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Incubation of Craving: An Analysis of Short-Access Self-Administration Models Without Food Pre-Training

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by

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ABSTRACT

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Incubation of craving is a behavioral phenomenon in which patients experience a time-dependent increase in cue-elicited drug craving as they progress through abstinence. Currently, the precise neurobiological mechanisms which drive incubation of craving have not yet been confirmed. Furthermore, a large proportion of the literature that encompasses current knowledge on these mechanisms uses sucrose or food pre-training procedures prior to cocaine self-administration. Literature shows that sucrose/food self-administration induces both overlapping and opposing neurochemical changes in comparison to cocaine self-administration. Therefore, the use of sucrose/food pre-training to facilitate drug self-administration could lead to confounding results for an analysis of the neurobiological underpinnings of drug craving. The experiments detailed in this thesis serve to compare the ability of two different short access (2-hour) rat models of cocaine self-administration, to elicit an incubation of craving, without the use of sucrose/food pre-training. The purpose of using a short-access (2-hour) model for cocaine self-administration rather than classical long-access (6-hour) models is to use a higher throughput procedure to study incubation of cocaine craving in rats, for the purpose of a future biochemical analysis for changes in neurobiological correlates for incubation of cocaine-craving. Additionally, this study explores the use of lower-dose short-access procedures than those described in existing literature, without the use of food training to elicit incubation of craving. The results demonstrate that both cocaine self-administration models are able to elicit comparable

incubation of craving, in spite of procedural differences. Thus, relatively simple models of cocaine self-administration can be employed to study the neurobiological underpinnings of the incubation of craving.

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1. Introduction

1.1 Prevalence of Cocaine Addiction

The National Survey on Drug Use and Health (NSDUH) reports that in 2015, 968,000 people age 12 or older initiated cocaine use, higher than in any previous year between 2008 -2015, and illustrating the increasing prevalence of this disease in our society. In 2014, it was reported that there were currently 1.5 million current cocaine users in the United States. Additionally, in 2011, data from the Drug Abuse Warning Network (DAWN) showed that cocaine was involved in 40% of all drug-related emergency room visits. Cocaine addiction is a chronic brain disease, which involves a cycle of binging, withdrawal, craving and resumption of drug-taking (Goldstein & Volkow, 2011). Currently, while behavioral therapies exist to treat cocaine use disorder there are no FDA approved pharmacotherapeutics available (NIDA, 2015). High relapse rates during abstinence are a common feature of drug addiction, and a central problem toward developing effective treatment. Statistics reveal that 24% of cocaine addicts relapse back to weekly cocaine use within a year following treatment, and only 18% of those individuals end up returning for treatment (NIDA, 2012). In abstinent drug addicts, exposure to drug-associated cues, stress, short-term withdrawal symptoms, and re-expsure to the drug itself often lead to relapse (Li, Venniro, & Shaham 2016).

1.2 Clinical Evidence For Incubation of Craving

1.2.1 Incubation of Cocaine-Craving: Human Evidence

Drug cravings are the subjective desire for drug taking reported by addicts and these desires appear to intensify upon exposure to drug associated cues during the course of drug

abstinence (Gawin & Kleeber, 1986; Parvaz et al., 2016). An incubation of craving is operationally defined as a time-dependent increase in responding for the drug-associated cue during as withdrawal progresses (Kerstetter et al., 2008; Grimm, Evall, & Osincup, 2005). Some examples of these cues include locations or individuals previously associated with drug use and imagery of drug paraphernalia (Gawin & Kleeber, 1986; Bossert, Merchant, Calu, & Shaham, 2013). Visual presentation of the drug itself can also act as an interoceptive cue to elicit incubation of cocaine-craving (Gawin & Kleeber, 1986, Shaham et al., 2013). This phenomenon was first reported in a clinical observation study conducted by Gawin and Kleeber (1986), which tracked human symptoms from early (9-hours) to protracted withdrawal (28-weeks). During this study, it was noted that patients go through a variety of different emotions and behaviors from early to late withdrawal, and these behaviors were categorized into three phases. Phase 1 was described as the crash phase, which occurred for a maximum of three days after drug abstinence. During this crash phase, addicts experience an extreme sense of dysphoria including depression, insomnia, irritability and anxiety (Gawin & Kleeber, 1986). After a crash phase, patients enter phase 2, what the researchers referred to as "the withdrawal phase" and which could last anywhere from 1-10 weeks. During the withdrawal phase, patients begin with 1-5 days of normal functioning and then begin to feel a sense of dysphoria, milder than that experienced in the crash phase. During this phase, patients also often report feeling an intense sense of boredom, to which cocaine use seems like the only remedy. If individuals are able to continue abstinence past this phase, they then enter phase 3: the extinction phase. During phase 3, patients report having an emotional state similar to that which they experienced before they began cocaine use. However, they also report feelings of intense cravings when exposed to drug-related

cues. It is phase 3, which describes what has come to be termed the incubation of craving (Grimm, Hope, Wise, & Shaham, 2001) and will be the primary focus of this thesis.

In Parvaz et al. (2016), a more quantitative analysis was conducted, showing the existence of incubation of craving in human cocaine addicts as they progress from early to protracted withdrawal. This study was conducted using event-related brain potentials (ERPs) which were described in a previous publication as an objective marker of incubation of craving (Franken et al., 2008). In Franken et al. (2008) the response of one particular ERP called the late-positive potential (LPP) was measured from three groups of people: cocaine-abstinent individuals, current cocaine users, and people with no cocaine experience. ERPs and consequently LPPs are a component of the electroencephalogram (EEG), which is a widely used, temporally precise, marker of motivated attention to salient stimuli, and therefore, is has utility as an objective marker for incubation of craving (Hajcak et al., 2013; Franken et al., 2008). Findings show elevated LPP levels in cocaine addicts, but most importantly, report an increase in LPP amplitudes as self-reported craving increased for patients in withdrawal (Franken et al., 2008). The methods used in Franken et al. (2008) provide an objective measure of craving, independent of self-report, which was applied in the study conducted by Parvaz to see if a change in craving is observed with increased withdrawal time. In the study by Parvaz and colleagues (2016), 76 individuals with cocaine use disorder were tested for cue-elicited craving by both LPP measurements and self-report at 2 days, 1 week, 1 month, 6 months and 1 year after ceasing cocaine use. Individuals showed a progressive increase in craving that peaked between 1 month and 6 months into drug abstinence and tapers off at 1 year of abstinence (Parvaz 2016). Along with craving, individuals show an increase in LPP amplitudes which also peaked between 1 and 6 months

of drug abstinence, and taper off at 1 year of abstinence, providing further quantitative support for the self-reported craving (Parvaz et al., 2016). This study shows quantitative and objective evidence of incubation of craving during cocaine withdrawal in human addicts.

1.2.2 Incubation of Methamphetamine, Nicotine and Heroin Craving: Human Evidence

Incubation of craving has also been reported for various other drugs of abuse such as methamphetamine (Wang et al., 2013), nicotine (Bedi et al., 2011), and heroin (Wang, GB. et al., 2011). Nicotine users described an increase in drug-craving from 7 days withdrawal to 35 days withdrawal, after exposure to smoking cues (Bedi et al., 2011). A study conducted on heroin addicts revealed that as they progress further into protracted withdrawal, baseline craving in response to neutral cues decreases. However, after exposure to drug-associated cues, patients report increases in craving, heart rate, and systolic blood pressure at all withdrawal days examined, out to 24 months (Wang, GB et al., 2011). In methamphetamine-dependent individuals, self-reported baseline (i.e., unprovoked) craving decreased with the duration of abstinence, while, cue-elicited craving showed a progressive increase until 3 months of abstinence. At 6 months of methamphetamine abstinence, patients reported a decline in cue-elicited craving, and even lower levels of cue-elicited craving were reported at 1 year of abstinence (Wang et al., 2013).

The evidence presented above confirms the existence of incubation of craving in cocaine addicts through both self-report and ERP measurements. The literature also indicates that this phenomenon is not specific to cocaine addiction, and is experienced

during withdrawal from many drugs of abuse. Thus, it is of clinical relevance to understand the neurological mechanisms underlying incubation of craving in rats.

