethSA/SalineT
8	MethSA/MethT vs SalineSA/MethT
9	MethSA/MethT vs SalineSA/SalineT
10	MethSA/Saline T vs SalineSA/MethT
11	MethSA/Saline T vs SalineSA/SalineT
12	SalineSA/Meth T vs SalineSA/SalineT
Between Sex	
13	Female MethSA/MethT vs Male MethSA/MethT
14	Female MethSA/SalineT vs Male MethSA/SalineT
15	Female SalineSA/MethT vs Male SalineSA/MethT
16	Female SalineSA/SalineT vs Male SalineSA/SalineT

Results

Self-administration

Figure 2.

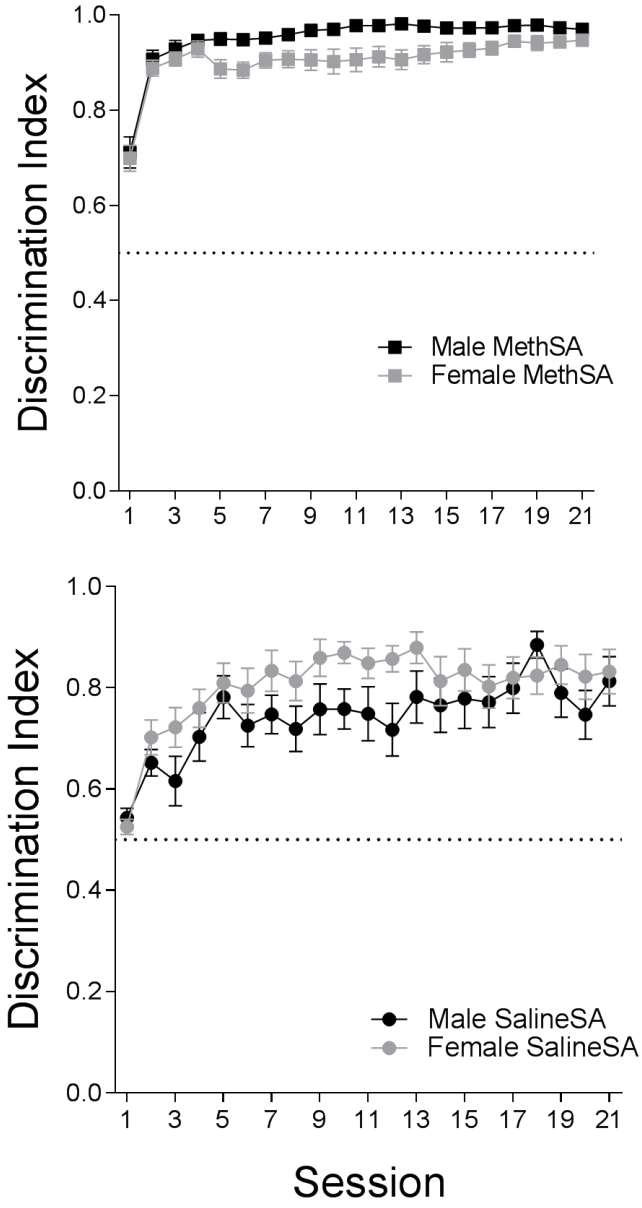


Active lever pressing (± SEM) during self-administration sessions for males (black) and females (grey) in the meth (square) and saline (circle) conditions.

Rats in the meth groups demonstrated robust active lever pressing (Figure 2). Analysis of active lever pressing revealed significant main effects of Group

[F(1,108)=240.338; p<0.001], Session [F(20,2160)=10.655; p<0.001], and a Group x Session interaction [F(20,2160)=14.467; p<0.001]. Rats responded significantly more for meth compared to saline in all 21 self-administration sessions. Lever pressing in the meth groups escalated slightly as early self-administration responding was slightly lower than subsequent responding, demonstrated by numerous significant post-hocs during sessions 1-9 compared to sessions 15-21. Lever pressing in the saline groups was quickly attenuated (bottom panel of figure 2). Active lever presses on session 1 were significantly higher than presses recorded on the subsequent 20 sessions, with no other significant post-hoc tests. This finding was not surprising given the preliminary training with food. Females and males responded similarly on the active lever as neither the main effect of Sex (F<1; p=0.982), the Sex x Group interaction [F(1,108)=2.838; p=0.095], the Sex x Session interaction [F(20,2160)=1.012; p=0.443], nor the Sex x Group x Session interaction [F(20,2160)=1.329; p=0.159] was significant.

Figure 3.



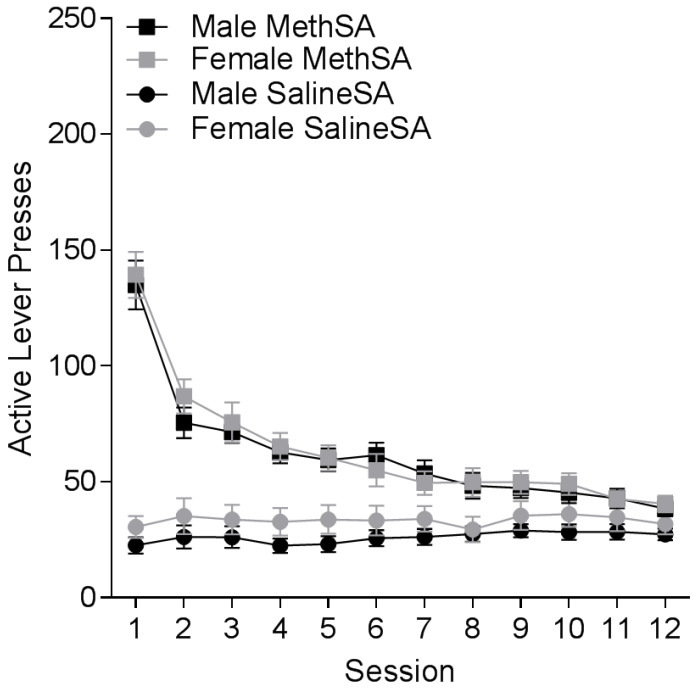
Discrimination Index (± SEM) during self-administration sessions for male (black) and female (grey) rats in the meth (square) and saline (circle) conditions.

Males and females displayed clear discrimination between the active and inactive lever in the meth and the saline conditions (Inset Figure 3; Discrimination Index well above 0.5). Analysis of the discrimination index did reveal significant main effects of Group [$F(1,108)=74.728$, $p<0.001$] and Session

[F(20,2160)=20.749, p<0.001], as well as significant Sex x Group [F(1, 108)=8.713, p=0.004] and Group x Session [F(20,2160)=1.759, p=0.020] interactions. Rats displayed better discrimination when receiving meth vs saline infusions. Lever discrimination increased in the meth and saline conditions, however it increased more quickly in the meth infusions condition. Notably, in the saline condition, females showed statistically better discrimination compared to the males. In the meth condition, however, this effect was reversed, with males tending to show better discrimination than their female counterparts, although this effect did not reach significance (p=0.074). The Sex x Group x Session [F(20,2160)=1.256, p=0.198] and Sex x Session [F<1, p=0.926] interactions were not significant.

Extinction

Figure 4.



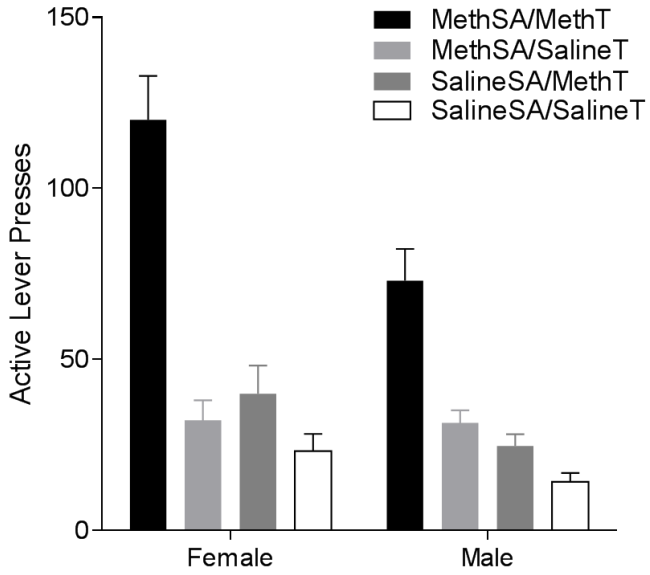
Active lever presses (± SEM) in extinction sessions for male (black) and female (grey) rats in the meth (square) and saline (circle) conditions.

Active lever pressing in the meth condition was attenuated during the extinction phase of this experiment (Figure 4). Analysis of active lever pressing during extinction revealed significant main effects of Group [$F(1,108)=64.885$; $p<0.001$] and Session [$F(11,1188)=45.431$; $p<0.001$], as well as a significant Group x Session interaction [$F(11,1188)=53.318$; $p<0.001$]. Responding in the meth condition was elevated compared to saline responding for the first 11 sessions, but was reduced to saline levels by session 12. Responding in the meth condition was higher during initial extinction sessions compared to the later extinction sessions, while responding in the saline condition remained stable throughout extinction. This outcome was expected, given that extinction sessions contained the timeout cues that were presumably maintaining a modest levels of

responding in the saline condition. Responding was again similar between males and females. The main effect of Sex ($F(1,108)=1.255$ $p=0.265$), the Sex x Group interaction ($F<1$; $p=0.506$), the Sex x Session interaction ($F<1$; $p=0.5$), and the Sex x Group x Session interaction ($F<1$; $p=0.957$) were not significant.

Reinstatement

Figure 5.



Active lever presses (±SEM) for female (left side) and male (right side) rats during the reinstatement session. Groups are delineated by color.

Analysis of active lever pressing during the reinstatement test (Figure 5) revealed significant main effects of Sex [$F(1,104)=12.569$; $p<0.001$], Self-Administration Drug [$F(1,104)=57.931$; $p<0.001$], and Reinstatement Drug [$F(1,104)=59.43$; $p<0.001$]. The Sex x Reinstatement Drug interaction [$F(1,104)=6.665$; $p=0.012$] and the Self Administration Drug x Reinstatement Drug interaction [$F(1,104)=25.297$; $p<0.001$] were both significant. The overall

3-way Sex x Self-Administration Drug x Reinstatement Drug interaction just missed significance [$F(1,104)=3.879$; $p=0.051$]. The Sex x Self-Administration Drug interaction was also not significant [$F(1,104)=1.347$; $p=0.248$].

Post-hoc tests on the planned comparisons revealed several interesting findings. In both sexes, the groups that received meth during self-administration and received meth as a trigger (MethSA/MethT) showed more reinstatement than the groups receiving meth in self-administration and not receiving a drug prime (MethSA/SalineT), receiving a meth trigger without prior meth experience (SalineSA/MethT), or the groups that never got meth at any point (SalineSA/SalineT). The females and males responded similarly when compared between sex in the MethSA/SalineT, SalineSA/MethT, and SalineSA/SalineSA groups. Notably, the only difference between the males and females was in the groups that received meth-primed reinstatement following meth self-administration. Females in this MethSA/MethT group responded significantly more than their male counterparts. The significance of these findings is quite clear, the differences in reinstatement behavior between the sexes were not a result of general differences following long-term meth self-administration (MethSA/SalineT), acute meth administration (SalineSA/MethT), nor basal behavioral differences (SalineSA/SalineT). Females only responded more during meth-primed reinstatement of meth-seeking (MethSA/MethT).

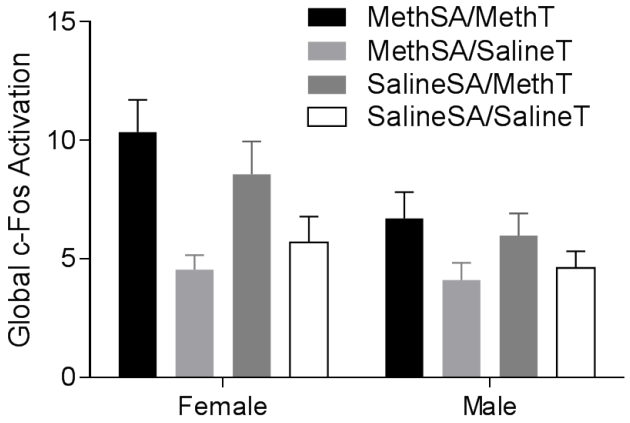
c-Fos Immunohistochemistry

Global Activation

Figure 6.

Global Activation

Within Females	Corrected p Value
MethSA/MethT > MethSA/SalineT	<0.001
MethSA/MethT > SalineSA/SalineT	0.011
SalineSA/MethT > MethSA/SalineT	0.035
Between Sex	
Female MethSA/MethT > Male MethSA/MethT	0.014



Significant post-hoc tests on the planned group comparisons are summarized (top). Global c-Fos activation (± SEM) following reinstatement testing in the female (left) and male (right) rats (bottom). Groups are delineated by color.

To assess global neuronal activation, the 20 brain areas were collapsed to provide a single measure of c-Fos activation for each rat (Figure 6). Examination of this data revealed that global c-Fos activation showed a somewhat similar pattern to the behavioral result during reinstatement (see Figure 5). There was a main effect of Sex [$F(1,104)=7.002$, $p=0.009$] with more activation in females and a main effect of Reinstatement Drug [$F(1,104)=18.677$, $p<0.001$] with meth inducing more neuronal activity. The 3-way Sex x Self-Administration Drug x Reinstatement Drug interaction ($F<1$, $p=0.566$) and the Sex x Self-Administration Drug interaction ($F<1$, $p=0.771$) were not significant. The main effect of Self-Administration Drug ($F<1$, $p=0.773$), the Sex x Reinstatement Drug

[F(1,104)=2.604; p=0.110], and the Self-Administration x Reinstatement Drug [F(1,104)=2.024; p=0.158] were also not significant.

There were significant group differences (Figure 6) in the planned comparisons. In the females, a meth prime following meth self-administration (MethSA/MethT) increased neuronal activation compared to the saline trigger groups (MethSA/SalineT, SalineSA/SalineT). Additionally, acute meth injection (SalineSA/MethT) in the females actually increased activation when compared to the long-term self-administration followed by abstinence group (MethSA/SalineT). In fact, although not statistically significant, visual inspection of the data reveals that the group that received meth self-administration, but was drug free during reinstatement and brain extraction, actually had the lowest amount of neuronal activation in both the females and males. This tendency could possibly be a sign of neuronal hypofunction following long-term drug taking. While no group differences reached significance in the males, visual inspection does show the groups that received a meth trigger in reinstatement were higher than the groups that received saline (i.e., no drug prime). Notably, the only difference between the sexes was that females that received a meth trigger following meth self-administration (MethSA/MethT) show more neuronal activation than males in the MethSA/MethT group. This finding corresponds nicely with the behavioral result found during reinstatement with increased meth-seeking in the Female MethSA/MethT compared to Male MethSA/MethT (cf. Figures 5 and 6).