1.3 A Forced Abstinence Model is Used to Study Incubation of Cocaine-Craving in Rats

This study used a forced-abstinence model to study incubation of craving. In rats, drug-craving is operationally defined as responding for a drug-associated cue during withdrawal (Pickens et al., 2011). This is a model of in-patient treatment- or incarceration-induced abstinence, that has been widely employed for studying incubation of cocaine craving in rats. During in-patient treatment or incarceration, patients are typically physically removed from the drug of abuse and drug-related paraphernalia for their initial abstinence. To study incubation of craving in a forced abstinence rat model, animals first undergo a period of self-administration in which they are allowed daily access to the reinforcer (be it a drug or sucrose). The duration of daily reinforcer access varies, depending upon the study. Some studies employ a long-access model (e.g., 6 h-access) (e.g.,; Ben-Shahar et al., 2013; Grimm et al., 2001; Miller et al., 2017; Shin et al., 2016; Shin et al., 2017; Lu et al., 2007; Conrad et al., 2008; Warner et al., 2015; Li et al., 2013; Werner, Stefanik, Milovanovik, Caccamise, and Wolf, 2018), while others employ a short-access model (e.g., 1-3 h-access) (e.g., Tran-Nguyen et al., 1998; Neisewander et al., 2000; Kerstetter, Aguilar, Parrish, & Kippin, 2008; Lee et al., 2013; Ma et al., 2014; Suska, Lee, Huang, Dong & Schluter, 2013; Halbout, Bernardi, Hansson, & Spanagel, 2014; Sorge & Stewart, 2005). To conduct self-administration procedures, rats are placed in operant chambers that contain an “active” and “inactive” lever. Upon pressing the active lever, rats are presented with discrete cues (eg.

light and tone) and concurrently receive an infusion of drug or the delivery of a non-drug reinforcer. Upon pressing the inactive lever, rats receive no reinforcer or associated cues. Following the initial self-administration phase, animals enter the abstinence phase in which they are placed back in their home cages and left undisturbed for a designated period of days or weeks.

Following the abstinence phase, animals enter the testing phase in which they are tested for cue-reinforced responding on the previously drug-reinforced lever in the absence of any drug reinforcer (i.e., under extinction conditions), which serves as the operational definition of drug-seeking behavior or craving. Under these procedures, an incubation of cocaine-craving in rats has been consistently observed across various studies in the Szumlinski laboratory (Ben-Shahar et al., 2013; Miller et al., 2017; Shin et al., 2016; Shin et al., 2017), as well as across many other laboratories studying this phenomenon (e.g., Grimm et al., 2001; Neisewander et al., 2000; Lu, Grimm, Hope, & Shaham, 2004; Lu et al., 2005; Lee et al., 2006; Lu, Uejima, Gray, Bossert, & Shaham, 2007; Werner, Stefanik, Milovanovik, Caccamise, and Wolf, 2018; Halbout et al., 2014;).

For this thesis, withdrawal days were determined based on prior research establishing that the increase in cue-reinforced behavior reaches its maximal point by 30-days withdrawal (Grimm et al., 2001; Neisewander et al., 2000, Kerstetter et al., 2008). However, it should be noted that cue-reinforced behavior has been reported to persist at 60-days withdrawal, and at 180-days withdrawal either tapers off (in males rats) or continues to

persist (in females rats) (Kerstetter et al., 2008; Anker & Carroll, 2010; Carroll & Anker, 2010).

1.4 Procedural Factors That Impinge Upon Incubation of Craving

1.4.1 Self-Administration Duration (Hours/Day)

An extended-access (6 hour day/ 10 day) cocaine self-administration protocol is a popular model used in the field (Ben-Shahar et al., 2013, Miller et al., 2017, Shin et al., 2016, Shin et al., 2017, Lu et al., 2007, Conrad et al., 2008, Werner, Milovanovic, Christian, & Loweth, 2015; Pickens et al., 2011; Lu et al 2004; Venniro, Caprioli, & Shaham, 2016), as prior literature has demonstrated that incubation of craving is robustly observed following an extended-access self-administration procedure. This being said, an incubation of craving is reported to occur under short-access (e.g., 2 hour/day) self-administration procedures (Sorge and Stewart, 2005; Kerstetter et al., 2008; Hollander & Carelli, 2007; Ma et al., 2014; Lee et al., 2013, Suska et al., 2013). Thus, this present study applies two short-access (2-hour) cocaine self-administration models, to establish a higher throughput model (compared to 6-hour models) for cocaine self-administration in the Szumlinski laboratory. This model will be applied toward the future analysis of neurobiological correlates of incubation of cocaine-craving.

1.4.2 Number of Days of Self-Administration

The majority of the previous studies cited above use a multi-day self-administration period (ranging from 6 to 10 days) prior to tests for incubated craving during withdrawal. However, a study has been conducted in mice using a single (6-hour) session of cocaine

self-administration with a dose of 0,5mg/kg/infusion of cocaine in which incubation of craving was observed (Halbout et al., 2014). Additionally, this study reports decreases in AMPA/NMDA receptor binding at 9-days withdrawal compared to saline controls. However, at 43-days withdrawal, cocaine-experienced rats exhibit increased AMPA/NMDA receptor binding, compared to saline controls. This increase in AMPA receptor availability is comparable to previous findings showing incubation related-changes in AMPA receptor availability after a 6-hour model of self-administration (Conrad et al., 2008; Loweth, Tseng, & Wolf, 2014). Thus, a single session procedure for cocaine self-administration may be sufficient to produce both the behavioral and neurobiological changes associated with incubation of craving.

1.4.3 Different Cocaine Doses Can Induce an Incubation of Craving in Rats

The dose of cocaine employed in the current study was selected based on the results of a report by Ahmed & Koob, (1998). This study examined a wide range of cocaine doses, and found that a dose of 31.25 ug/infusion was the minimum necessary to maintain cocaine self-administration behavior. The results also illustrated that a 0.25mg/0.1ml/infusion dose induced stable cocaine intake in a short-access (1-hour) self-administration model, while intake can escalate when rats are allowed 6-hour access to this drug dose (e.g., Ben-Shahar et al., 2012; Ahmed & Koob, 1998). Of relevance to this thesis, the 0.25mg/0.1ml/infusion dose reliably elicits an incubation of cocaine-craving in animals allowed 6-hour access to the drug, as demonstrated by a series of reports from the Szumlinski laboratory (Ben-Shahar et al., 2013, Gould et al., 2015, Miller et al., 2017, Shin et al., 2016, Shin et al., 2017;

Szumlinski et al., 2018). Thus, a dose of 0.25mg/0.1ml/infusion was used in this present study. However, literature shows that other short-access (2-hour) self-administration models have used higher doses to induce incubation. Kerstetter et al. (2008) employed a dose of 0.5 mg/kg/0.1 ml infusion and Lee et al. (2013) used a dose of 0.75 mg/kg/0.1ml infusion of cocaine in their respective self-administration studies. These results suggest that a wide range of doses can be used to elicit incubation of cocaine-craving in rats. Thus, the present study aims to test the effects of a short-access (2-hour) self-administration model, with a lower cocaine dose than existing literature, to elicit incubation of cocaine-craving in rats.

1.4.4 Sucrose or Food-Training is Often Use to Entice Drug-Taking Behavior

Food or sucrose training is commonly used in order to encourage rats to respond on the active lever prior to the onset of self-administration training (Tran-Nguyen et al., 1998; Grimm et al., 2001; Neisewander et al., 2000; Ben-Shahar et al., 2012; Ben-Shahar et al., 2013; Miller et al 2017; Shin et al 2016; Shin et al 2017). Briefly, this procedure involves food-restricting animals prior to surgery, then training these animals to respond on the active lever in order to receive a food reward. Although this is a popular method, there are potential consequences to the use of food training. As detailed in section 1.5 below, there are overlapping neurobiological mechanisms for incubation of cocaine-craving and incubation of sucrose-craving (Bassareo, Cucca, Frau, & Di Chiara, 2017). Although, not all neurobiological effects are the same for drug and non-drug reinforcers. Additionally, food training introduces a confound in which on day one of cocaine self-administration, animals are initially motivated to press on the lever providing cocaine (active lever) because of the previous association with receiving food rewards. Several laboratories have been able to

successfully train animals to self-administer cocaine, behaviorally demonstrate an incubation of craving, and show differences in neurobiological correlates for incubation in rats without the use of food training (Lee et al., 2013; Halbout et al., 2014; Ma et al., 2013). Halbout et al. (2014) specifically tested for an incubation of craving following a single (6-hour) session of cocaine self-administration (0.5mg/kg/infusion of cocaine) with and without the use of food training and found no effect of this manipulation upon behavior. Thus, it is possible to model the incubation of cocaine-craving in rats without the use of food training. Further, these aims to determine whether or not the incubation of cocaine-craving can be elicited by short-access, low-dose cocaine procedures in the absence of food training.

1.5 Incubation of Sucrose Craving

1.5.1 Behavioral Effects of Sucrose Self-Administration

Literature shows that an incubation of craving can be observed after the self-administration of non-drug or natural high-incentive rewards such as sucrose (Grimm et al., 2002; Grimm, Eyall, & Osincup, 2005; Counotte, Scheifer, & Shaham, 2014; Aoyama, Barnes, & Grimm, 2014; Di Ciano & Everitt, 2004; Van den Oever et al., 2006) or saccharin (Grimm et al., 2014), illustrating that the phenomenon is not specific to drug rewards. In a study by Grimm and colleagues (2002), rats were trained to lever-press for 10% sucrose (0.2 ml/reinforcer delivered into a liquid drop receptacle) for a period of 10 days. Following operant training, rats were undisturbed for either a 1-day or 15-day period. On their respective test days, rats were allowed to lever press on the previously active lever for 6-7 hours with no programmed consequences (i.e., no sucrose reinforcer or paired cue was provided) to extinguish responding. After this extinction period, rats were tested for cue-

induced reinstatement for one hour and an increase in cue-induced sucrose-seeking behavior was observed between 1 and 15 days (Grimm et al., 2002). Similar results were reported after saccharin administration (Aoyama et al., 2014) suggesting that the caloric intake of sucrose is not required to show a behavioral incubation of sucrose-craving.

There have also been studies conducted in which sucrose self-administration did not lead to an incubation of sucrose-craving. In one such study, conducted by Shin and colleagues (2017), rats were trained to self-administer sucrose pellets and showed a time-dependent increase in active lever responding during withdrawal that did not reach statistical significance. This study differed from Grimm et al., (2002) by capping reinforcers on day 1 of operant responding, employing pellets rather than liquid sucrose, and allowing *ad libitum* home cage feeding, all factors that might have contributed to the reported a lack of behavioral incubation of craving. In spite of these behavioral results, time-dependent changes in vmPFC extracellular glutamate levels were observed as detailed below in 1.5.2 below.

1.5.2 Molecular effects of Sucrose Self-Administration

Literature shows that there are overlapping molecular effects of sucrose and drug self-administration. Addictive drugs (including cocaine) and palatable food share a property of preferentially increasing extracellular dopamine levels in the NAc shell rather than the NAc core (Di Chiara & Bassareo, 2007). Studies have also shown opposing molecular effects in animals responding for cocaine and sucrose. One study showed that sucrose-reinforced responding was associated with decreased AMPA/NMDA receptor ratios within

the NAc during abstinence (Counette et al., 2014), while the AMPA/NMDA receptor ratio increases after withdrawal from cocaine self-administration (Conrad et al., 2008; Loweth et al., 2014). Similarly, Shin and colleagues (2017) have described opposing or different effects of cocaine and sucrose self-administration upon PFC dopamine and glutamate levels. In that study, separate groups of rats were trained to lever-press for either sucrose or cocaine, and vmPFC glutamate and DA levels were measured during abstinence using *in vivo* microdialysis. Results demonstrate that responding for drug-associated cues elicited a rise in vmPFC dopamine in early withdrawal and a rise in vmPFC glutamate in late withdrawal. Results also demonstrate that responding for sucrose associated cues elicited an increased in vmPFC extracellular glutamate that was greater in early versus later withdrawal; however, responding for sucrose cues elicited no increases in vmPFC dopamine (Shin et al., 2017).

Human evidence also exists supporting neurobiological changes following administration of non-drug reinforcers (Tomasi & Volkow, 2013) A neuroimaging study also reveals overlap in brain circuitry between addiction disorders and food intake disorders such as binge eating disorder and obesity (Tomasi & Volkow, 2013). In this study, Tomasi and Volkow reported fMRI evidence of an involvement of ventral striatal and dorsal striatal networks in both addiction and obesity. The evidenced neurochemical changes following sucrose self-administration suggest that the use of food training prior to cocaine self-administration has the potential to introduce confounding effects on neurobiological findings.

1.5.3 Summary of Sucrose Literature

In summary, the literature detailed above first establishes that responding for both sucrose and non-caloric sweeteners can induce an incubation of craving (Grimm et al., 2002; Grimm et al., 2014). Studies describe preferential extracellular dopamine increases in the NAc shell compared to the NAc core after administration of both palatable food and cocaine (Di Chiara & Bassareo, 2007). Additionally, overlapping effects of sucrose and drug administration are observed in fMRI imaging studies of humans (Tomasi & Volkow, 2013). Such findings illustrate the potential for confounding effects when using sucrose training to encourage active lever responses for cocaine self-administration.

However, there are also molecular effects that are exclusive to drug self-administration (Bassareo et al., 2017). Several studies describe opposing neurobiological mechanisms following drug and sucrose self-administration (Shin et al., 2017; Counette et al., 2014; Conrad et al., 2008; Loweth et al., 2014). Although not all molecular effects of drug and sucrose self-administration are overlapping, the presence of potential confounds supports eliminating food training for this thesis.

1.6. Specific Aim

To expand on this previous work and develop a more simplistic rat model of incubated craving, this study aimed to determine whether or not a low-dose (0.25 mg/infusion), short-access cocaine self-administration model can induce the incubation of craving in rats in the absence of food-training procedures. This study compares two different short-access term administration models, based on existing models in the literature that have

successfully shown an incubation of cocaine-craving using higher cocaine doses. The first self-administration model is based on a 2-hour, daily self-administration model used by Kerstetter et al., (2008), with some modifications that simplified the testing procedures. The data from the first cohorts of rats tested under the 2-hour cocaine self-administration procedures indicated a relatively high degree of variability in cue-reinforced responding, compared to historical data in the laboratory collected under 6-hour procedures. Thus, I examined whether or not allowing the animals more training time on the first day of cocaine self-administration might reduce the variability in cue-reinforced responding and facilitate the detection of incubated responding. To do this, I developed a a "mixed-access" self-administration model based off of the procedures used in Lee et al., (2013) and involved one day of long-access (6-hour) administration, followed by 2-hour short-access sessions. My findings demonstrate that prior food-training is not necessary to induce an incubation of craving in rats trained to self-administer a relatively low dose of cocaine under short-access procedures. Further, the employ of an initial 6-hour self-administration session does not significantly impact subsequent cocaine intake nor does it produce obvious effects upon the variability in cue-reinforced responding during either short or longer-term withdrawal. Further, although the magnitude of the incubated response (i.e., the difference in the mean active lever responses observed between rats tested in early versus later withdrawal) was not statistically different between rats trained under the different procedures, visual inspection of the data suggested that the magnitude of the incubated response was larger in rats in the mixed-access model. From these data, I conclude that while both procedures are sufficient to induce an incubation of craving, allowing the animals more time to engage in cocaine-related learning during initial self-administration training facilitates the expression of

incubated cocaine-craving of relevance to future studies of the neurobiological underpinnings of this phenomenon.

Materials and Methods

2.1 Subjects.

The subjects for this study were 77 male Sprague-Dawley rats, which weighed approximately 250-275 g upon arrival. The rats were obtained from Charles River Laboratories (Hollister, CA, USA). Rats were housed in a colony room, controlled for temperature and humidity, under a 12-hr day/ 12-hr night cycle (lights off from 07:00 to 19:00) hours. Animals were given *ad libitum* access to food and water throughout the duration of the study. The animals were allowed to acclimate to the colony room for 48 hours following arrival. All experimental protocols were consistent with the guidelines of the *NIH Guide for Care and Use of Laboratory Animals* (NIH publication No. 80-23, revised 2014) and were reviewed and approved by the University of California, Santa Barbara Institutional Animal Care and Use Committee. N=22 rats were excluded from the study due to failure to meet the acquisition criteria during self-administration.