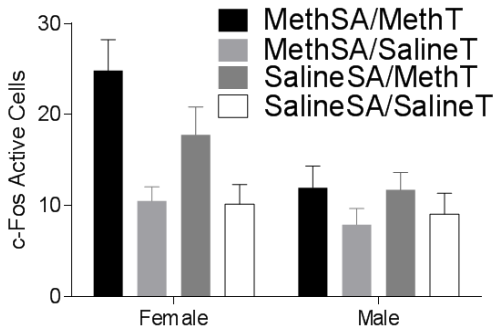
Cortical Regions

Cg1

Figure 7.

Cg1

Within Females	Corrected p Value
MethSA/MethT > MethSA/SalineT	<0.001
MethSA/MethT > SalineSA/SalineT	<0.001
SalineSA/MethT > MethSA/Saline T	0.006
SalineSA/MethT > Saline/Saline T	0.012
Between Sex	
Female MethSA/MethT > Male MethSA/MethT	>0.001



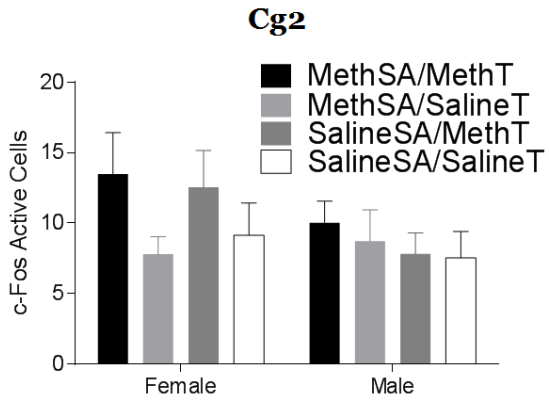
Significant post-hoc tests in the Cg1 are summarized (top). c-Fos active cells (±SEM) during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

In the Cg1 (Figure 7), there were main effects of Sex [F(1,104)=11.199; p=0.001] and Reinstatement Drug [F(1,104)=17.923; p<0.001], with a significant Sex x Reinstatement Drug interaction [F(1,104)=5.061, p=0.027]. The main effect of Self-Administration Drug (F<1, p=0.341), Sex x Self-Administration Drug x Reinstatement Drug interaction (F<1, p=0.433), Sex x Self-Administration interaction [F(1,104)=1.531; p=0.219], and Self-Administration Drug x Reinstatement Drug Interaction [F(1,104)=1.428; p=0.235] were not significant. In the planned comparisons, female rats that received meth during reinstatement had more c-Fos activation than the rats that received saline during reinstatement (see Figure 7 for details on significant group comparisons). These group

differences were not detected in the males. Additionally, the females the received meth-primed reinstatement were higher than the males that received meth-primed reinstatement (MethSA/MethT). This data pattern was very similar compared to the reinstatement behavioral data.

Cg2

Figure 8.

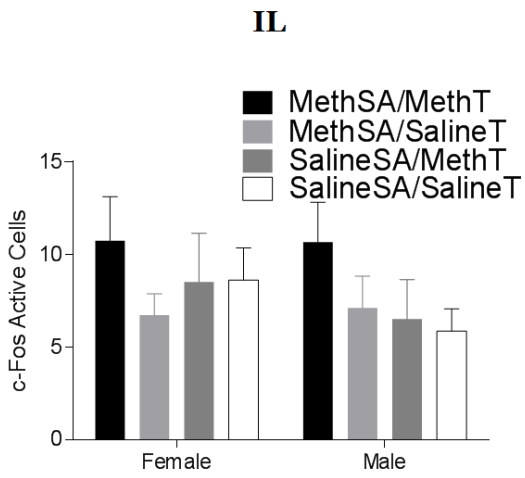


c-Fos active cells (±SEM) during reinstatement in the Cg2 in females (left) and males (right) are displayed. Groups are delineated by color.

There were no significant main effects [Sex: $F(1,104)=2.230$, $p=0.138$; Self-Administration Drug: $F<1$, $p=0.618$; Reinstatement Drug $F(1,104)=3.232$, $p=0.075$], nor significant interactions [Sex x Self-Administration Drug x Reinstatement Drug interaction: $F<1$, $p=0.832$; Sex x Self-Administration interaction: $F<1$, $p=0.521$; Sex x Reinstatement Drug: $F(1,104)=1.591$, $p<0.210$; Self-Administration Drug x Reinstatement Drug Interaction: $F<1$, $p=0.579$] in c-Fos activation in the Cg2 (Figure 8). None of the planned comparisons were significant.

IL

Figure 9.



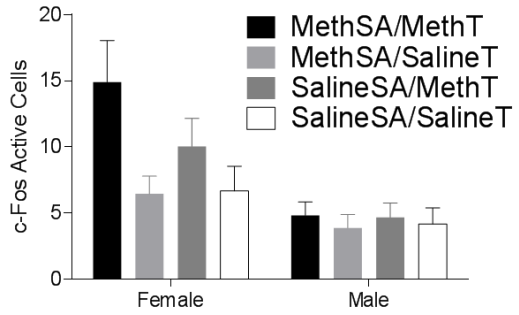
c-Fos active cells (±SEM) during reinstatement in the IL in females (left) and males (right) are displayed. Groups are delineated by color.

For the IL (Figure 9), there were no significant main effects [Sex: $F<1$, $p=0.423$; Self-Administration Drug: $F(1,104)$, $p=0.302$; Reinstatement Drug $F(1,104)=2.156$, $p=0.145$], nor significant interactions [Sex x Self-Administration Drug x Reinstatement Drug interaction: $F<1$, $p=0.826$; Sex x Self-Administration interaction: $F<1$, $p=0.357$; Sex x Reinstatement Drug: $F<1$, $p=0.962$; Self-Administration Drug x Reinstatement Drug Interaction: $F(1,104)=1.613$, $p=0.207$]. Post-hoc tests on the planned comparisons were not significant.

LO

Figure 10.

Within Females	Corrected p Value
MethSA/MethT > MethSA/SalineT	0.005
MethSA/MethT > SalineSA/SalineT	0.006
Between Sex	
Female MethSA/MethT > Male MethSA/MethT	<0.001
Female SalineSA/MethT > Male SalineSA/MethT	0.032



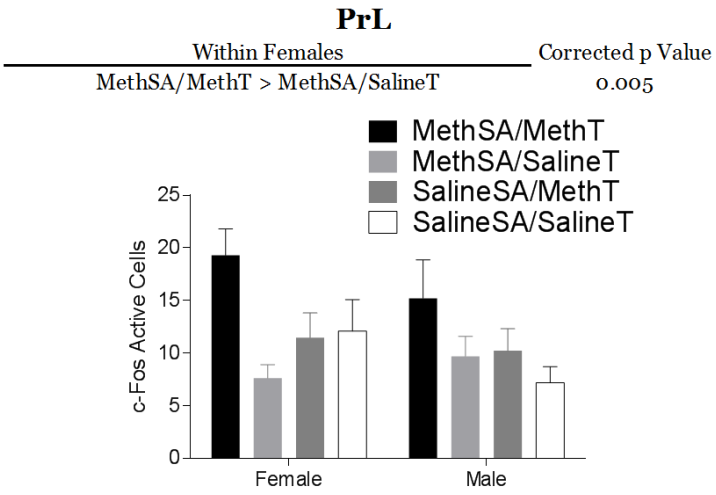
Significant post-hoc tests in the LO are summarized (top). c-Fos active cells (±SEM) during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

For the LO (Figure 10), there were main effects of Sex [F(1,104)=17.256; p=0.001] and Reinstatement Drug [F(1,104)=7.231;p=0.008], as well as a significant Sex x Reinstatement Drug interaction [F(1,104)=4.415; p=0.038]. The main effect of Self-Administration Drug (F<1, p=0.361), Sex x Self-Administration Drug x Reinstatement Drug interaction (F<1, p=0.352), Sex x Self-Administration interaction [F<1, p=0.337], and Self-Administration Drug x Reinstatement Drug Interaction [F(1,104)=1.250; p=0.266] were not significant. In the planned comparisons, the female group that received the meth trigger following meth self-administration had higher neuronal activation than the groups that received saline in reinstatement. Interestingly, the female group that received acute meth did not show activation levels that were significantly different than the saline groups. That is, the c-Fos activation induced by meth

was only higher when females had a previous history with meth. There was no significant group difference detected in the males. Additionally, both of the female groups that received meth during reinstatement showed more c-Fos activation than their male counterparts, suggesting amplified neuronal activation induced by meth injection in the females.

PrL

Figure 11.



Significant post-hoc tests in the PrL are summarized (top). c-Fos active cells (±SEM) during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

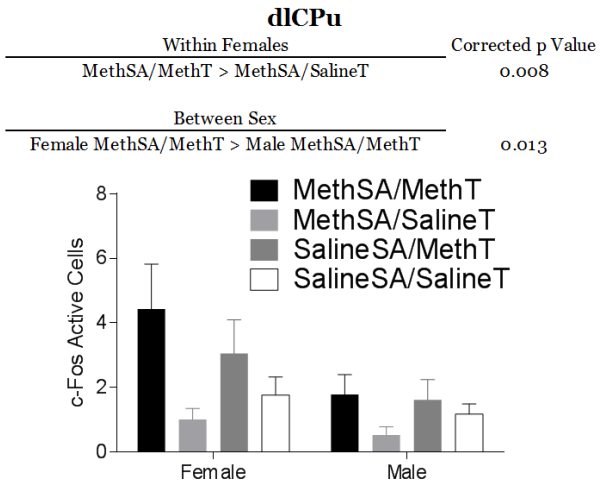
In examination of activation in the PrL (Figure 11), there was a main effect of Reinstatement Drug [F(1,104)=8.392, p=0.004] and a significant Self-administration Drug x Reinstatement Drug Interaction [F(1,104)=4.729, p=0.0319]. The main effects of Sex [F(1,104)=1.456, p=0.230] and Self-Administration Drug [F(1,104)=2.558, p=0.112] were not significant. Nor were the Sex x Self-Administration Drug x Reinstatement Drug interaction [F(1,104)=2.108, p= 0.150], the Sex x Self-Administration Interaction (F<1,

p=0.543), and the Sex x Reinstatement Drug interaction (F<1, p=0.716). Interestingly, the only significant group difference was between the female group that received meth self-administration and a meth trigger and the female group that received meth self-administration and then did not receive a meth injection (Figure 11). Visual inspection of the data again hints that this may be because the female group that is in meth abstinence (MethSA/SalineT) was showing neuronal hypofunctioning. This difference was not seen in the males.

Caudate-Putamen Regions

dlCPu

Figure 12.



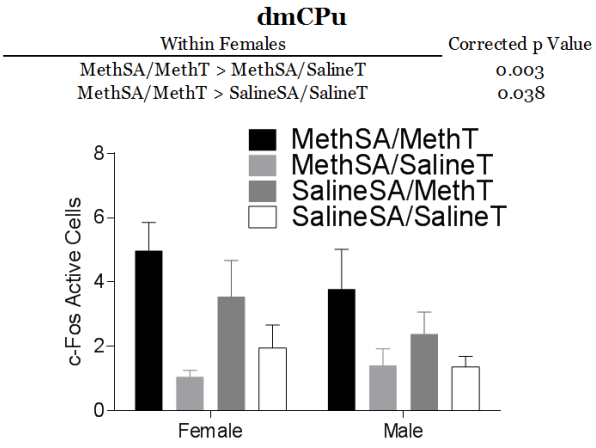
Significant post-hoc tests in the dlCPu are summarized (top). c-Fos active cells (±SEM) during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

In the dlCPu (Figure 12), there were main effects of Sex [F(104)=6.001, p=0.016] and Reinstatement Drug [F(104)=9.377, p=0.002]. The main effect of Self-Administration Drug was not significant (F<1, p=0.946) and none of the

interactions reached significance [Sex x Self-Administration Drug x Reinstatement Drug interaction: F<1, p=0.527; Sex x Self-Administration interaction: F<1, p=0.603; Sex x Reinstatement Drug: F(1,104)=2.042, p=0.156; Self-Administration Drug x Reinstatement Drug Interaction: F(1,104)=1.978, p=0.163]. For the planned comparisons, the females that received meth injection for reinstatement following meth self-administration had more activation than the meth self-administration group that was in abstinence (Figure 12). Again, the pattern points to hypofunction in the abstinence group. This MethSA/MethT group of females was also higher than their male counterparts, suggesting increased activation in the females when meth is administered following meth self-administration.

dmCPu

Figure 13.



Significant post-hoc tests in the dmCPu are summarized (top). c-Fos active cells (±SEM) during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

In the dmCPu (Figure 13), there was a significant main effect of Reinstatement Drug [F(1,104)=16.082, p<0.001]. The Sex [F(1,104)=1.359, p=0.246] and Self-Administration Drug (F<1, p=0.377) main effects were not significant. The interactions were also not significant [Sex x Self-Administration Drug x Reinstatement Drug interaction: F<1, p=0.662; Sex x Self-Administration interaction: F<1, p=0.677; Sex x Reinstatement Drug: F<1, p=0.343; Self-Administration Drug x Reinstatement Drug Interaction: F(1,104)=2.748, p=0.100]. In the females, the group that received meth self-administration and a meth-prime had more c-Fos activation than the female groups that received a saline injection before reinstatement.

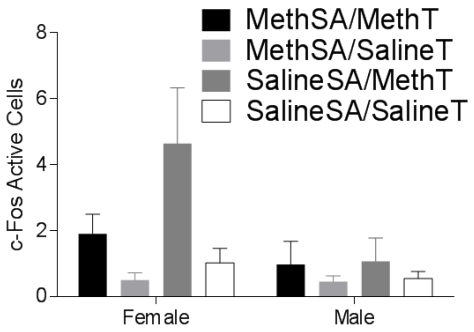
vlCPu

Figure 14.

vlCPu

Within Females	Corrected p Value
SalineSA/MethT > MethSA/Saline T	0.001
SalineSA/MethT > Saline/Saline T	0.005

Between Sex	
Female SalineSA/MethT > Male SalineSA/MethT	0.001



Significant post-hoc tests in the vlCPu are summarized (top). c-Fos active cells (±SEM) during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

In the vlCPu (Figure 14), there were significant main effects of Sex [F(1,104)=5.605, p=0.020] and Reinstatement Drug [F(1,104)=8.816, p=0.005]. The main effect of Self-Administration Drug [F(1,104)=2.647, p=0.107] and the interactions were not significant [Sex x Self-Administration Drug x Reinstatement Drug interaction: F(1,104)=1.088, p=0.299; Sex x Self-Administration interaction: F(1,104)=2.095, p=0.151; Sex x Reinstatement Drug: F(1,104)=3.511, p=0.064; Self-Administration Drug x Reinstatement Drug Interaction: F(1,104)=1.088, p=0.299]. Curiously, the female group that received an acute meth injection showed more activation than the saline reinstatement groups. This group of females also responded more than their male counterparts. This finding may imply in the vlCPu, an acute injection of meth robustly activates c-Fos; even more than meth following meth self-administration. However, analysis of the data revealed that this effect was driven by one SalineSA/MethT female rat that had an average vlCPu c-Fos score of 23.66. This is more than 3 standard deviations from the mean (group mean= 4.643, SD= 6.310), so caution may be warranted when evaluating this finding.