2.2 Jugular Vein Catheterization Surgery.

Following the 48-hour acclimation period, animals underwent a surgery to implant chronic intravenous (IV) catheters as previously described (Ben-Shahar et al. 2008; 2009). In short, the procedures were as follows: animals were placed under ketamine/xylazine anesthesia (11/76 mg/ml xylazine and 88.23 mg/ml ketamine, Abbot Laboratories, North Chicago, IL, USA) and then each implanted with a chronic silastic catheter (13 cm long; 0.3 mm inner diameter, 0.64 mm outer diameter) into the right jugular vein. Banamine (2 mg/kg) and buprenorphine (0.3 mg/ml) were administered subcutaneously to treat post-surgical pain. Bupivicaine (5 mg/ml) was administrated subcutaneously at the site of each

incision prior to the start of the surgery, to act as a local analgesic. Each catheter ran subcutaneously around the shoulder to the back where it lay perpendicular to the dorsal surface and was secured to a threaded 22-gauge metal guide cannula (Plastics One, Roanoke, VA, USA). A guide cannula protruded through a small hole on the animal's back and was capped (when not in use) to protect against infection. The cannula was held in place by being cemented to a small (approximately 0.5 x 0.5 inch) swatch of bard mesh (C. R. Bard Inc., Cranston, RI, USA). After the IV catheterization procedure, the catheters were immediately flushed with 0.1 ml of sterile cefazolin/ heparin (100 mg/ml cefazolin and 70 U/ml heparin) and 0.1 ml of sterile gentamicin (2 mg/ml). During the first two-days of post-operative care, animals received sub-cutaneous banamine twice a day. For the remainder of the study, animals received daily IV injections of gentamicin and cefazoin/heparin to maintain catheter patency. Prior to the beginning of self-administration training, catheter patency was verified through IV administration 0.1 ml of sodium Brevital (10 mg/ml), which produces rapid loss of muscle tone when administered intravenously. Catheter patency was verified again at the end of self-administration prior to conducting tests for cue-reinforced behavior.

2.3 Sham Surgery.

While prior studies in the Szumlinski laboratory employed saline self-administering controls (e.g., Ben-Shahar et al., 2013; Shin et al., 2016; Shin et al., 2017), sham surgeries were conducted on the control animals in this study. The rationale for this decision related to the fact that saline self-administering animals exhibit very low levels of non-selective lever-responding during testing that are comparable to that exhibited by surgery-naïve

animals trained to respond for the presentation of the neutral tone/light cue alone (see Ben-Shahar et al., 2013 vs. Shin et al., 2016). Thus, I rationalized that sham surgeries would serve to control for the effects of exposure to anesthesia and post-operative medication, while avoiding subject attrition due to loss of catheter patency/development of infection.

For the sham surgery, rats were injected with the same anesthesia and analgesics as described above for the the jugular vein catheterization surgery. However, they received one dorsal and one ventral incision, which were then immediately stapled closed. With the obvious exception of the catheter treatment, the post-operative care was identical between sham and catheterized rats and all animals were given a minimum of four days to recovery prior to the start of the operant conditioning sessions.

2.4 Cocaine self-administration training.

On each trial, for both the Mixed design and the 2-hours design, each rat's catheter was connected by a liquid swivel to a motorized pump, that was located outside of the operant chamber (e.g., Ben-Shahar et al., 2008; Kerstetter et al., 2008). Briefly, the pump was linked to an active lever inside the operant chamber, and pressing of this lever resulted in 5-s activation of the infusion pump (i.e., which delivered the cocaine reinforcer) paired with a 20-s presentation of a tone (78 dB, 2 kHz), and a conditioned stimulus light positioned above the active lever. Rats were also given access to an inactive lever, which had no programmed consequences when pressed. During the 20-s period when the tone/light stimulus was on, responses on the active lever were recorded but had no consequences (i.e., time-out period).. Rats were trained to self-administer cocaine for 10 2-hour daily sessions. To prevent over-dose, the number of cocaine infusions during the first day was capped at

100 infusions (Ben-Shahar et al., 2008; Ben-Shahar et al., 2009) and rats had to meet a minimum criterion of at least 15 infusions and over 75% of responding on the active lever over the last 3 days of self-administration in order to be included in the study (n=22 rats excluded from study).

2.5 2-Hour Self-Administration Design.

This 2-hour self-administration protocol is based on that described in Kerstetter et al. (2008), but with procedural modifications to render the testing conditions more similar to our prior work (e.g., Ben-Shahar et al., 2013; Shin et al., 2016, Shin et al., 2018). In contrast to Kerstetter et al. (2008), prior food-training was also not conducted to eliminate this interpretational confound. As conducted in Kerstetter et al., rats in my 2-hour model underwent 10 days of self-administration training for 2 hours/day. However, we maintained the dose at 0.25 mg/0.1 ml/infusion (vs. 0.5 mg/kg/0.1 ml infusion) to be consistent with the prior work in the laboratory (e.g., Ben-Shahar et al., 2013, Shin et al., 2016, Shin et al., 2018). Following the 10 days of self-administration, animals were randomly assigned to either the 3WD or 30WD groups, ensuring that the rats tested at either withdrawal period exhibited equipment cocaine intake over the last 3 days of self-administration. Animals remained in their home cages for their assigned period of forced abstinence after which time the tests for cue-reinforced responding were conducted.

2.6 Mixed Self-Administration Design

Our Mixed model of self-administration is based off the procedure described in Lee et al. (2013) with some procedural modifications. In the Lee study, rats were placed in

operant chambers for one overnight session of drug self-administration. During this session each cocaine infusion resulted in a 0.75 mg/kg/0.1 ml of drug paired with the illumination of a 3 to 6-second conditioned stimulus light and a 20-second house light. While the house light was illuminated, additional active lever responding did not result in an infusion. After the overnight session, rats that met a criterion of 40 or more self-administered infusions of cocaine were allowed to continue in the study. These rats were then given 2-hour access to cocaine over 5 consecutive days of self-administration. Following self-administration, animals were tested in a 1-hour session for cue-reinforced responding at 1, 10 or 45-days of drug withdrawal. During these tests, responding on the active lever resulted in presentation of the cocaine-associated cue only and produced a withdrawal-dependent increase in incubated craving.

Prior work in the Szumlinski laboratory indicated that, on average, rats will self-administer 40 or more infusions of 0.25 mg/0.1 ml/infusion during their first 6-hour access session (e.g., Ben-Shahar et al., 2013; Shin et al., 2016, Shin et al., 2018). Thus, I opted to train the rats in the Mixed model by allowing them 6-hour access to cocaine on their first day of self-administration, *in lieu* of conducting an over-night session. As a sub-goal of this thesis was to simplify the cocaine self-administration procedures, capping the initial training session at 6 hours also eliminated the need to provide the animals with non-contingent food and water in the operant chambers (IACUC-mandated for an overnight operant session), which may have interfered with the formation of drug-cue associations and operant-learning in general. As our prior studies of incubated craving employed a 10-day self-administration procedure (e.g., Ben-Shahar et al., 2013; Shin et al., 2016, Shin et al., 2018), the rats in the

Mixed model then underwent an additional 9 days of self-administration training under 2-hour access procedures. However, unlike Lee et al. (2015), no minimum acquisition criterion was in place for day 1 of self-administration. Consistent with prior work (e.g., Ben-Shahar et al., 2013), the number of reinforcers received ranged from 24 – 101 during the initial 6-hour session, with the animals earning an average of 64.55 reinforcers Following self-administration, animals were randomly assigned to either a 3-day withdrawal group (3WD) or a 30-day withdrawal group (30WD), ensuring that the two groups exhibited comparable cocaine intake over the last 3 days of self-administration. Rats were placed in their home cages for their designated period of forced abstinence, after which tests for cue-reinforced responding was conducted as described above.

2.7 Sham Operant Procedures.

Sham rats were placed in operant chambers for 2-hour sessions for a period of 10 days. At the beginning of each session, rats were placed in the same operant chambers as the cocaine self-administering rats; however, they were not attached to the drug-delivery system. Upon active lever responding, rats were presented with a 20 second light and tone, but received no reinforcer upon pressing the lever. Responding on the inactive lever had no consequences. There were no minimum criteria of active lever responding, or selectivity of responding for sham rats.

2.8 Tests for Cue-Reinforced Responding Under Extinction Conditions

As conducted in prior work (e.g., Ben-Shahar et al., 2013, Shin et al, 2016, Shin et al., 2018), rats were placed in the operant chambers on either 3 days of withdrawal (3WD)

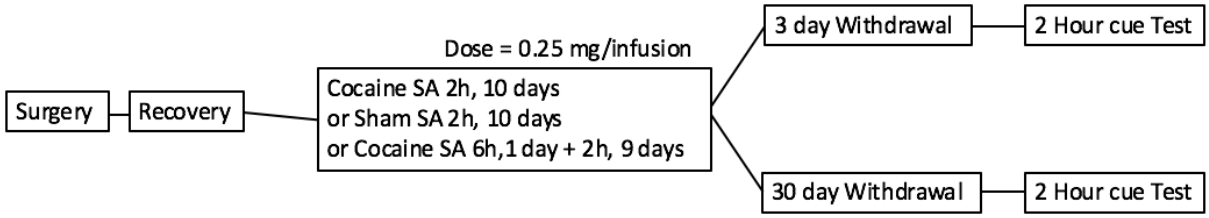
or 30 days of withdrawal (30WD), depending on their group assignment. During this period, rats were given 2-hour access to both levers in the chamber, and pressing of the active lever resulted in the same 20 second tone and light cue as during the self-administration sessions. However, during the cue test, no cocaine reinforcer was available for the cocaine-trained rats. Following cue-testing, rats were decapitated, their brains removed and frontal cortex tissue was dissected over ice for future immunoblotting studies (see Chapter 5).

2.9 Experimental Design.

Seventy male Sprague-Dawley rats were separated into six different groups that were tested at either 3 days withdrawal or 30 days withdrawal (Figure 1). The table below summarizes the final number of animals in each group. (N=22 rats excluded due to failure to meet the acquisition criterion for self-administration).

Treatment Group (2-hour or mixed or sham)	Withdrawal Day (3WD or 30WD)	Number of animals
2-hour	3WD	12
2-hour	30WD	9
Mixed	3WD	12
Mixed	30WD	11
Sham	3WD	10
Sham	30WD	10

Figure 1. Schematic of methods for 2-hour self-administration, mixed self-administration and sham operant responding designs.



2.10 Statistics

A three-way Analysis of Variance (ANOVA) omnibus test [Treatment Group (2-hour vs. Mixed vs. Sham) x Lever (active vs. inactive) x Self-Administration Day (D1 vs 10)] was conducted on the number of lever presses emitted by the rats during the self-administration period. A two-way ANOVA [Treatment Group (2-hour vs. mixed vs. sham) x Self-Administration Day (D1 vs 10)] was conducted on the data for reinforcers earned during the self-administration period. A three-way ANOVA [Treatment Group (2-hour, mixed, sham) x Withdrawal (3WD vs 30WD) x Lever (active vs inactive)] was conducted on the lever-pressing data from the 2-hour tests. For all analyses, significant interactions were followed up with tests for simple effects, followed by Student Newman Keuls post-hoc tests, when appropriate. α=0.05 for all analyses.

3. Results

3.1 Self-Administration Training.

The lever-pressing data were analyzed using a mixed factors ANOVA with the between-subject factor of Treatment (2-Hour, Mixed, Sham), and the within-subjects factors of Lever (active vs. inactive), and Self-Administration Day (D1-D10). The results of the ANOVA failed to indicate a significant 3-way Treatment x Lever x Self-Administration Day interaction (Figure 2A,B) [$F(18,114) = 0.872$, $p=0.613$]. However, a significant Lever X Treatment interaction was observed [$F(2,114) = 18.671$, $p<0.001$]. Collapsing the data across self-administration day, a re-analysis of the results using a Student Newman Keuls post hoc test shows that both Mixed ($p<0.05$) and 2-hour ($p<0.05$) groups showed greater responding on the active lever compared to the inactive lever during self-administration testing. In contrast, no significant difference in responding on the active versus inactive levers was observed in sham controls ($p=0.476$). These findings indicate that the responding exhibited by both cocaine self-administering groups was selective for the active, cocaine-reinforced lever, while that for the sham control group was not.

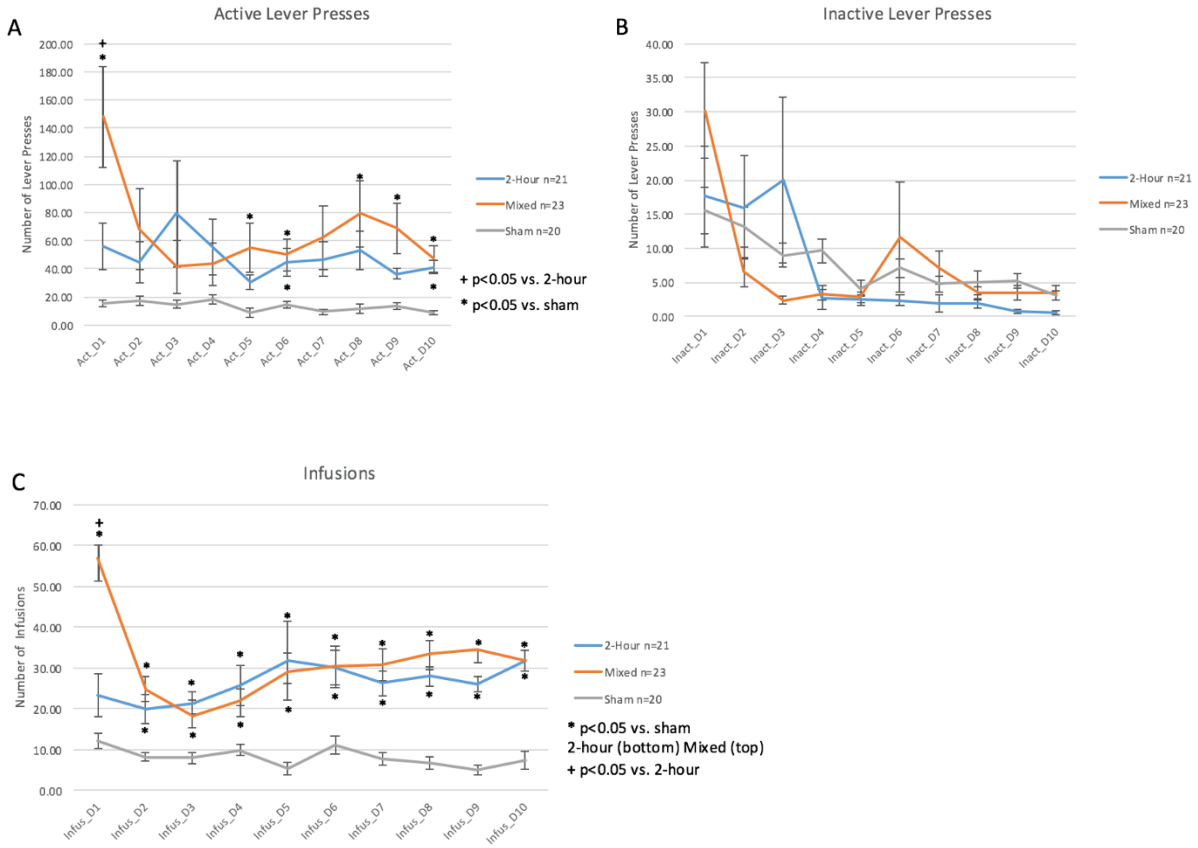
A significant Self-Administration Day x Treatment interaction was also observed for lever-pressing behavior [$F(18,1114) = 1.636$, $p=0.045$]. Given the group differences in response allocation described above, the interaction was deconstructed along the Self-Administration Day factor and re-analyzed along the Treatment factor, separately for the active and inactive levers. Results revealed significant treatment group differences on D1, D5, D6, D8, D9, and D10 for active lever data [one-way ANOVAs: $F's(2,59) > 4.029$, $p's <0.023$]. No significant treatment group differences were observed for responses on the

inactive lever [one-way ANOVAs: F's(2,59)<2.134, p's> 0.127] on any self-administration day. Significant effects were followed up with Student Newman Keuls tests to determine specific treatment group differences for active lever responses on D1, D5, D6, D8, D9, and D10. Not surprisingly, given that the rats in the mixed group were allowed 6-h access to cocaine, the mixed group showed significantly greater active lever responding than both the 2-hour group ($p<0.05$) and sham group ($p<0.05$) on D1 of self-administration (Figure 2A). There were no significant differences in active lever responding between the two cocaine self-administering groups for any other day of self-administration (p's>0.051). Significant differences between 2-Hour and Sham groups were observed on D6 and D10 (p's<0.05). Significant differences between Mixed and Sham groups were observed on D1, D5, D6, D8, D9 and D10 (p's<0.05).