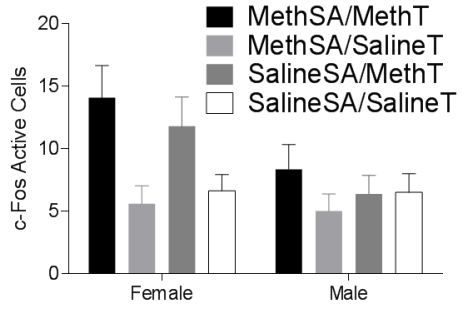
vmCPu

Figure 15.

vmCPu

Within Females	Corrected p Value
MethSA/MethT > MethSA/SalineT	0.006
MethSA/MethT > SalineSA/SalineT	0.021

Between Sex	
Female MethSA/MethT > Male MethSA/MethT	0.027
Female SalineSA/MethT > Male SalineSA/MethT	0.036



Significant post-hoc tests in the vmCPu are summarized (top). c-Fos active cells (±SEM) during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

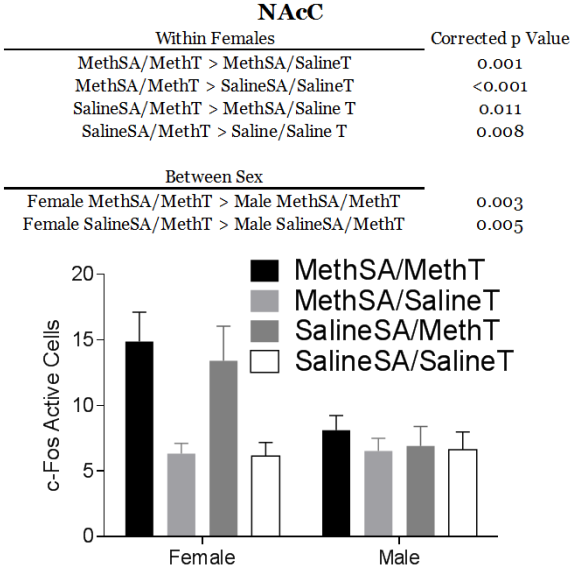
In the vmCPu (Figure 15), there were main effects of Sex [F(1,104)=5.408, p=0.022] and Reinstatement Drug [F(1,104)=10.978, p=0.001], as well as a significant Sex x Reinstatement Drug interaction [F(1,104)=4.185, p=0.043]. The main effect of Self-Administration Drug (F<1, p=0.734), Sex x Self-Administration Drug x Reinstatement Drug interaction (F<1, p=0.974), Sex x Self-Administration interaction [F<1, p=0.878], and Self-Administration Drug x Reinstatement Drug Interaction [F(1,104)=1.764; p=0.187] were not significant. Post-hocs on the planned comparisons revealed findings that again correspond with the activation in other brain regions and the behavioral results. Females in the MethSA/MethT group showed more activation than the female groups that did not receive a meth injection. There were no group differences in the males.

Additionally, both female groups that received meth during reinstatement showed more activation than their male counterparts. This outcome suggests that females show greater activation by a meth injection and the female group that received a meth injection following meth self-administration show the greatest activation.

Nucleus Accumbens Regions

NAcC

Figure 16.



NAcC

Within Females	Corrected p Value
MethSA/MethT > MethSA/SalineT	0.001
MethSA/MethT > SalineSA/SalineT	<0.001
SalineSA/MethT > MethSA/Saline T	0.011
SalineSA/MethT > Saline/Saline T	0.008

Between Sex	
Female MethSA/MethT > Male MethSA/MethT	0.003
Female SalineSA/MethT > Male SalineSA/MethT	0.005

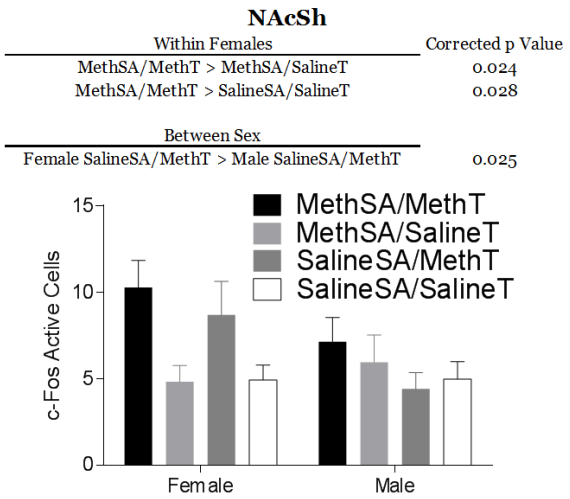
Significant post-hoc tests in the NAcC are summarized (top). c-Fos active cells (±SEM) during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

For c-Fos activation in the NAcC (figure 16), there were main effects of Sex [$F(1,104)=7.917$, $p=0.005$] and Reinstatement Drug [$F(1,104)=15.737$, $p<0.001$], as well as a significant Sex x Reinstatement Drug interaction [$F(1,104)=9.755$, $p=0.002$]. The main effect of Self-Administration Drug ($F<1$, $p=0.541$) and other

interactions were not significant [Sex x Self-Administration Drug x Reinstatement Drug interaction (F<1, p=0.996); Sex x Self-Administration interaction [F<1, p=0.894]; Self-Administration Drug x Reinstatement Drug Interaction [F<1, p=0.562]. In the females, both groups that received a meth injection during reinstatement showed more activation than either of the groups that received saline during reinstatement. These differences were again not found in the males. Females that received meth in reinstatement had higher c-Fos amounts than their male counter parts in the NAcC (Figure 16).

NAcSh

Figure 17.



Significant post-hoc tests in the NAcSh are summarized (top). c-Fos active cells (±SEM) during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

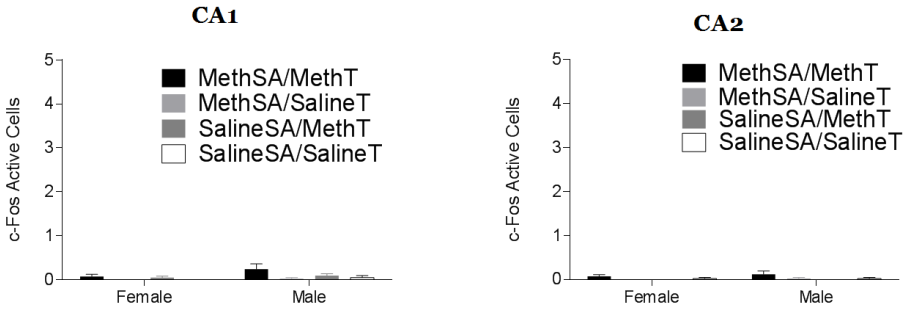
In the NAcSh (Figure 17), there was a main effect of Reinstatement Drug [F(1,104)=6.787, p=0.011] and a significant Sex x Reinstatement Drug interaction [F(1,104)=5.189, p=0.025]. The main effects of Sex [F(1,104)=2.752, p=0.100]

and Self-Administration Drug (F=1.901, p=0.170) were not significant. Nor were the other interactions [Sex x Self-Administration Drug x Reinstatement Drug interaction (F<1, p=0.985); Sex x Self-Administration interaction (F<1, p=0.559); Self-Administration Drug x Reinstatement Drug Interaction (F<1, p=0.362)]. In the females, the group that received a meth injection following meth self-administration showed more activation than the groups that did not receive meth during reinstatement. No differences were again found in the males. Notably, the females that received an acute injection of meth, responded more than the males that received meth for the first time during reinstatement. This pattern suggests that there may be sex differences in NAcSh activation with injection of meth, but only during initial administration.

Hippocampal Regions

CA1

Figure 18.



c-Fos active cells (±SEM) during reinstatement in the CA1 are displayed in the left graph. c-Fos active cells (±SEM) during reinstatement in the CA2 are displayed in the right graph. Groups are delineated by color.

Neuronal activation in the CA1 was nearly non-existent (Figure 18). Despite the nearly non-existent activation, a main effect of Reinstatement Drug

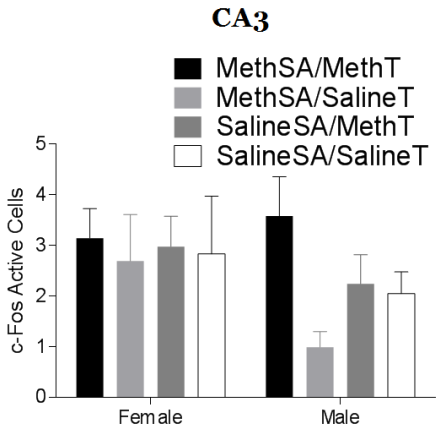
was found (F=6.156, p=0.015), with rats that received meth reinstatement higher than animals that received saline during reinstatement. There were no other significant main effects [Sex: F(1,104)=3.476, p=0.065; Self-Administration Drug: F<1, p=0.353;], nor significant interactions [Sex x Self-Administration Drug x Reinstatement Drug interaction: F<1, p=0.355; Sex x Self-Administration interaction: F<1, p=0.535; Sex x Reinstatement Drug: F<1, p=0.355; Self-Administration Drug x Reinstatement Drug Interaction: F(1,104)=1.537, p=0.217]. Additionally, there were no group differences in the planned comparisons.

CA2

c-Fos activation was also near zero levels in the CA2 (Figure 18). There were no significant main effects [Sex: F<1, p=0.450; Self-Administration Drug: F(1,104)=2.970, p=0.088; Reinstatement Drug F(1,104)=1.49, p=0.225] and the Sex x Self-Administration Drug x Reinstatement Drug interaction (F<1, p=0.811), Sex x Self-Administration interaction (F<1, p=0.460), Sex x Reinstatement Drug interaction (F<1, p=0.811) were also not significant. There was a Self-Administration Drug x Reinstatement Drug Interaction [F(1,104)=4.85, p=0.030], however none of the post-hoc tests reached significance.

CA3

Figure 19.

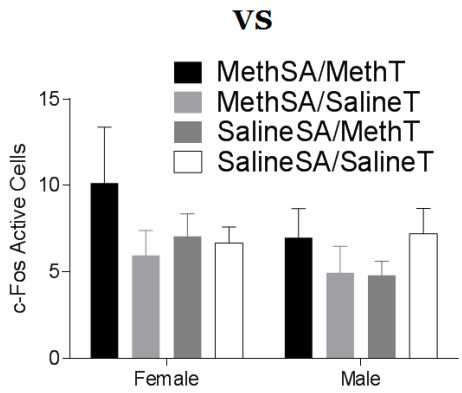


c-Fos active cells (±SEM) in the CA3 during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

In the CA3 (Figure 19), there were no significant main effects [Sex: $F(1,104)=1.932$, $p=0.168$; Self-Administration Drug: $F<1$, $p=0.883$; Reinstatement Drug $F(1,104)=2.861$, $p=0.094$] nor significant interactions in c-Fos activation [Sex x Self-Administration Drug x Reinstatement Drug interaction: $F(1,104)=1.109$, $p=0.295$; Sex x Self-Administration interaction: $F<1$, $p=0.902$; Sex x Reinstatement Drug: $F(1,104)=1.211$, $p=0.273$; Self-Administration Drug x Reinstatement Drug Interaction: $F(1,104)=1.851$, $p=0.177$]. Similar to other regions of the hippocampus, there were no significant differences in the planned comparisons.

VS

Figure 20.



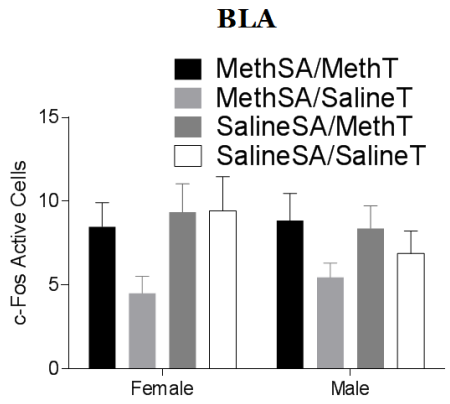
c-Fos active cells (±SEM) in the VS during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

There were no significant main effects [Sex: $F(1,104)=1.492$, $p=0.224$; Self-Administration Drug: $F<1$, $p=0.639$; Reinstatement Drug $F<1$, $p=0.383$] nor significant interactions [Sex x Self-Administration Drug x Reinstatement Drug interaction: $F<1$, $p=0.894$; Sex x Self-Administration interaction: $F<1$, $p=0.612$; Sex x Reinstatement Drug: $F(1,104)=1.069$, $p=0.304$; Self-Administration Drug x Reinstatement Drug Interaction: $F(1,104)=2.911$, $p=0.091$] in c-Fos activation in the VS (Figure 20). There were again no group differences in the planned comparisons.

Amygdala Regions

BLA

Figure 21.



c-Fos active cells (±SEM) in the BLA during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

In the BLA (Figure 21), there was a main effect of Reinstatement Drug [$F(1,102)=4.478$, $p=0.036$], with the rats that received saline in reinstatement lower than the animals that received meth. This effect was largely driven by low activation in the meth abstinence rats (MethSA/SalineT), again, possibly suggesting hypofunction in this condition. Despite the main effect, none of the post-hoc tests reached significance. Additionally, the main effects of Sex ($F<1$, $p=0.600$) and Self-Administration Drug [$F(1,102)=2.621$, $p=0.109$] were not significant. None of the interactions were significant [Sex x Self-Administration Drug x Reinstatement Drug Interaction: $F<1$, $p=0.605$; Sex x Self-Administration Interaction: $F(1,102)=1.360$, $p=0.246$; Sex x Reinstatement Drug: $F<1$, $p=0.814$;

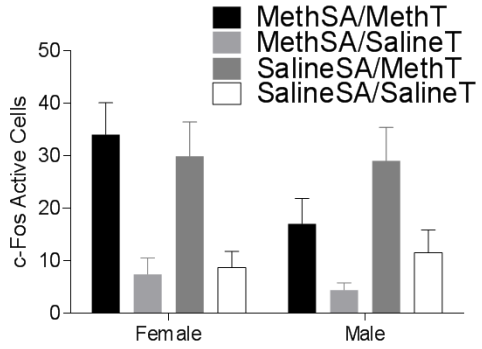
Self-Administration Drug x Reinstatement Drug Interaction: F(1,102)=2.045, p=0.156].