A Self-Administration Day x Treatment ANOVA was conducted on the data for the number of reinforcers earned during self-administration training. Results showed a significant 2-way interaction [$F(18,608) = 2.9$, $p<0.001$] (Figure 2C). Deconstruction of this interaction along the Self-Administration Day factor, followed by re-analysis of the data between the Treatment groups revealed significant differences in the number of infusions earned on all self-administration days [F's(2,59)>4.751, p's<0.012]. Student Newman Keuls post hoc tests were then conducted to determine specific group differences in infusions on each day of self-administration. As expected, given that rats in the mixed group were allowed 6-hour access to cocaine, the mixed group earned significantly more infusions on D1 of self-administration, compared to both 2-hour and sham groups ($p<0.05$). For the remainder of self-administration, there were no significant differences in infusions between

the two cocaine self-administering animals (p's>0.397). On D1 of self-administration, the 2-hour group showed no significant difference in the number of reinforcers earned, compared to sham controls (p= 0.144). However, for the remainder of self-administration, both cocaine self-administering groups earned more reinforcers compared to the sham group (p's<0.05). These findings indicate that both cocaine self-administering groups self-administered a comparable amount of cocaine, with the exception of the first day of self-administration.

Figure 2. Acquisition data for sham, 2-hour, and mixed administration groups. Data from rats with 2-hour daily access to cocaine (2-Hour), 1-day of 6-hour access followed by 9-days of 2-hour access to cocaine (Mixed), or 10-days of 2-hour placement in operant cages with access to no drug reinforcer (Sham) over the 10-day course of self-administration training. (A) Active lever responses during self-administration training. (B) Inactive Lever presses during self-administration training. (C) Number of infusions administered during self-administration training. All sample sizes are reported in figure legends. Significance was determined using a criteria of $p<0.05$. Symbols for significance are as follows: (*) represents significance at $p<0.05$ vs. Sham and (+) represents significance at $p<0.05$ vs. 2-Hour.



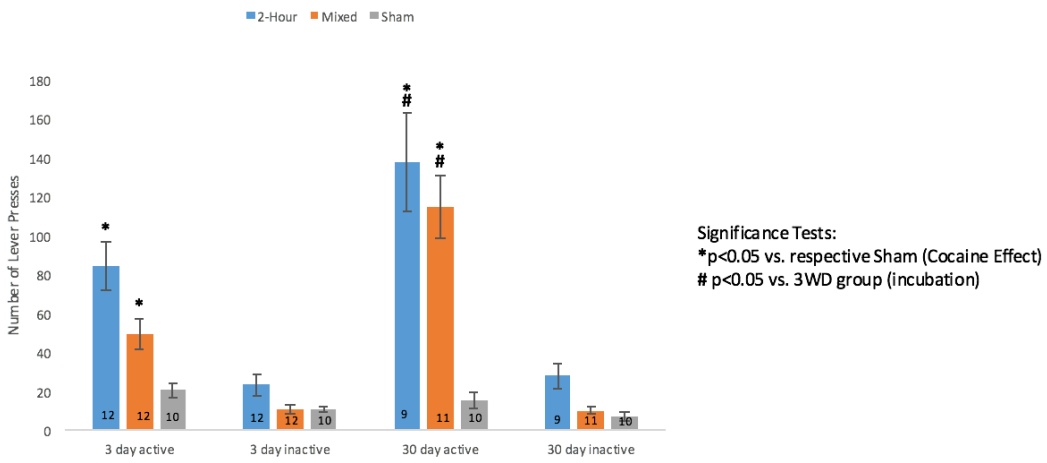
3.2 Time-dependent increases in cue-reinforced responding are observed in cocaine self-administering animals during protracted withdrawal.

The lever-pressing data from the tests for cue-induced behavior were analyzed using a mixed factor ANOVA, with the between-subjects factors of Treatment and Withdrawal (3 versus 30 days) and the within-subjects factor of Lever. Analysis revealed a significant Treatment x Lever Interaction [$F(2,115) = 6.795$, $p=0.002$]. Collapsing the data across the two withdrawal days, a re-analysis of the results was conducted using a Student Newman Keuls post-hoc test along the Treatment factor. Results indicated that both the mixed ($p<0.05$) and 2-hour ($p<0.05$) groups exhibited greater active lever responding than inactive lever responding during the cue-test (Figure 3). In contrast, no significant difference in responding on the active and inactive lever was observed in sham controls ($p=0.433$). These findings indicate that both cocaine self-administering groups were engaged in cue-reinforced responding during testing.

The ANOVA also indicated a significant Withdrawal x Lever interaction [$F(1,115) = 6.798$, $p = 0.010$]. Thus, the data were collapsed across the Treatment factor and re-analyzed along the Withdrawal factor, separately for the active and inactive levers. The results of this re-analysis indicated a time-dependent increase in active lever-pressing [$t(61) = 2.325$, $p=0.006$], but not in inactive lever-pressing [$t(62) = 0.101$, $p=0.679$], consistent with an incubation of cue-reinforced responding. However, the ANOVA failed to indicate a significant Withdrawal x Treatment x Lever interaction [$F(2,115) = 1.257$, $p = 0.288$]. While this negative result suggests that all 3 self-administration groups exhibited an incubation of cue-reinforced behavior, inspection of Figure 3 argued otherwise. Thus, to confirm the

presence/absence of incubated responding, the number of active lever-presses emitted by each treatment group was compared across the two withdrawal time-points. A simple effects test for incubated responding show significant time-dependent increases in active lever responding in the 2-hour group ($p<0.05$), and the Mixed group ($p<0.05$), but not in the sham group ($p=0.175$) (Figure 3). Thus, both cocaine self-administration procedures were sufficient to elicit an increase in cue-induced responding on 30WD compared to 3WD, a result consistent with the presence of an incubation in cocaine-seeking behavior for both treatment procedures.

Figure 3. Comparison of cue-test data for 2-hour self-administration, mixed self-administration schemes, and sham self-administration groups. (A) Number of active and inactive lever responses for 2-Hour, Mixed, and Sham groups during cue-test at 3WD or 30WD. Significance was determined based on $p<0.05$ (*) denotes significance compared to respective sham group and (#) denotes significance compared to respective 3WD group.