CEA

Figure 22.

CEA

Within Females	Corrected p Value
MethSA/MethT > MethSA/SalineT	<0.001
MethSA/MethT > SalineSA/SalineT	0.002
SalineSA/MethT > MethSA/Saline T	0.006
SalineSA/MethT > Saline/Saline T	0.012
Within Males	
SalineSA/MethT > MethSA/SalineT	0.003
Between Sex	
Female MethSA/MethT > Male MethSA/MethT	0.015



Significant post-hoc tests in the CEA are summarized (top). c-Fos active cells (±SEM) during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

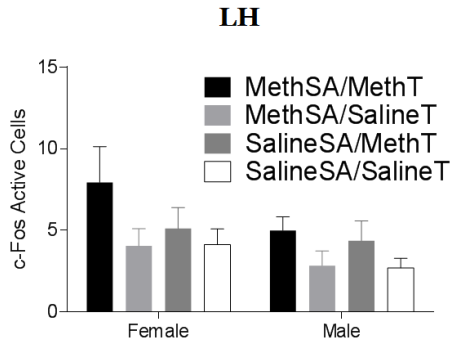
In the CEA (Figure 22), there was a significant main effect of Reinstatement Drug [F(1,102)=32.584, p<0.001]. The other main effects [Sex: F(1,102)=1.751, p=0.189; Self-Administration Drug: F(1,104)=1.417, p=0.237] and all of the interactions [Sex x Self-Administration Drug x Reinstatement Drug interaction: F<1, p=0.451; Sex x Self-Administration Interaction: F(1,102)=2.589, p=0.111; Sex x Reinstatement Drug: F(1,102)=1.689, p=0.197; Self-

Administration Drug x Reinstatement Drug Interaction: F<1, p=0.975] were not significant. For the females, both groups that received meth during reinstatement showed more activation than the 2 groups that received saline during reinstatement (Figure 22). The CEA was the only region examined that had significant group differences in the males with the group that received an acute injection of meth higher than the group that was given meth self-administration, but was in abstinence (MethSA/SalineT). The lower activation in the MethSA/SalineT group suggests possible neuronal hypofunction following self-administration in the absence of a drug prime. Additionally, the female MethSA/MethT group was higher than their male counterparts in this region as well.

Other Regions

LH

Figure 23.



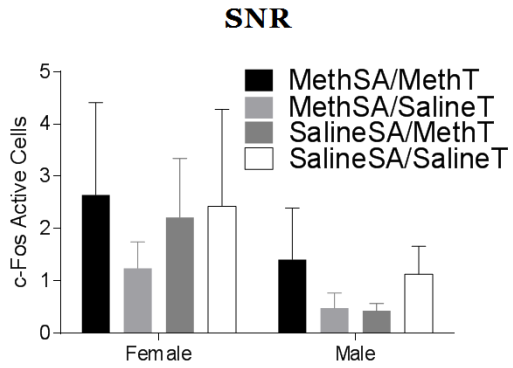
c-Fos active cells (±SEM) in the LH during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

In the LH (Figure 23), there was a main effect of Reinstatement Drug [F(1,104)=6.378, p=0.013] with rats receiving meth showing more activation

compared to saline rats. The main effects of Sex [F(1,104)=3.417, p=0.067] and Self-Administration Drug [F(1,104)=1.024, p=0.314] were not significant, nor were any of the interactions (all Fs<1). Additionally, none of the post-hoc tests reached significance.

SNR

Figure 24.

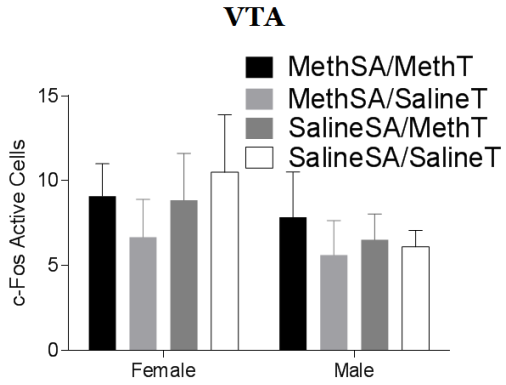


c-Fos active cells (±SEM) in the LH during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

In the analyses of c-Fos levels in the SNR (Figure 24), there were no significant main effects [Sex: F(1,103)=2.696, p=0.104; Self-Administration Drug: F<1, p=0.888; Reinstatement Drug F<1 p=0.648] nor significant interactions [Sex x Self-Administration Drug x Reinstatement Drug: F<1, p=0.998; Sex x Self-Administration: F<1, p=0.727; Sex x Reinstatement Drug: F<1, p=0.757; Self-Administration Drug x Reinstatement Drug: F(1,103)=1.099, p=0.297]. None of the planned comparisons were significant.

VTA

Figure 25.



c-Fos active cells (±SEM) in the VTA during reinstatement in the females (left bottom) and males (right bottom) are displayed. Groups are delineated by color.

In the VTA (Figure 25) there were no significant main effects [Sex: $F(1,103)=1.890$, $p=0.172$; Self-Administration Drug: $F<1$, $p=0.676$; Reinstatement Drug $F<1$, $p=0.601$]. There were also no significant interactions (all $Fs<1$) and no significant planned comparisons.

Summary

Using the standard procedures in our lab, female and male rats self-administered meth. Active lever responding was also maintained by saline with timeout cues, albeit at levels significantly lower than that maintained by meth. Females and males did not differ in active lever responding in the self-administration phase. When meth was removed in the extinction phase, responding was attenuated to saline levels. An unsurprising finding, given that extinction sessions contained the timeout cues that were presumably maintaining the modest levels of responding in the saline groups. Responding during

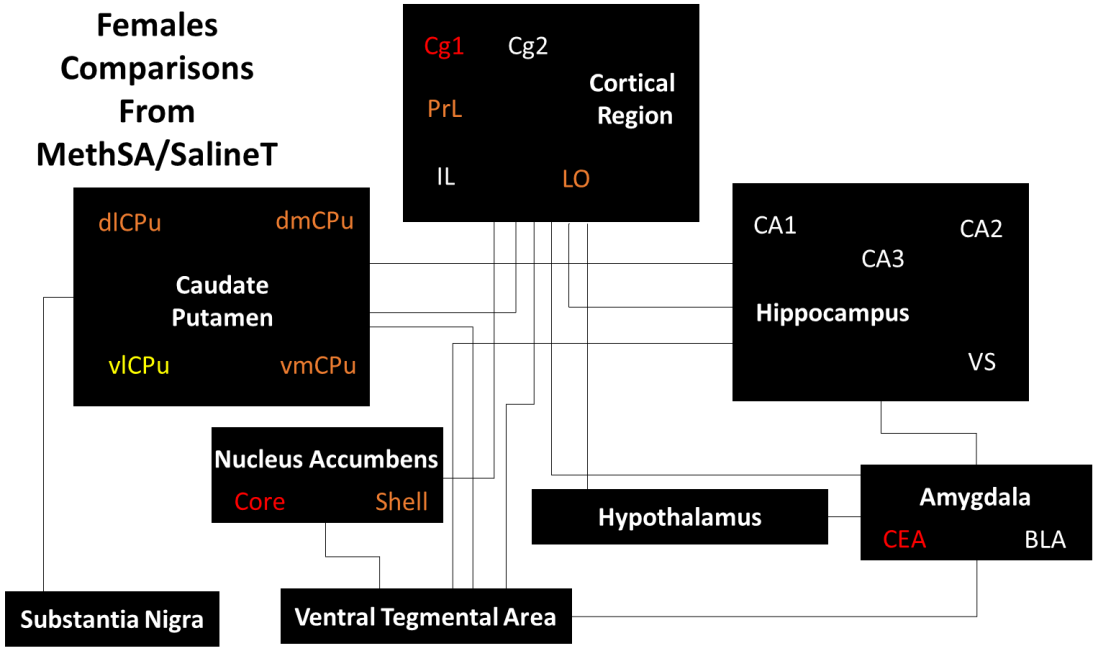
extinction was again similar between males and females. Meth-primed reinstatement of meth-seeking behavior was found in both males and females, although this effect was potentiated in females.

Investigation of c-Fos activation in the brain following reinstatement showed several notable findings. Generally speaking, the pattern of activation was in the same direction as the behavioral responding during reinstatement, with c-Fos activation more prevalent in the brains of the females that received a meth-prime following meth self-administration. More specifically, the most common differences in the planned comparisons were found between the females that received a meth trigger following self-administration (MethSA/MethT) and the females that had received long-term meth self-administration and did not receive a meth-prime (i.e., were in a period of abstinence from meth; MethSA/SalineT). This was largely a product of the high levels of c-Fos activation in the MethSA/MethT group and the low levels of activation in the MethSA/SalineT group. This MethSA/SalineT group often trended lower than the baseline group that never received meth (SalineSA/SalineT), although these differences did not reach statistical significance.

In the female rats, the MethSA/MethT group showed more activation than the MethSA/SalineT group in the Cg1, LO, PrL, dlCPu, dmCPu, vmCPu, NacC, NAcSh, and CEA (see Graphic 6). The group that received acute meth exposure (SalineSA/MethT) also showed higher c-Fos activation levels compared to the long-term self-administration with no prime group in several regions. These regions were the Cg1, vlCPu, NAcC, and CEA (Graphic 6). With the exception of

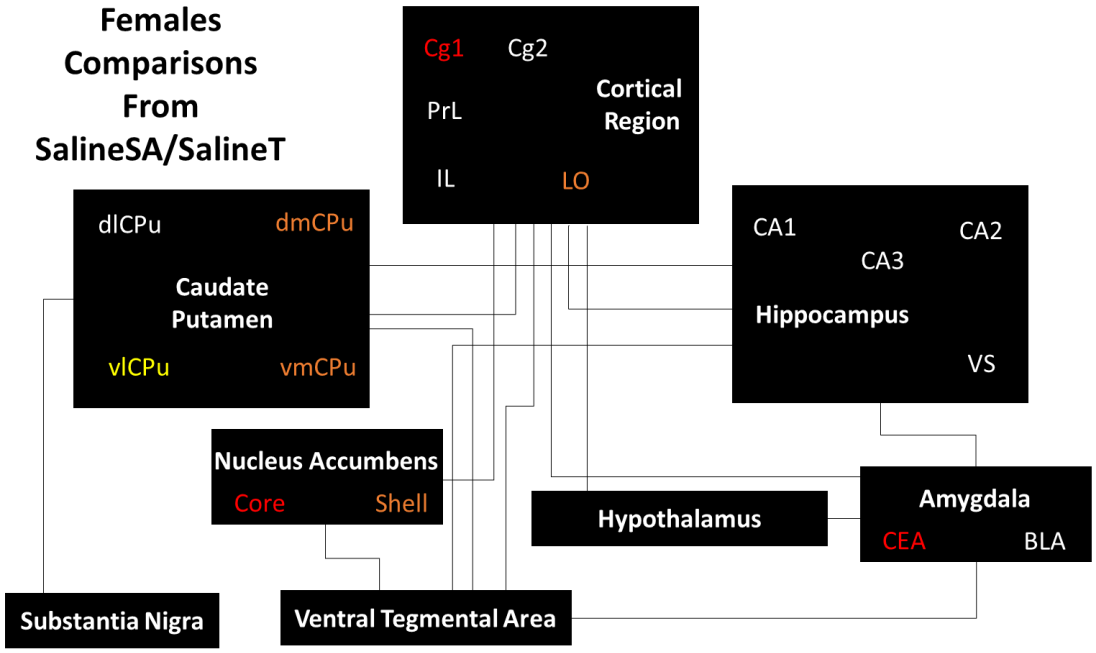
the vlCPu, the activation following an acute meth injection was less robust than the activation in the group that received a meth injection following a self-administration history with meth. This data pattern suggests that the learning history with meth self-administration further potentiated neural activity correlated with meth administration, except in the vlCPu which shows more activation following acute meth administration (see Graphic 6; region identified in yellow). Differences in the females between the baseline group (SalineSA/SalineT) and the reinstatement group (MethSA/MethT) were found in the Gg1, LO, dmCPu, vmCPu, NAcC, NAcS, and CEA (Graphic 7), while differences between the baseline group and an acute meth injection group were only detected in the Cg1, vlCPu, NAcC, and CEA (Graphic 7).

Graphic 6.



Comparisons from long-term self-administration with no prime group (MethSA/SalineT) are displayed. Significant differences of both MethSA/MethT AND SalineSA/MethT (vs MethSA/SalineT) are displayed in red. Significant differences of MethSA/MethT alone (vs MethSA/SalineT) are displayed in orange. Significant difference of SalineSA/MethT alone (vs MethSA/SalineT) is displayed in yellow. Areas with no differences are displayed in white.

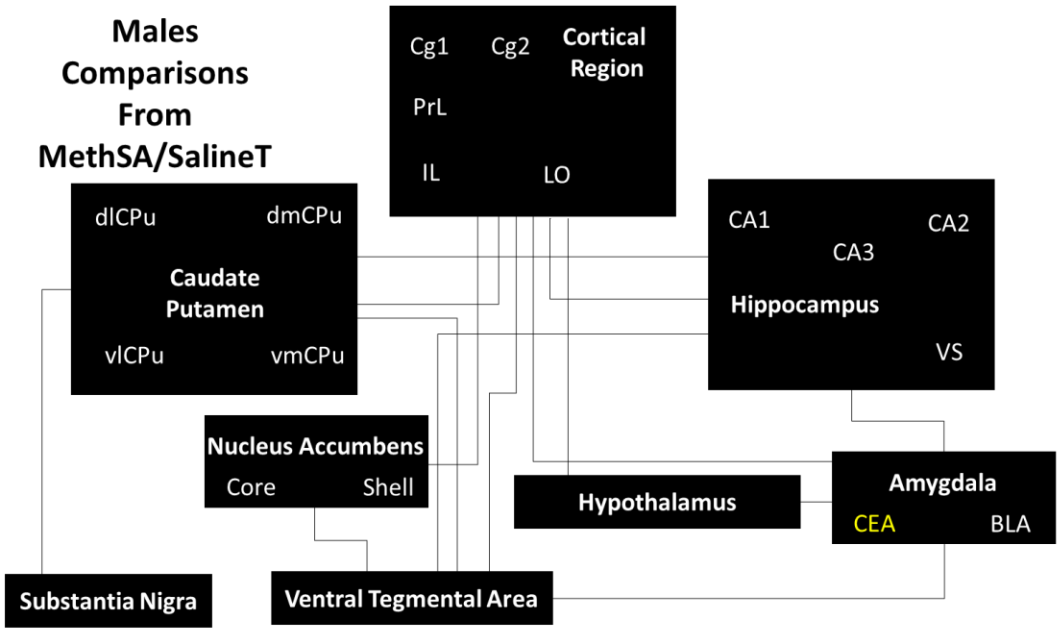
Graphic 7.



Comparisons from the baseline group with no meth (SalineSA/SalineT) are displayed. Significant differences of both MethSA/MethT AND SalineSA/MethT (vs SalineSA/SalineT) are displayed in red. Significant differences of MethSA/MethT alone (vs SalineSA/SalineT) are displayed in orange. Significant difference of SalineSA/MethT alone (vs SalineSA/SalineT) is displayed in yellow. Areas with no differences are displayed in white.

There were substantially fewer differences between the male groups. In fact, the only significant difference in the males was found in the CEA with the group that received acute meth injection showing higher c-Fos activation than the group that was drug free following long-term self-administration (SalineSA/MethT>MethSA/SalineT; see Graphic 8).

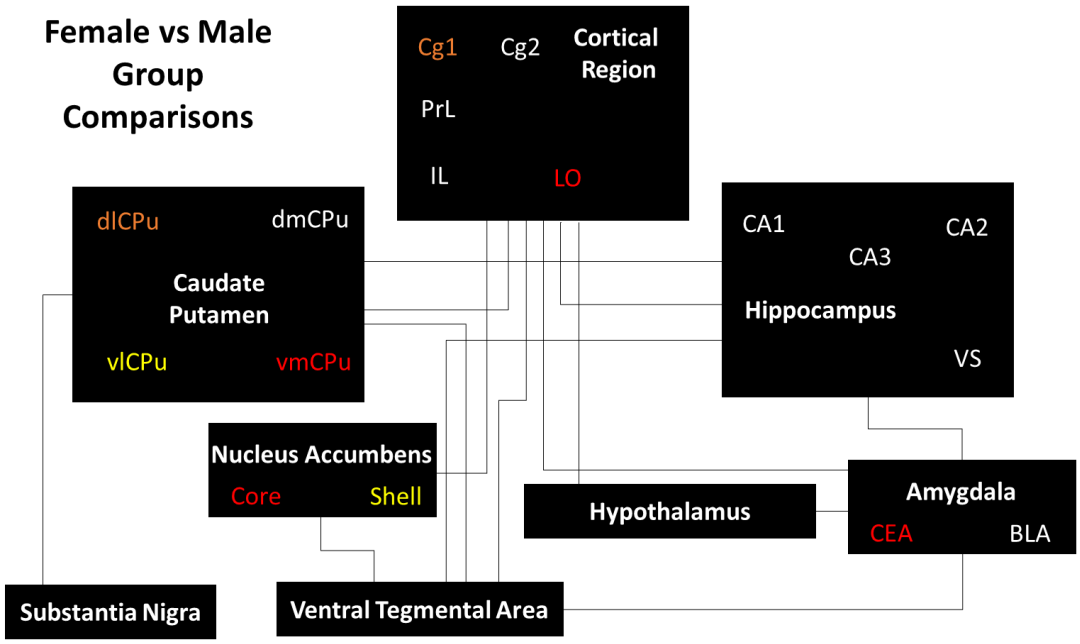
Graphic 8.



Comparisons from long-term self-administration with no prime group (MethSA/SalineT) are displayed. Significant difference of SalineSA/MethT alone (vs MethSA/SalineT) is displayed in yellow. Areas with no differences are displayed in white.

There were also significant differences detected in the planned comparisons between sexes. Females in the reinstatement group (MethSA/MethT) had higher c-Fos activation than their male counterparts in the Gg1, LO, dlCPu, vmCPu, NAcC, and CEA (Graphic 9). There were also differences between males and females following acute meth administration (SalineSA/MethT). Females had more c-Fos immunoreactivity following acute meth in the LO, vlCPu, vmCPU, NAcC, NAcSh, and CEA (Graphic 9).

Graphic 9.



Group comparisons between female and male c-Fos activation are displayed. Significant difference between females and males (in all cases females>males) in both the MethSA/MethT condition AND SalineSA/MethT condition are displayed in red. Significant difference between females and males in only the MethSA/MethT condition are displayed in orange (females>males). Significant difference between females and males in only the SalineSA/MethT condition are displayed in yellow (females>males).

CHAPTER 4

EXPERIMENT 2

INVESTIGATION OF SEX DIFFERENCE IN NICOTINE- AND COCAINE-TRIGGERED METH REINSTATEMENT

Introduction

Drug-primed Reinstatement with Alternate Drug Type

The majority of meth-dependent individuals that seek drug treatment return to meth use within 6 months of treatment (Brackins et al., 2011; Brecht et al., 2004). These individuals are presumably motivated to stop meth dependence, report a strong desire to quit, but return to use regardless. The inability to maintain meth cessation following treatment highlights the insufficiency of current behavioral and pharmacological interventions. Understanding the factors that may influence a return to drug use following cessation (i.e., relapse) is crucial to increasing the efficacy of current interventions. To this end, Experiment 2 examined sex differences in an innovative relapse model: meth reinstatement triggered by alternative drugs that show high comorbidity with meth use (i.e., nicotine and cocaine).

Experiment 2 was precipitated by notable findings from several novel studies; one of which was conducted in our lab. Pittenger et al. (females published in 2016; males In Preparation) used our standard meth self-administration procedures to train male and female rats to self-administer meth. This training was followed by extinction and then meth-primed reinstatement.

The primary goal of the study was to examine the effects of varenicline (Chantix®), a partial α4β2 and full α7 nicotinic acetylcholine receptor (nAChR) agonist (Coe et al., 2005a; Coe et al., 2005b; Gonzales et al., 2006; Mihalak et al., 2006; Smith et al., 2007), on meth self-administration and reinstatement in male and female rats. Varenicline has been suggested as a potential meth abuse disorder pharmacotherapy (Verrico et al., 2014; Zorick et al., 2010). Of relevance to this report was the reinstatement testing. Rats received IP varenicline administration (0, 0.3, 1.0, 3.0 mg/kg) prior to meth-primed reinstatement. Notably, and contrary to speculation that suggest the effective use of varenicline in the treatment of meth dependence, the lower doses of meth (0.3 and 1.0 mg/kg) actually increased drug seeking behavior during meth-primed reinstatement, but only in the females (Pittenger et al., 2016; Pittenger et al., In Preperation).

This finding engendered further inquiry on drug-induced reinstatement. If varenicline could increase meth-induced drug seeking, could nicotine, also a nicotinic acetylcholine receptor agonist, alter meth drug seeking? Further, could nicotine alter meth reinstatement on its own? While the drug-primed model of reinstatement does serve as a useful tool for the evaluation of behavioral and pharmacological intervention, as well identification of the neurobiology underlying relapse, it does have a limitation. If an individual administers a small amount of meth, it can be argued that relapse has already occurred. Therefore we would be studying the behavior and mechanisms following a relapse and not

precipitating it. This notion may make the investigation of other drugs that facilitate relapse to meth more translationally relevant.

Nicotine is of particular interest given its legality, availability, and social acceptance. Recall our meth addict from the General Introduction and imagine in addition to abusing meth, she is also a smoker; not hard, as 97% of meth addicts also smoke (Brecht et al., 2004). While attempting to abstain from meth use following treatment, she continues smoking cigarettes. Under the right circumstances, could the nicotine found in cigarettes be serving as a drug prime, triggering the persistent and chronic relapse she is suffering? This was the central question of interest in Experiment 2.

Research suggests that under certain parameters nicotine can indeed trigger meth reinstatement. In a study conducted by Neugebauer et al. (2010), male rats were repeatedly injected with nicotine or saline, unpaired (temporally separate) from meth self-administration sessions. Rats then underwent extinction of meth-maintained lever pressing followed by reinstatement testing. Rats were administered nicotine before the reinstatement session (no meth available). Interestingly, rats that had a prior history with repeated nicotine administration demonstrated meth-seeking behavior (lever pressing on the previously meth-maintained lever). Reinstatement was not triggered in male rats that had no previous experience with nicotine (Neugebauer et al., 2010). However, others have found acute nicotine can induce meth reinstatement (Hiranita et al., 2006). These findings clearly demonstrate that nicotine can serve as a trigger for meth reinstatement in male rats.

The effect of acute and repeated nicotine on reinstatement in female rats has not been studied. This is quite surprising given the high comorbidity of meth and nicotine dependence and robust sex differences associated with pre-clinical models of nicotine addiction (Caldarone et al., 2008; Chaudhri et al., 2005; Gentile et al., 2011; Hamilton et al., 2009; Harrod et al., 2004; Kanyt et al., 1999; Rhodes et al., 2004; Rhodes et al., 2001; Torres et al., 2013, Torres et al., 2015). For example, Caldarone et al. (2008) found that anxiety-like behaviors were increased in female, but not male mice following chronic exposure to nicotine. Other studies also suggest that female rats have greater withdrawal symptoms from nicotine than males (Gentile et al., 2011; Torres et al., 2013; Torres et al., 2015), show greater stress response following acute nicotine administration (Gentile et al., 2011), and show more nicotine sensitization (Harrod et al., 2004; Kanyt et al., 1999).

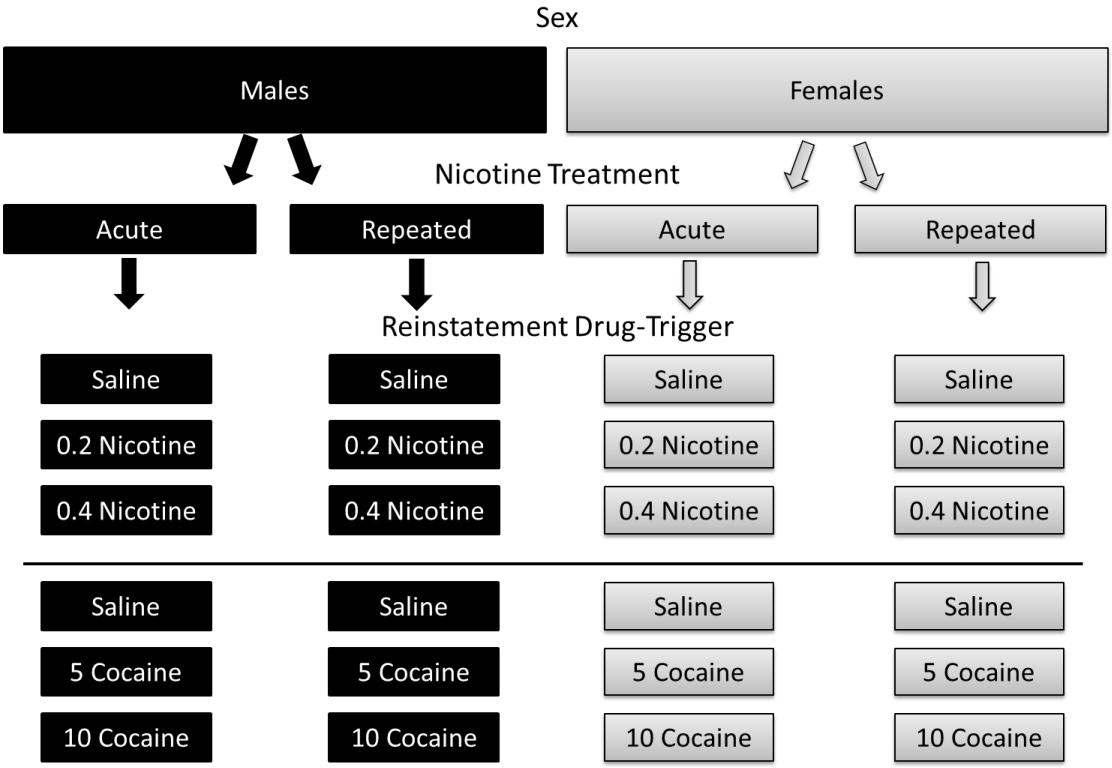
Studies have also demonstrated that nicotine differentially alters dopaminergic systems in female and male rats (Dluzen and Anderson, 1997; Harrod et al., 2004; Pittenger et al., 2016; Pogun, 2001). For example, Dluzen and Anderson (1997) showed that peak nicotine-evoked (10 μM *in vitro* infusion of nicotine) dopamine release tended to be lower in striatal regions of ovariectomized females compared to castrated male rats. Estrogen treatment resulted in differential effects, increasing nicotine-evoked dopamine release in the females and decreasing release in the males. Additionally, extracellular dopamine concentrations in the nucleus accumbens have also been reported to be higher in female rats compared to male rats following systemic nicotine

injections (Pogun, 2001). Harrod and colleagues (2004) found that females exhibited an increase in the number of dopamine transporters in the NAcC following 21 days of nicotine infusions (50 μg/kg/ml). Finally, some research shows that nicotine does trigger reinstatement of nicotine seeking differentially between the sexes (Swalve et al., 2016). These reinstatement effects, however, are not ubiquitous with others finding no differences between the sexes in nicotine-triggered reinstatement of nicotine seeking (Feltenstien et al., 2012).

Given the robust sex differences in nicotine sensitivity highlighted above and the increased vulnerability to reinstatement found in females detailed in the General Discussion, determining if nicotine could differentially reinstate meth drug-seeking in males and females was the primary goal of Experiment 2. Additionally, we wanted to examine the generality of drug-primed reinstatement using alternative drugs as triggers, namely cocaine. That is, using the procedures detailed below, can other drugs of abuse (i.e., cocaine) also function as a drug trigger for meth-seeking behavior, or is this an effect specific to nicotine? Cocaine in particular was interesting in that it activates similar brain regions during drug-primed reinstatement (cf. Experiment 1 Results and Neisewander et al., 2000). This experiment begins to fill a gap in the literature regarding sex differences in a novel behavioral model of relapse by examining if females are more sensitive to both acute and repeated nicotine-triggered meth reinstatement, as well as cocaine-triggered meth reinstatement.

Design

Graphic 10.