4. Discussion

4.1 Increased Duration of Initial Drug-Taking has Minimal Effects on the Remainder of Self-Administration

Cue-elicited incubation of craving is modeled in rats by a time-dependent, selective increase in operant responding on the operandum that previously delivered a positive reinforcer. In both models used for this thesis, cocaine self-administering rats were placed in operant chambers for a period of 10 days and tested for cue-induced responding (i.e., incubation of craving) at 3-day or 30-day withdrawal time points. In the 2-hour self-administration model, animals were given 2-hour daily access to cocaine for the duration of the study. The results from tests for cue-elicited responding displayed a higher degree of variability and a lower magnitude of incubation than those observed after the use of prior 6-hour self-administration models in the Szumlinski laboratory (Ben-Shahar et al., 2013, Gould et al., 2015, Miller et al., 2017, Shin et al., 2016, Shin et al., 2017; Szumlinski et al., 2018) Thus, we applied a Mixed model for self-administration based off of the protocol in Lee et al., 2013, with procedural modifications. In the Mixed self-administration model, animals were given 6-hour access on their first day of self-administration, followed by 2-hour access for the remainder of the self-administration period. This model was used to test if increasing the number of hours of self-administration on day 1 increases the magnitude of incubation of craving during withdrawal. A control group was included that underwent sham surgical procedures and was placed into the operant-chambers to respond for the light-tone stimulus complex that was paired with each cocaine reinforcer delivery for the animals undergoing cocaine self-administration procedures. As expected, given the longer initial self-administration session, the Mixed group displayed significantly greater active lever

responses and earned significantly more reinforcers than both 2-hour and sham groups on day 1 of self-administration training. The Mixed group earned over twice the number of reinforcers as the 2-Hour group on day 1 of self-administration. Thus, they were exposed to the cue-reinforced self-administration training environment over twice as long as the 2-Hour group and had a greater duration to learn the cue-reinforced behavior. Further, we predict that this would likely increase their subsequent intake, and perhaps increase the magnitude of the incubated response. However, there were no significant differences in either active lever responses or reinforcers received between the two cocaine self-administering groups for the remainder of self-administration. These data indicate little effect of the longer initial self-administration upon subsequent drug-taking. During self-administration, both 2-hour and Mixed groups also showed significant increases in responses on the active lever compared to the inactive lever, and sham groups did not show any significant lever preference. This indicates that the cocaine self-administering groups were engaged in drug-reinforced behavior during self-administration, while the sham animals were not. Thus, the results demonstrate that lever-pressing for cocaine was in fact a comparable reinforcing event across each of the cocaine self-administering groups, while the light/tone complex along was not sufficient to engender comparable behavior in the sham animals.

4.2 Time-dependent Increases in Cue-Reinforced Responding are Observed in Both Cocaine Self-Administering Groups

Results of the cue-test revealed significant increases in cue-elicited responding on the active lever between groups tested at 3WD and 30WD for 2-Hour and Mixed groups; while, sham controls showed no time-dependent increases in lever responding. Thus, the

current results are consistent with the conclusion that both short-access protocols were sufficient to elicit an incubation of craving during cocaine withdrawal. Although the Mixed self-administration group appeared to exhibit a larger magnitude of incubation than the 2-Hour group (Figure 3), the statistical analyses of the data did not support a significant group difference. Furthermore, the results suggest that offering animals increased drug-access and duration to learn drug-cue association on day one of self-administration is not critical to observe an incubation of craving during cocaine withdrawal and has marginal effects on the magnitude of the incubation of craving. During cue-testing, both cocaine self-administering groups showed selective responding on the active lever compared to the inactive lever, while sham groups did not show significant lever preference. Thus, both cocaine self-administrating groups were engaged in cue-reinforced behavior during the cue-test. The resulting increase in the number of active lever responses exhibited by the cocaine-experienced groups cannot be explained by increased non-specific motor activity in late withdrawal, as there was no corresponding increase in the number of responses on the inactive lever between the early and late withdrawal conditions. Overall, the present results demonstrate that, consistent with prior literature (Lee et al., 2013; Ma et al., 2014; Suska et al., 2013; Sorge & Stewart, 2005; Halbout et al., 2014), incubated cocaine-seeking is observed in two different short-access models of self-administration and importantly, that an incubation of cocaine-craving was observed in the absence of any food/sucrose pre-training. Literature describes various short-access (2-hour) self-administration models that have employed higher doses of cocaine than this study. Some examples include a dose of 0.5mg/kg/infusion (Kerstetter et al., 2008) and a dose of 0.75mg/kg/infusion (Lee et al., 2013). Additionally, the study which used a single, 6-hour, session employed a dose of 0.5

mg/kg/infusion to elicit an incubation of cocaine-craving in mice (Halbout et al., 2014). To the best of my knowledge, the results of this present study are the first to demonstrate that daily, 2-hour access to a lower cocaine dose (0.25 mg/infusion) can elicit an incubation of cocaine-craving in rats, suggesting that this procedure is sufficient to induce plasticity within neural circuits driving the conditioned reinforcing properties of cocaine-associated cues.

4.3 Analysis of Procedural Factors That Impinge Upon Incubation of Craving

This study combined with existing reports in literature implies that incubation of craving can be observed following the use of different self-administration models with a variety of procedural modifications. The 2-hour model employed in this thesis were based on that described in Kerstetter et al. (2008). A comparison of the results from this prior study and those present indicate that an incubation of cocaine craving can be observed during protracted withdrawal, despite major differences in pre-training, cocaine dose and the conditions under which the rats are tested for incubated craving. In Kerstetter et al., (2008), the active lever-responding was extinguished in cocaine-abstinent rats prior to the test for cue-reinforced incubated craving, while the present study did not extinguish behavior prior to testing. Furthermore, in Kerstetter et al., (2008), food training was conducted prior to cocaine self-administration, while no food-training was employed herein. This fact supports the claim that a relatively simple self-administration model can be sufficient to elicit incubation of craving in rats, and is sufficient to elicit similar changes in neuroplasticity to more complex self-administration models.

Circumstantial evidence from the literature argues that the number of days of cocaine self-administration does not impinge upon the ability to detect an incubation of cocaine-seeking. For example, Tran Nguyen et al. (1998) used a 14-day self-administration period, while several studies from the Szumlinski laboratory (Ben-Shahar et al., 2013; Miller et al., 2017; Shin et al., 2016; Shin et al., 2017; present study) used a 10-day self-administration period and others have observed incubated craving following a 5-day self-administration period (Lee et al., 2013; Ma et al., 2014; Suska et al., 2013). Furthermore, in mice, a single 6-hour session is sufficient to elicit an incubation of cocaine craving in withdrawal (Halbout et al., 2014). Thus, it would appear that the number of days of cocaine self-administration is not a critical factor for the manifestation of incubated craving. Thus, the present data implies that the neuroplasticity induced by these various different procedures would also be similar.

The present study differs in the number of hours/day of self-administration from several other incubation of craving studies in the literature. A 6-hour self-administration paradigm has been extensively used in literature, while other studies have used 3-hour self-administration periods (Tran-Nguyen et al., 1998; Calu et al., 2007). Studies have also used 2-hour self-administration models (Sorge & Stewart, 2005; Kerstetter et al.., 2008;; Neisewander et al., 2000) and "mixed" self-administration models involving initial over-night training sessions (Lee et el., 2013; Ma et al., 2014; Suska et al., 2013). While the magnitude of our incubated response tended to be higher in rats trained initially in a 6-hour session versus those trained only in 2-hour sessions, the difference in magnitude was not statistically significant. Taken together, the above data argue that the duration of the self-administration session is not a major factor affecting the ability to induce an incubation of

craving. Thus, the present data implies that low amounts of cocaine-exposure are sufficient to elicit an incubation. Further, these data suggest that changes in neuroplasticity observed during an incubation of craving can be elicited by low doses of cocaine self-administration.

Variable magnitudes for incubation of craving have been reported across various studies in the literature (Ben-Shahar et al., 2013; Shin et al., 2016, Lee et al., 2013; present study). In studies conducted by the Szumlinski laboratory using a 6-hour model for self-administration rats displayed approximately 90 more responses on the active lever after 30WD compared to 3WD (Ben-Shaher et al., 2013) and in a different study displayed 65 more responses on the active lever at 30WD compared to 3WD (Shin et al., 2016). The results from these studies indicate that there is variability in the magnitude of incubation of craving, when the factors of laboratory and self-administration method are held constant. Studies conducted by the Wolf laboratory rats have displayed 15 more responses at 30WD compared to 3WD (Conrad et al., 2008) and in another study displayed 17 more responses at 30WD compared to 3WD (Werner et al., 2018). Although both the Wolf laboratory and Szumlinski laboratories used a 6-hour model of self-administration, the Wolf laboratory shows a lower magnitude for incubation of craving in the studies presented. In a study from the Shaham laboratory, which have also used a 6-hour model for cocaine self-administration, rats display an increase of 25 lever responses from early to late withdrawal when they were returned to their home cages after self-administration; while, when rats were chronically housed in their self-administration chambers they displayed an increase of 50 active lever responses from early to late withdrawal (Lu et al., 2005). However, in another study from the Shaham laboratory, rats displayed an increase of 54 active lever responses

from early to late withdrawal and were returned to their home cages after self-administration (Koya et al., 2009). Another study conducted by Lee and colleagues, (2013) uses a mixed short-access self-administration model and rats exhibited approximately 40 more responses on the active lever after 45WD compared to 1WD. In this present study, using a 2-hour model of self-administration rats displayed 50 more responses at 30WD compared to 3WD. Using the Mixed self-administration model, rats responded on the active lever 69 more times at 30WD compared to 3WD. Overall, this literature suggests that there are no distinct trends between duration of self-administration and magnitude of incubation of craving. Thus, the results imply that changes in neuroplasticity observed across all models described, with various magnitudes of incubation of craving, should be similar.