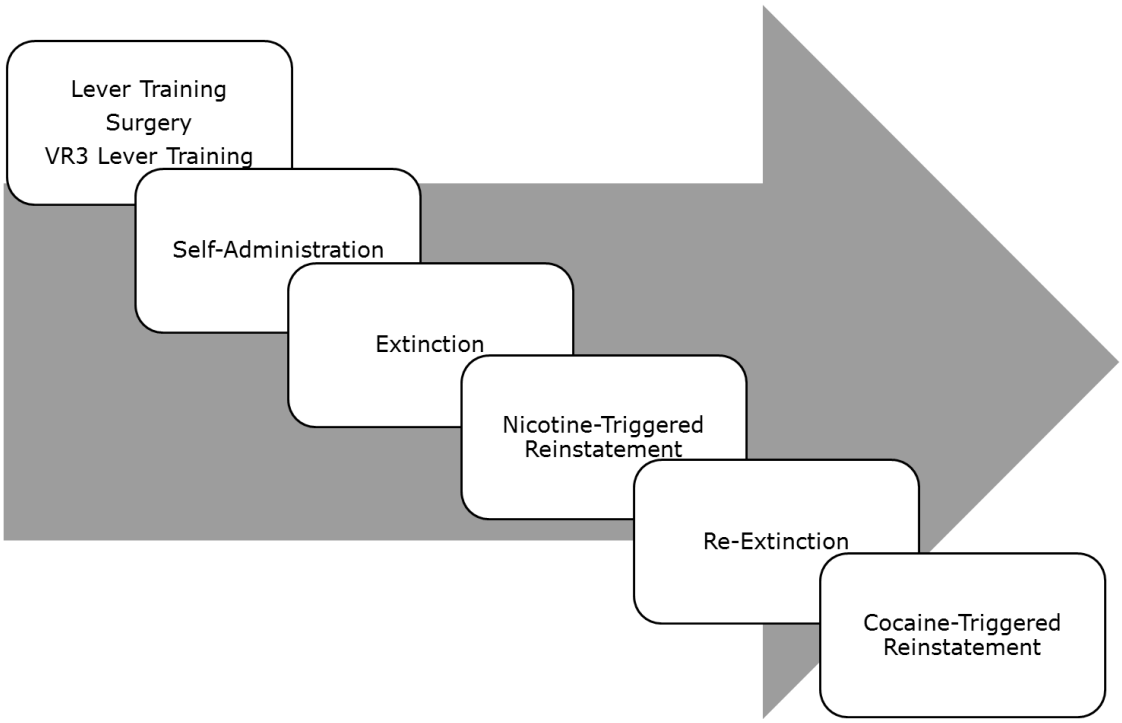


Experiment 2 used a 2 x 2 x 3 mixed factorial design with sex (male or female) and nicotine condition (acute or repeated) as between-subjects variables and nicotine reinstatement dose (0.0 [saline], 0.2 , and 0.4 mg/kg) as a within-subjects factor (see Graphic 10). Additionally, to assess if a different drug of abuse (cocaine) could trigger meth reinstatement, a separate 2 x 2 x 3 mixed factorial design with sex (male or female) and nicotine condition (acute or repeated) as between-subjects variables and cocaine reinstatement dose (0.0 [saline], 5 mg/kg , and 10 mg/kg) as a within-subjects factor was also used. The design of this experiment allowed for the examination of sex differences in nicotine-triggered reinstatement over multiple doses of nicotine in rats

repeatedly administered nicotine (repeated condition) or were first injected with nicotine during the reinstatement tests (acute condition). The follow-up investigation of cocaine assessed the generality of drug-primed reinstatement.

Procedures

Graphic 11.



Preliminaries and Surgery

Rats received preliminary lever training following acclimation to the colony room and food restriction to maintain 90% of free-feeding weight. Indwelling jugular catheters were then implanted using our standard protocol and rats were allowed to recover for 7 days. Rats were then place on a variable ratio 3 (VR3) schedule of sucrose reinforcement in standard post-surgery lever training (refer to GENERAL PROCEDURES for details).

Self-administration

Following preliminary training and surgery, male and female rats were split into 2 nicotine treatment conditons prior to meth self-administration: repeated or acute (Female Repeated: n=14; Female Acute: n=14; Male Repeated: n=12; Male Acute: n=13). Rats in the repeated condition received nicotine injections (SC; 0.4 mg/kg) 4 h after their daily meth self-administration session (cf. Neugebauer et al., 2010). This temporal arrangement was selected so that the nicotine stimulus could not serve as a drug-context that may have induced reinstatement later in the experiment. Previous research suggests that nicotine can serve as a reinstatement trigger through non-associative mechanisms in male rats. That is, repeated nicotine administration does not need to co-occur with the self-administration session (Neugebauer et al., 2010). Rats in the acute condition received saline injections 4 h after their self-administration sessions. Meth self-administration sessions and assigned injections continued for 21 days (see General Procedures for details).

Experiment 2 utilized a new MedPC program to administer meth. The program was designed to use a stock dose of meth (0.05 mg/kg/infusion) and alter the volume infused to account for slight differences in body weight using the equation Meth Dose = [1-sec*(Current Weight/ Average Weight Used to Calculate Meth Stock)]. While the program worked, the infusion pumps were not set for a variable infusion time, so all rats received a 1-sec infusion of the stock dose. The outcome of this oversight was that females with a slightly lower body weight than

males received an average meth dose of 0.059 mg/kg/infusion; the males received an average meth dose of 0.042 mg/kg/infusion.

Extinction

Extinction sessions commenced 24 h after the last self-administration session. Extinction sessions were identical to self-administration sessions except meth was no longer infused. Requisite VR3 responding on the active lever still produced the same cues and the timeout. To match the procedures of Neugebauer and colleagues (2010), all injections administered following the sessions were saline (i.e., nicotine was no longer administered). Extinction was conducted daily for 14 sessions.

Nicotine-Triggered Reinstatement

Twenty-four hours after the last extinction session, rats began nicotine-triggered reinstatement testing. Reinstatement testing proceeded over 3 days. Reinstatement sessions were identical to extinction sessions (i.e., meth not available). Five min prior to reinstatement sessions, rats were administered 0.0 (saline), 0.2, or 0.4 mg/kg nicotine. Random assignment for each rat was used to construct the order in which each dose was tested.

Re-Extinction

Three additional days of extinction were then given. These re-extinction sessions were identical to earlier extinction sessions and occurred across consecutive days.

Cocaine-Triggered Reinstatement

Following re-extinction, rats began cocaine-triggered reinstatement testing. Similar to reinstatement with nicotine, testing proceeded over 3 days. Reinstatement sessions were identical to extinction sessions (i.e., meth not available). Fifteen min prior to reinstatement sessions, rats were administered 0.0 (saline), 5, or 10 mg/kg cocaine IP. Random assignment for each rat was again used to construct the order in which the doses were tested.

Dependent Measures

While lever-Pressing was the primary dependent measure during the behavioral phases of Experiment 1, the small difference in dose (recall Experiment 2 Self-administration Procedures) created a situation where this measure may not be optimal. That is, the females that received a slightly higher meth dose (0.059 mg/kg) titrated meth intake with fewer lever presses than the males that received a lower meth dose (0.042 mg/kg). Accordingly, a better measure for acquisition may be meth intake. Intake was calculated for each rat in each self-administration session using the equation Drug Received = [((Average Weight Used to Calculate Meth Stock/Current Weight)*Stock Meth Dose)*Infusions Earned]. The amount of drug received then served as the primary dependent measure during acquisition. Active lever presses served as the primary dependent measure when meth was not available (i.e., extinction, nicotine- and cocaine- induced reinstatement). To show inactive lever responding relative to active lever responding during self-administration, a discrimination index was again calculated using the following formula: Discrimination Index = [Active Lever Presses/ (Inactive Lever Presses + Active Lever Presses)]. A

Discrimination Index value of 0.5 indicates equal responding on the active and inactive lever (i.e., no discrimination between levers); a value >0.5 indicates more pressing on the active lever. Lever pressing on the inactive lever was near zero following early acquisition and remained for the rest of the experiment (data not displayed).

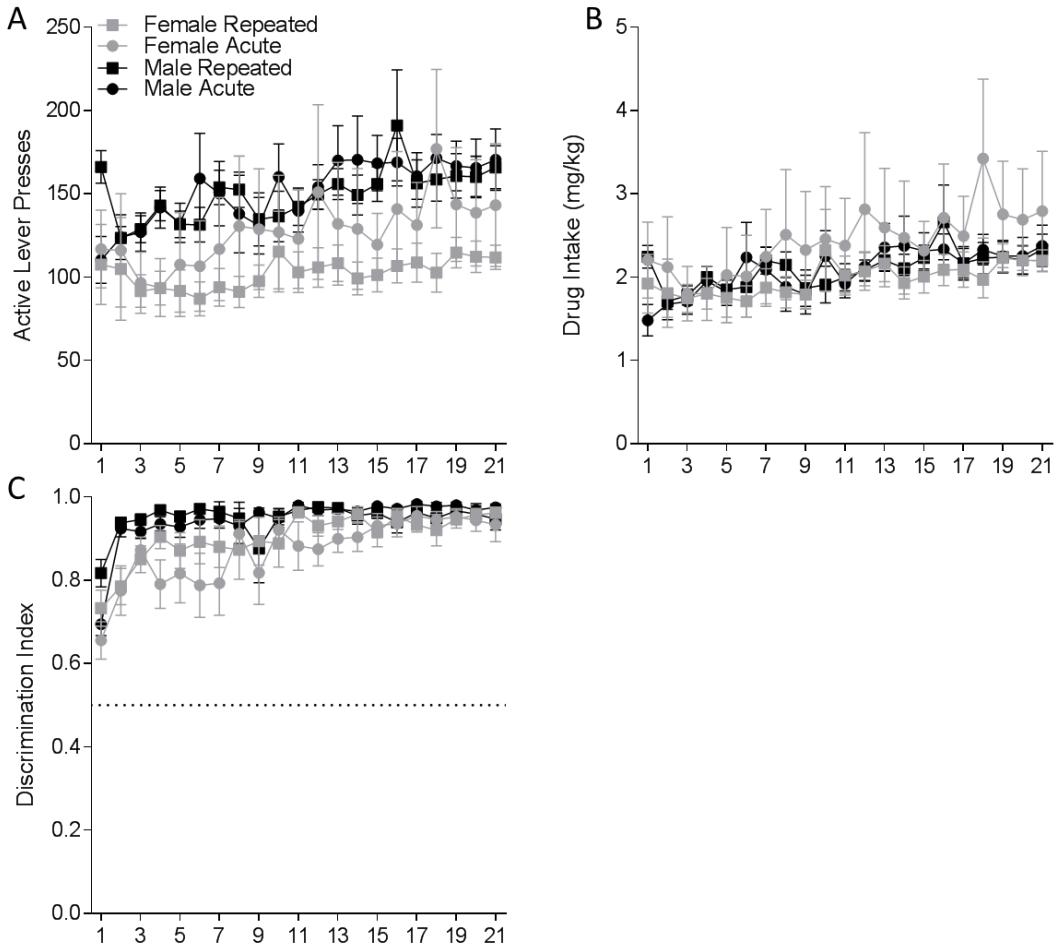
Statistical Analyses

Active lever responding, drug intake, and discrimination index in acquisition were analyzed by 2 separate 3-way mixed measures analysis of variance (ANOVA) with Sex (Female vs Male) and Nicotine Treatment (Acute vs Repeated) as between-subjects factors and Session as a within-subjects factor. Active lever responding in extinction and re-extinction were analyzed by the same 3-way mixed measures. Nicotine- and cocaine-primed reinstatement were analyzed by 2 separate 3-way mixed measures ANOVA with Sex (Female vs Male) and Nicotine Treatment Group (Acute vs Repeated) as between-subjects factors and Reinstatement Dose [0 (saline), 0.2 , 0.4 mg/kg for nicotine reinstatement; 0 (saline), 5, 10 mg/kg for cocaine reinstatement] as a within-subjects factor. To adjust for multiple comparisons, Tukey HSDs were utilized for post-hoc analysis of behavioral data. Statistical significance was declared at $p<0.05$.

Results

Self-Administration

Figure 26.



A: Active Lever presses (±SEM) during self-administration sessions for females (grey) and males (black) in the repeated (square) and acute (circle) conditions is displayed. B: Total meth intake (±SEM) accounting for body weight during each self-administration session is displayed. C: Discrimination Index (±SEM) during self-administration sessions is displayed.

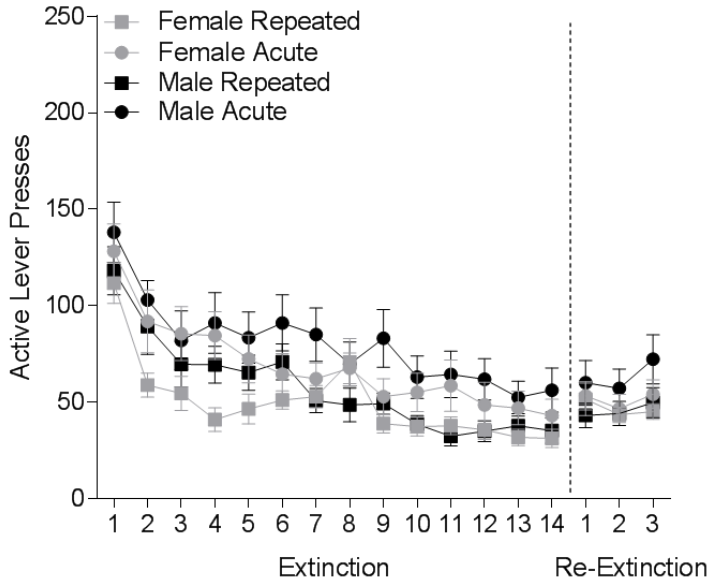
Rats again showed robust active lever responding (Figure 26A). Analysis of active lever pressing, unsurprisingly given females received a larger meth dose, had a significant main effect of Sex [$F(1, 49)=4.057$; $p=0.049$] with females responding less than the males (Figure 26A). There was also a main effect of Session [$F(20, 980)=3.670$; $p<0.001$] with responding escalating during the phase. The main effect of Nicotine Treatment ($F<1$; $p=0.464$) and all of the interactions (all $Fs<1$) were not significant. To account for the difference in meth

dose between the sexes (detailed in Self-Administration Procedures) analysis of the drug intake in acquisition was conducted (Figure 26B). There was no longer a main effect of Sex ($F<1$, $p=0.727$). That is, females and males titrated consumption of meth to similar levels. There was again a main effect of Session [$F(20, 980)=2.073$; $p=0.025$] with drug intake at the end of self-administration higher than levels in early sessions. Nicotine treatment 4 h after each session did not alter the amount of meth received ($F<1$, $p=0.499$) and again none of the interactions were significant ($Fs<1$).

Rats quickly discriminated between the active and inactive lever (Figure 26C) showing better discrimination as self-administration progressed [main effect of Session: $F(20,980)=13.500$, $p<0.001$]. There was a main effect of Sex [$F(1,49)=7.745$, $p=0.007$] and a significant Sex x Session interaction [$F(20,980)=1.810$, $p=0.016$]. The females showed statistically lower lever discrimination during initial self-administration sessions; females reached male levels consistently by session 12. This finding parallels the findings of Experiment 1, that also showed a trend (correct $p=0.074$) for male lever discrimination to be better. Nicotine treatment did not have a significant effect on the discrimination index [$F<1$, $p=0.441$] and there were no other significant interactions [Sex x Nicotine Treatment Group x Session: $F<1$, $p=0.609$; Sex x Nicotine Treatment Group: $F<1$, $p=0.497$; Nicotine Treatment Group x Session $F(20,980)=1.401$, $p=0.112$].

Extinction and Re-extinction

Figure 27.



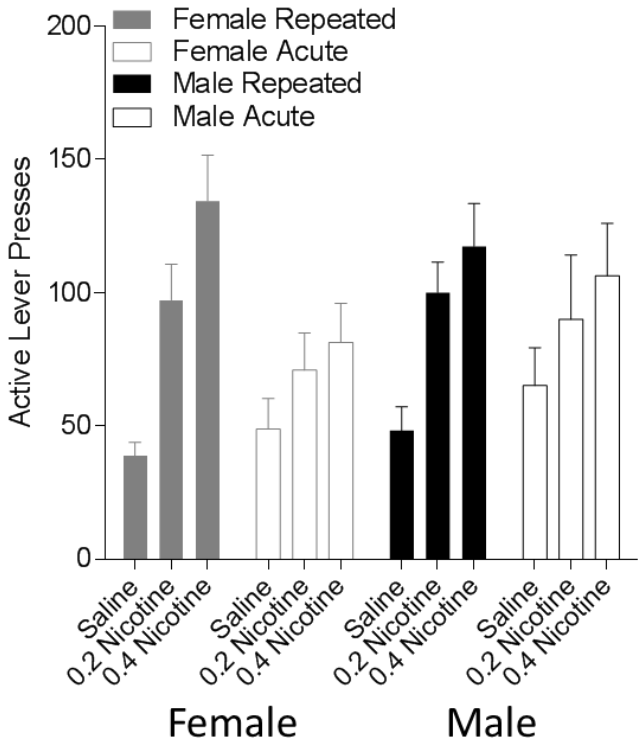
Active lever presses (± SEM) in extinction (left side) and re-extinction (right side) for female (grey) and male (black) rats in the repeated (square) and acute (circle) nicotine treatment conditions.

Active lever pressing was attenuated in extinction (Figure 27). There was a significant main effect of Session [$F(13,637)=38.783$, $p<0.001$], with responding in the early sessions significantly higher than responding in the subsequent session. Notably, there was a significant main effect of Nicotine Treatment [$F(1,49)=6.959$, $p=0.011$]. Rats that received nicotine repeatedly throughout the self-administration phase of the experiment responded less than rats treated with saline. Extinction in females and males was similar with no main effect of Sex [$F(1,49)=1.559$, $p=0.217$]. In re-extinction (Figure 27), the main effect of Sex ($F<1$, $p=0.479$) and group [$F(1,49)=2.170$, $p=0.147$] were not significant, but there was an effect of session [$F(1,98)=3.826$, $p=0.0251$]. Session 2 was slightly lower than session 3, with no other differences in re-extinction sessions. Visual

inspection revealed this variation was quite small, and as groups did not differ, not a major concern.

Nicotine-Triggered Reinstatement

Figure 28.



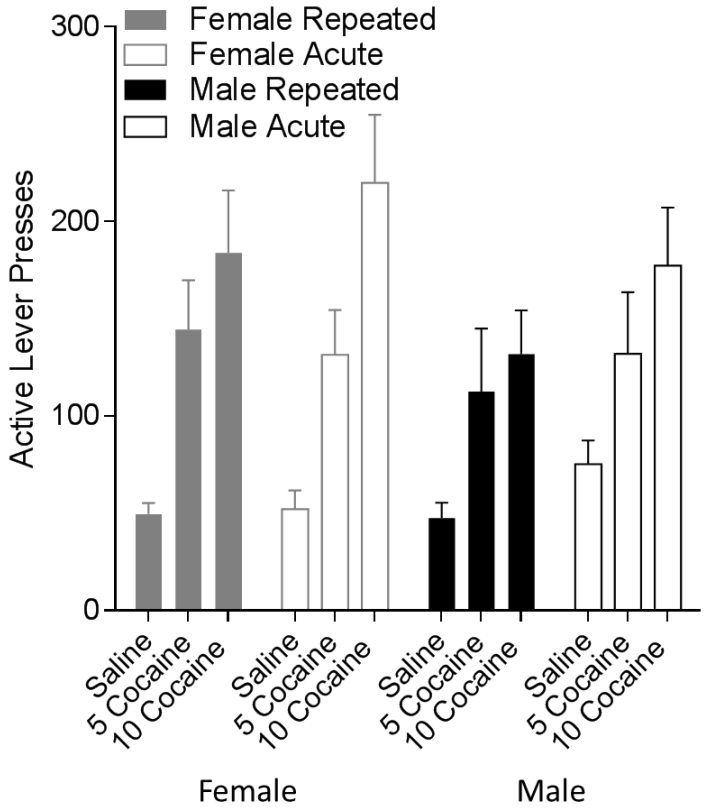
Active lever presses (±SEM) for female (grey) and male (black) rats during nicotine-triggered reinstatement. Filled bars delineate the repeated condition and open bars delineate the acute condition.

Overall, nicotine reinstated robust meth-seeking behavior (Figure 28). Analysis of nicotine-induced reinstatement of meth-seeking revealed a main effect of Reinstatement Dose [$F(2, 98)=38.311$, $p<0.001$], with no significant effect of Sex or Nicotine Treatment Group ($Fs<1$). Notably, there was a significant Nicotine Treatment x Reinstatement Dose interaction [$F(2,98)=5.691$, $p=0.005$. In the Nicotine Repeated and Nicotine Acute groups, 0.2 mg/kg and 0.4 mg/kg

nicotine administration induced more active lever pressing compared to no nicotine injection (Saline). However, reinstatement following a 0.4 mg/kg administration was potentiated in rats that had received nicotine repeatedly during self-administration when compared to rats that had no previous experience with nicotine (Acute groups). That is, when nicotine was previously experienced more reinstatement of meth-seeking was induced by 0.4 mg/kg nicotine. There were no other significant interactions (Fs<1).

Cocaine-Triggered Reinstatement

Figure 29.



Active lever presses (±SEM) for female (grey) and male (black) rats during cocaine-triggered reinstatement. Filled bars delineate the repeated condition and open bars delineate the acute condition.

During cocaine-triggered reinstatement of meth-seeking (Figure 29) there was a significant main effect of Reinstatement Dose [$F(2,980=45.753$, $p<0.001$]. Cocaine robustly induced meth-seeking behavior. A dose effect was determined with 10 mg/kg cocaine inducing the most reinstatement responding, 5 mg/kg triggering an intermediate amount. The main effect of Sex and Nicotine Treatment were not significant ($Fs<1$). Additionally, none of the interactions were significant [Sex x Nicotine Treatment Group x Reinstatement Dose: $F<1$, $p=0.901$; Sex x Nicotine Treatment Group: $F<1$, $p=0.574$; Sex x Reinstatement Dose: $F(2,98)=2.531$, $p=0.085$; Group x Reinstatement Dose: $F(2,98)=1.114$, $p=0.333$].

Summary

Under our procedures, females and males readily self-administered meth. While females did press the active lever less than males (females<males) during acquisition, the lower responding in the females was likely an effect of the slightly greater meth dose (0.059 mg/kg/inf for females vs 0.042 mg/kg/inf for males). Females and males administrated equivalent amounts of meth, accordant with the self-administration results of Experiment 1. Males discriminated between the active and inactive lever sooner than the females, matching a similar trend that did not reach significance in Experiment 1. Repeated, unpaired nicotine administration did not affect meth self-administration.

Extinction attenuated active lever pressing. Males and females decreased responding during extinction similarly. Interestingly, rats that were repeatedly

treated with nicotine during the self-administration phase showed more persistent active lever responding in the face of non-reinforcement.

Nicotine reinstated meth-seeking behavior in male and female rats with no difference between the sexes. While 0.2 and 0.4 mg/kg nicotine induced reinstatement in both nicotine treatments (i.e., acute and repeated), a prior history of nicotine administration did potentiate this effect even further. Cocaine also reinstated meth-seeking behavior. This effect was again similar in males and females. A significant dose effect was detected with 5 mg/kg triggering more responding than 0 (saline) and 10 mg/kg triggering more than 5 mg/kg. Nicotine treatment during self-administration did not alter cocaine-primed meth-seeking.

CHAPTER 5

DISCUSSION

Experiment 1

In Experiment 1, rats readily self-administered meth. This matches the preliminary experiment conducted as the basis of this work, previous work in our lab (Charntikov et al.,2015; Pittenger et al., 2016; Reichel et al., 2008; Reichel et al., 2009), and the findings of other labs (Beardsley et al., 2010; Cornish et al., 2012; Cox et al., 2013; Holtz et al., 2012; Hofford et al., 2014; Reichel et al., 2012; Roth and Carroll, 2004; Rubio et al., 2015; Shepard et al., 2004; Sobieraj et al., 2016). Females and males robustly administered meth and no difference in meth taking was detected. This lack of sex difference during self-administration is common, but not ubiquitous. Differences in sex, with females taking more than males, are often established when self-administration sessions are longer in duration than the 2 h protocol used in this study (Roth and Carroll, 2004; Reichel et al., 2012). The lack of difference in our procedures can be viewed as a strength of this study. As meth intake was similar during this study, we are not concerned with differential intake complicating interpretation of the sex differences found during the subsequent reinstatement phase.

The inclusion of the saline self-administration conditions was primarily to assure equivalent learning histories in the conditions that would receive acute meth administration (SalineSA/MethT) and the baseline conditions (Saline SA/SalineT) with the meth self-administration conditions. Their use also served as a methodological strength of Experiment 1. Recall that the only difference

between the meth condition and the saline condition is the type of infusion (meth or saline); pre-training, infusion/timeout cues, progression through the study, etc. were similar. Thus, this saline benchmark allowed for not only the detection of c-Fos differences specific to drug type in self-administration, but also careful analysis of the behavior controlled by meth compared to that controlled by the weak reinforcing effects of infusion cues (Caggiula et al., 2009; Chaudhri et al., 2006b; Palmatier et al., 2006). Indeed, the groups that received saline during self-administration differentially responded on the active vs inactive lever. However, this responding was significantly lower than responding for meth. This finding supports the notion that the timeout cues (i.e., light illumination and lever extraction for 20 sec) have weak reinforcing value that maintains modest levels of responding (Caggiula et al., 2009; Chaudhri et al., 2006b; Palmatier et al., 2006).

In extinction, responding in the meth groups was attenuated to levels comparable to the saline benchmarks, an expected outcome, as extinction sessions included the timeout cues that were presumably maintaining the low levels of responding in the saline groups. Responding during extinction was similar between females and males. While some work has found females may be more resistant to extinction of meth self-administration [e.g. our preliminary experiment and Cox et al., (2013)], the current finding matches others that do not report differences in extinction (Reichel et al., 2012; Holtz et al., 2012). The discrepancy between this work and our preliminary study was somewhat surprising and the cause of this variation remains unknown. However, the lack of

sex differences in extinction allowed the assessment of possible sex differences in subsequent reinstatement.

Males and females showed significant meth-seeking behavior following a meth-prime injection. Responding in the male and female groups that had a learning history with meth and received a meth trigger (MethSA/MethT) was higher than the group that had a learning history with meth and did not receive a meth prime (MethSA/SalineT), the group that received an acute injection of meth (SalineSA/MethT), and the group that never received meth. Notably, this meth-primed reinstatement effect was potentiated in the females. Responding in the Female MethSA/MethT group was significantly higher than responding in the Male MethSA/MethT group; this was the only group that differed between males and females. The difference in these groups alone suggests that differences in reinstatement behavior between the sexes were not a result of general differences following long-term meth self-administration, acute meth administration, nor basal behavioral differences. Females only responded more during meth-primed reinstatement of meth-seeking. This finding is accordant with our preliminary experiment, as well as work by others that also show amplified reinstatement behavior (Cox et al., 2013; Holtz et al., 2012; Reichel et al., 2012).

There were numerous exciting findings in the examination of c-Fos as a marker of neuronal activation (Curran and Morgan, 1985; Curran and Morgan, 1995; Kovacs, 1998; Greenberg and Ziff, 1984). Generally speaking, c-Fos immunoreactivity showed a similar pattern to the behavioral reinstatement data, particularly in females. c-Fos was higher in females with a learning history with

meth and received a meth-prime compared to females that received long-term meth self-administration and no prime in the Cg1, LO, PrL, dlCPu, dmCPu, vmCPu, NacC, NAcSh, and CEA (refer Graphic 6). These differences were a result of both the high activation levels following a prime and lower levels following long-term meth self-administration (i.e., levels in the MethSA/SalineT group were marginally lower than even the baseline SalineSA/SalineT group). These results suggest that hyperactivation was prevalent after a meth prime in females and, notably, there may be hypofunctioning in the female rat brain following long-term meth self-administration and extinction. As extinction and reinstatement in the MethSA/SalineT groups were conducted drug free, this could be conceptualized as a withdrawal period. Accordantly, some work does show hypofunction in critical areas associated with drug addiction during withdrawal (Parsegian and See, 2014). With research suggesting females show heightened sensitivity to withdrawal symptoms in some drug paradigms (for a review see O'Dell and Torres, 2014), future work using female subjects to examine not only the hyperfunction found during reinstatement, but also possible hypofunction during meth withdrawal will be of interest.

The increased c-Fos expression in the females in cortical, striatal, and amygdala regions during reinstatement is concordant with several studies investigating c-Fos expression during reinstatement (Bossert et al., 2012; Ciccocioppo et al., 2000; Cornish et al., 2012; Hamlin et al., 2008; Kufahl et al., 2009; Miller et al., 2005; Neisewander et al., 2012; Recinto et al., 2012; Zavala et al., 2007). These increases in c-Fos during drug-seeking paradigms are not

universal (Sobieraj et al., 2016; Zahm et al., 2010; Zhou et al., 2014). In fact, the only other study to examine possible sex dependent neural correlates in reinstatement actually found c-Fos expression in the NAcC and NAcSh lower in reinstating male and female rats compared to control animals that did not receive reinstatement. These discrepancies in neuronal activation have been explained by inhibitory GABAergic and dopaminergic neurotransmission in these regions, as well as significant differences between the studies in self-administration drug and reinstatement trigger (Sobieraj et al., 2016; Zahm et al., 2010; Zhou et al., 2014). The increased neuronal activation in both the NAcC and NAcSh of reinstating females reported herein is supported by work specific to methamphetamine-triggered reinstatement. Parsegian and See (2014) reported that meth self-administration reduced basal levels of glutamate in cortical regions, as well as the nucleus accumbens. Notably, meth-primed reinstatement reversed this effect, increasing glutamate efflux in these regions. Dopamine was increased in the dorsal medial prefrontal cortex, but not in the nucleus accumbens (Parsegian and See, 2014).

Albeit speculative, our findings are consistent with decreased glutamatergic action following long-term self-administration and amplified glutamatergic neurotransmission during reinstatement in females. That is, glutamate is reduced following long term self-administration, possibly by opponent-process neuroadaptations (Koob and Le Moal, 2001; Parsegian and See, 2014). This results in marginally reduced neuroactivity in regions central to mediating the effects of the drug, including areas of the cortex, striatum, and

amygdala. When a drug-trigger is delivered, these brain structures, which are in a state of reduced basal activity, become hyperactive; thus mediating the resulting robust reinstatement behavior. An important note is that the study reported herein only examined the neural correlates of meth-primed reinstatement. Additionally, the current study did not utilize double-staining techniques to identify the specificity of neuronal activation. Future research will be crucial to test this glutamatergic account of reinstatement behavior in females. Functional examination of the regions that were identified to play a role in female meth-primed reinstatement via selective inactivation, as well as chemo- and optogenetics are in the queue.

While the mechanisms involved will need further inquiry, we successfully identified sex-dependent neural correlates of meth-primed reinstatement (refer Graphic 9). In fact, when the male groups were compared within sex, there was only one significant difference in a single area. Activation following acute meth (SalineSA/MethT) in the CEA was higher than activation in the group that received self-administration and then no meth (MethSA/SalineT). The significantly higher activation in the acute group, but not the meth-trigger group (MethSA/MethT), compared to the MethSA/SalineT group suggests that acute meth injection may be correlated with more activation than meth serving as a reinstatement trigger. Although there was no significant difference between the male SalineSA/MethT and MethSA/MethT groups when directly compared, visual inspection of the CEA data does suggest that the group that received acute meth does indeed show more activation than the group that received meth

injection and had a history of meth self-administration. While speculative, given that functionality was not examined in this study, the ability of meth to activate the CEA may actually decrease in males following a learning history with the drug.

We did find that in the males there were no detected differences in any regions following meth-triggered reinstatement of meth-seeking (MethSA/MethT) and any other group. Sobieraj et al. (2016) found similar null results in one of the few other examinations of the neural correlates of meth-reinstatement. Following meth self-administration and extinction, no c-Fos differences were detected following drug paired cue-induced reinstatement in the medial prefrontal cortex, VTA, or nucleus accumbens (Sobieraj et al., 2016). These null effects in meth reinstatement are not always found, as others did find differences in males in the medial prefrontal cortex (Recinto et al., 2012) and LH (Cornsh et al., 2012). While no difference reached significance in the male meth reinstatement group, it should be stated that visual inspection of the data does show patterns that match the females in a couple brain regions (e.g., see PrL and dmCPu). Although discussion of data patterns revealed by ocular inspection should certainly be limited, these patterns may suggest that the underlying neurobiology mediating meth-triggered reinstatement in males and females overlap in these areas.

Significant findings were found in group comparisons between the sexes. Females in the reinstatement group (MethSA/MethT) had higher c-Fos activation than their male counterparts in the Gg1, LO, dlCPu, vmCPu, NAcC, and, CEA.

There were also differences between males and females following acute meth administration (SalineSA/MethT). Females had greater c-Fos immunoreactivity following acute meth in the LO, vlCPu, vmCPu, NAcC, NAcSh, and CEA. This significant overlap in neural activation when meth was administered, regardless of self-administration drug type, suggests that many of the differences between the sexes may be a result of initial meth administration and not necessarily in differences in meth as a drug trigger for reinstatement of meth-seeking. The two exceptions to this notion were the Cg1 and dlCPu. The Cg1 is particularly significant as previous work suggests this region is of particular importance in multiple forms of reinstatement (Breiter et al., 1997; Childress et al., 1999; Ciccocioppo et al., 2001; Neisewander et al., 2000; Thomas and Everitt, 2001; Wexler et al., 2001). In fact, Neisewander et al. (2000) determined that the cingulate cortex was the sole region that was activated by a cocaine prime triggering reinstatement. Recinto et al. (2012) extended this work to a meth-prime model, also determining the cingulate cortex was integral in reinstatement. Given the accumulating data showing the Cg1 plays a central role in meth-primed reinstatement, this area will likely serve as the first region of interest for subsequent study.

Experiment 2

Experiment 2 was designed to extend behavioral reinstatement differences in meth-primed reinstatement to a novel model. Concordant with Experiment 1, males and females robustly self-administered meth. Differences in active lever pressing were detected, however these were likely due to the slight difference in

meth dose. When meth intake was adjusted for body weight, males and females did not differ. The lack of difference in meth intake between the sexes was similar to the findings from Experiment 1, as well as previous work in limited access self-administration sessions (Cox et al., 2013; Reichel et al., 2012). Repeated nicotine administration did not alter meth self-administration. This was not surprising as nicotine was explicitly unpaired with from the self-administration session (injected 4 h after). These findings replicate previous work with repeated nicotine unpaired from meth self-administration in males and extends them to females (Neugebauer et al., 2012).

In extinction, no differences were detected between males and female, again matching the findings from Experiment 1. This finding supports the notion that although responding was different due to the slight difference in meth dose in acquisition, these differences did not persist during non-reinforcement. Given that the reinstatement tests are non-reinforced, this is an important finding that allows comparison of the sexes without accounting for differences in responding during acquisition. Some consideration was given to evaluating reinstatement responding as measured by a percentage of responding at the end of acquisition. However, given comparable responding in extinction, similar levels of meth intake, and the lack of differences also in Experiment 1, we determined a percentage of acquisition responding measure was not necessary.

In extinction an interesting effect of nicotine was seen with the group that received nicotine during the self-administration phase (recall nicotine was not given during extinction) demonstrating increased extinction. That is, responding

in the repeated nicotine group was lower compared to responding in the group that had not received nicotine. One possible explanation for this effect is that nicotine enhanced learning during the extinction phase. Previous work does suggest that nicotine can function as a cognitive enhancer, augmenting learning, memory, and attention (Couey et al., 2007; Levin et al., 2006; Mansvelder et al., 2006; Newhouse et al., 2004) with effects demonstrated in extinction (Elias et al., 2010; Kaplan et al., 2011). As extinction is new learning (Bouton, 2004; Bouton and King, 1983, Rescorla, 2004), the nicotine treatment may be enhancing this learning.

This explanation does have a major issue; the enhancement of learning associated with nicotine is found when nicotine is administered concurrently with the learning (Tian et al., 2008; Elias et al., 2010; Gould and Higgins, 2003; Gould and Wehner, 1999). In the present report, nicotine was administered during the self-administration phase and halted prior to the extinction phase. Thus, nicotine would not be expected to enhance learning in this phase. This lack of enhanced extinction learning was found in previous work with nicotine administration during the acquisition of both meth self-administration and meth conditioned place preference (Berry et al., 2012; Neugebauer et al, 2010). The divergent findings reported here may reflect between study differences, including the drug addiction model (Berry et al., 2012) or reinforcement schedule (Neugebauer et al., 2010). Future work investigating if nicotine administered concurrently with extinction in a meth self-administration paradigm further facilitates learning will be of interest.

Nicotine successfully induced meth reinstatement in females and males. Reinstatement was induced by 0.2 and 0.4 mg/kg in the acute and repeated nicotine groups. However, reinstatement was potentiated following the 0.4 mg/kg dose in the group that had a prior history with nicotine. Meth reinstatement induced by acute nicotine did not align with the result of Neugebauer et al. (2012), but do match those of Hiranita et al. (2006). The potentiated ability of nicotine to reinstate meth-seeking following repeated administration is quite interesting. This effect may be a result of neurochemical sensitization following repeated nicotine administration. Repeated nicotine administration can increase reactivity in response to nicotine in overlapping neural circuitry known to play a role in the expression of meth-seeking behavior [for a review see Vezina et al. (2007)]. We hypothesize that this amplified reactivity following nicotine sensitization results in the modest meth-seeking reinstatement induced by acute nicotine and the robust meth-seeking reinstatement induced by repeated nicotine administration demonstrated herein and in other studies (Neugebauer et al., 2012; Hiranita et al., 2006).

Cocaine also successfully reinstated meth-seeking and no differences were detected between sexes. This finding suggests that nicotine is not unique in its ability to reinstate meth-seeking. The ability of nicotine and cocaine to serve as triggers for meth-seeking may be a result of their overlapping interoceptive stimulus effects with meth. As discussed, it is well established that a meth injection can reinstate meth-seeking behavior. While nicotine and meth, as well as cocaine and meth differ in biological mechanism, they also share significant

overlap. In fact, nicotine and cocaine can substitute for meth in a 2-lever discrimination task. In a 2-lever discrimination task, responding on 1 of 2 levers is reinforced on saline sessions. On intermixed drug sessions, the opposite lever is reinforced. Eventually, the majority of pressing is on the drug or no-drug appropriate lever depending on the injected solution (Meltzer et al., 1980; Stolerman, 1989; Stolerman et al., 1984). Stimulus similarity is then typically assessed by administration of a test ligand prior to a brief test [e.g., 5 min (Stolerman, 1989)] with the reinforcer unavailable. The greater the proportion of pressing on the drug-appropriate lever the more similar the substitution ligand is said to be. Under these test conditions, full substitution (>80% responding on the drug-appropriate lever) is found when nicotine (Desai et al., 2010a; Gatch et al., 2008) and cocaine (Czoty et al., 2004; Dasai et al., 2010b) were substituted for meth. Although the 2-lever discrimination paradigm can be limited by abbreviated test durations or cumulative dose procedures (see Reichel et al., 2012; Bevins et al., 2011), these findings do suggest that nicotine and cocaine may initially be perceived as the meth, thus inducing reinstatement behavior. While future work will be needed to elucidate the precise mechanisms by which nicotine and cocaine trigger meth reinstatement, Experiment 2 clearly shows that nicotine and cocaine induce meth-seeking in both females and males, yet the sex difference observed when meth served as the trigger was not seen with these other drug triggers. These findings implies that females are more sensitive to reinstatement when the original drug is used as a drug-trigger, but this amplified sensitivity does not remain when a different drug is used as a prime. Future work examining if this effect is specific to meth as a primary drug, or if this effect

generalizes to other drugs of abuse that are readily self-administered will be of interest.

General Discussion

The experiments reported herein did not examine gonadal hormone levels. However, previous work does show they likely play a role in the amplified vulnerability to drug addiction found in females. In humans, the positive subjective measures following administration of psychostimulants are increased during the follicular phases of the menstrual cycle compared to the luteal phase (Justice and de Wit, 1999; White et al., 2002). Namely, when estrogen levels are elevated and progesterone levels are low, the positive subjective effects are enhanced (Evans et al., 2007; Justice and de Wit, 1999; Justice and de Wit, 2000; White et al., 2002). These hormonal fluctuations also affect drug craving. Sinha et al. (2007) found that craving induced by stress or cue was lower when progesterone was elevated in cocaine-dependent women. Preclinical work parallels the human findings. In general, estrogen enhances and progesterone inhibits acquisition and escalation of self-administration, resistance to extinction, and reinstatement of drug-seeking (for a review see Anker and Carroll, 2011).

Specific to drug-primed reinstatement, multiple studies have shown that estrogen treatment enhanced cocaine-primed reinstatement in ovariectomized rats (Anker et al., 2007; Larson and Carroll 2007; Larson et al., 2005). Estrogen enhancement of reinstatement may involve activation of estrogen β receptors (ER- β). Larson and Carroll (2007) demonstrated that administration of diarylpropionitrile, an ER- β agonist, during cocaine-primed reinstatement

enhances responding; a notable finding as ER-β is found on striatal dopamine neurons and is known to influence dopamine neurotransmission (Laflamee et al., 1998; Morissette et al., 2008).

Conversely, progesterone, or its metabolite, allopregnanolone attenuates drug-primed reinstatement (Feltenstein and See, 2007; Anker et al., 2007). The metabolism of progesterone to allopregnanolone may be essential for this attenuation. When progesterone was administered with a 5-α reducatase inhibitor that prevents metabolism of progesterone to allopregnanolone, progesterone no longer attenuated cocaine-prime reinstatement (Anker et al., 2009). Congruently, depletion of allopregnanolone has been shown to potentiate increases in dopamine induced by stress (Dazzi et al., 2002), suggesting the inhibiting effects of allopregnanolone in reinstatement may be a result of dopaminergic inhibition.

The preclinical work specifically with meth reinstatement is not nearly as extensive as that with cocaine. With meth, studies have not detected differences in reinstatement based on phase of estrous cycle (Ruda-Kucerova et al., 2015; Cox et al., 2012). However, allopregnanolone does reduce meth-primed reinstatement in female, but not male rats, suggesting gonadal hormones may be a factor. Future work further elucidating the precise brain areas involved in these hormonal effects on meth-primed reinstatement will be useful. That is, does direct microinjections of estrogen and progesterone/allopregnanolone into the Cg1, LO, PrL, dlCPu, dmCPu, vmCPu, NacC, NAcSh, or CEA enhance or inhibit meth-primed reinstatement in female and male rats.

In addition to possible differences in meth pharmacodynamics as a result of gonadal hormones, differences in meth pharmacokinetics between the sexes have also been reported. Milesi-Halle et al. (2005) examined the pharmacokinetic profile of a range of experimenter delivered meth doses in female and male Sprague-Dawley rats. Significantly slower meth clearance and attenuated meth metabolism was found in females compared with males (Milesi-Halle et al., 2005). That is, meth remained in an unaltered form for longer in females. In a follow-up study, Milesi-Halle and colleagues (2015) replicated these results in a meth self-administration paradigm; finding decreased clearance and higher than expected serum concentration levels in females. These pharmacokinetic sex differences may contribute to differences in meth behavior in females and males (Milesi-Halle et al., 2005; Milesi-Halle et al., 2015.

As mentioned throughout this dissertation, the findings from the set of studies reported herein start to fill in gaps in the literature and reveal a bevy of follow-up studies that are of great interest. Successful identification of sex-dependent neural correlates associated with meth-primed reinstatement will allow a targeted approach for the utilization of cutting edge neuronal manipulation techniques (e.g., chemo- and opto-genetics) to further characterize possible sex differences in the cortex, striatum, and amygdala. Additionally, given the translational relevance of alternative drug-triggered reinstatement, this paradigm deserves further examination with both behavioral (e.g., altering self-administration drug types) and neurobiological techniques (e.g., identification of neural correlates). While these studies opened an abundance of additional

questions, they do significantly contribute to our understanding of meth reinstatement. Females and males differ in the neural correlates associated with meth-primed reinstatement and both females and males can have reinstatement triggered by alternate drug priming.

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