Several studies have used of food training as an initial step prior to the initiation of drug self-administration (Tran-Nguyen et al., 1998; Kerstetter et al., 2008; Grimm et al., 2001; Neisewander et al., 2000; Ben-Shahar et al., 2012; Ben-Shahar et al., 2013; Miller et al., 2017; Shin et al., 2016; Shin et al., 2017). We have suggested (see the Introduction section of the present thesis) that this may produce an interpretational confound that may not be necessary for initial cue-reinforced responding on the operandum. Indeed, the current study is methodologically consistent with other reports from other researchers who similarly demonstrated cocaine-self-administration without the need for prior food training (Sorge & Stewart, 2005; Halbout et al., 2014; Lee et al., 2013; Ma et al., 2013). One specific study, Halbout et al. (2014), tested for behavioral differences during withdrawal after a single 6-hour session of cocaine self-administration in mice, with and without the use of sucrose pre-training. Results showed incubation of craving in both groups, and no significant differences

were observed between the groups. Thus, while I did not explicitly compare the effects of food-training versus no food-training in this thesis, these published findings argue that lever-response training using non-drug reinforcers are not necessary for, nor do they alter the magnitude of, incubated responding. In line with this finding, I was able to observe the incubation of cocaine-craving with a magnitude comparable to that observed in other studies from the Szumlinski laboratory employing food-training procedures.

As already described, there is a considerable amount of literature showing that incubation of craving can be observed following sucrose administration alone (Grimm et al., 2002; Grimm et al., 2005; Counotte et al., 2014; Aoyama et al., 2014; Di Ciano et al., 2004; Van den Oever et al., 2006). The questions then become: are the underlying neurochemical changes responsible for sucrose craving the same or different from those underlying cocaine craving? Some researchers suggest that an incubation of craving for both sucrose and cocaine share the same neuronal underpinnings, while others suggest that the two reinforcers can produce their effects via opposing systems (Bassareo, Cucca, Frau, and Di Chiara, 2015; Bassareo et al., 2017; Conrad et al., 2008; Counette et al., 2014; Loweth et al., 2014; Shin et al., 2017). For example, in the study conducted by Shin and colleagues (2017), a cue-elicited increase in vmPFC extracellular glutamate was observed in late cocaine withdrawal, while exposure to sucrose-associated cues resulted in elevated extracellular glutamate levels early following self-administration (Shin et al., 2017). Some neurochemical changes are unique to drug self-administration. An example of this can be seen in the same study, Shin et al. (2017), as drug associated cues elicited an increase in vmPFC dopamine in early withdrawal and no changes in vmPFC dopamine were observed following exposure to

sucrose cues. However, if in fact the same results occur with regular rat chow (as opposed to sucrose) this potentially introduces an interpretational confound when attempting to identify drug-induced or drug cue-associated biological changes. As ultimately the goal of establishing a simple short-access self-administration model is to characterize and then study the neurobiological correlates of incubation, it is preferable to avoid these potential confounds by eliminating sucrose (and food) pre-training for the study of this phenomenon.

4.4 Relevance For The Human Condition

A large proportion of addiction research is conducted using animal models, as there are several barriers to studying this disease in the human brain. These animal models have been well established and various partial models exist that are useful in examining different aspects of addiction. The few human studies focused on the incubation of craving in cocaine-addicted individuals (Gawin & Kleeber, 1986; Parvaz et al., 2016) do not manipulate the cocaine history of their subjects and thus, there is variability in drug dose, frequency of drug taking and duration of drug taking across subjects. My survey of the incubation research in animals presented herein, coupled with the clinical observation that incubated cocaine-craving is observed in humans with highly varied drug-taking histories, argues that the time-dependent increase in the capacity of cocaine-associated cues to elicit craving during cocaine withdrawal depends more so on factors related to drug withdrawal, rather than factors related to drug self-administration as important for incubated craving.

4.5 Summary

In summary, this paper presents two procedurally different short-access self-administration models, which both showed incubation of craving during cocaine withdrawal. The discussion highlights several other models used in literature, which were also able to show an incubation of craving. Many factors were varied across these models including: the duration of self-administration on the first day of self-administration, the number of days over which animals self-administer drug, the cue-test procedures, and use of sucrose/food pre-training. This shows the incubation of craving can be reliably observed during cocaine withdrawal, regardless of variability in experimental manipulations. Thus, illustrating that incubation of craving is a robust phenomenon driven by cue-presentation during withdrawal from drug use. Further, the results of the present study demonstrate that relatively simple self-administration training procedures are sufficient to elicit incubation of craving of relevance to studying its neurobiological underpinnings.

5. Future Directions

5.1 Rationale

Although some patients will respond well to drug-counseling, a large portion of addicts in cocaine withdrawal require more than standard psychological treatment. Therefore, it is a current priority to develop effective therapeutics for cocaine dependence (Kampan, 2005; Willyard, 2015). Having now established more simple models for incubated cocaine-craving, a future goal of study is to conduct an analysis of its biochemical correlates, with the ultimate goal of identifying molecular targets for potential therapeutics.

5.2 Connections between Incubation of Craving Behavior and Changes in Neurobiological Correlates

5.2.1 Functional analysis of neurobiological markers in the PFC

There have been many reports of PFC activation during human cocaine withdrawal measured in fMRI studies (Mass et al 1998) and during cocaine withdrawal in rats (Luís, Cannella, Spanagel, & Kohr, 2017; Koya et al., 2009). Several studies analyzing neurobiological changes during protracted cocaine withdrawal have been reported in the pre-frontal cortex, alongside behavioral analysis, using a 6-hour long-access cocaine self-administration model (Ben-Shahar et al., 2013; Gould et al., 2013; Szumlinski et al., 2016; Miller et al., 2017; Koya et al., 2009). The first animal study to implicate the vmPFC in incubation of cocaine craving was conducted by Koya and colleagues (2009) found time-dependent increases in p-ERK positive cells in the vmPFC of rats which underwent cue-elicited testing for drug seeking behavior in late withdrawal compared to early withdrawal.

Literature shows that p-ERK is a marker for neuronal activity (Thomas & Huganir, 2004). Thus, the functional relevance of these findings were tested through administering GABAa and GABAb agonists which inhibit neural activity during late withdrawal and GABAa and GABAb antagonists which increase neural activity during early withdrawal (Koya et al., 2009). Results showed decreased and increased cue-elicited drug-seeking behavior at late withdrawal and early withdrawal time points, respectively, indicating the vmPFC neuronal activity plays a critical role in incubation of craving (Koya et al., 2009). Furthermore, literature shows that the opportunity to engage in drug-seeking behavior elevates vmPFC PKCe levels in cocaine-experienced animals during protracted withdrawal and the local infusion of a PKCe translocation inhibitor reduces cue-elicited drug-seeking behavior in late withdrawal (Miller et al., 2017). Literature also shows increased increases in Akt activity at 3 and 30 days withdrawal in the vmPFC of rats, which had been re-exposed to cocaine cues (Szumlinski et al., 2018). The functional relevance of this change in kinase activity was tested using intra-vmPFC administration of a PI3K inhibitor and reduced drug seeking behavior was observed in protracted withdrawal (Szumlinski et al., 2018). Thus, there appears to be a number of functionally relevant changes in intracellular signaling within the vmPFC that correlate with the incubation of cocaine-seeking.

5.2.2 Functional Analysis of Neurobiological Markers in the Nucleus Accumbens and Amygdala

Functional relevance of biomarkers for incubation of craving have also been studied in the amygdala and the nucleus accumbens (NAc). Literature shows that following a long-access (6-hour) cocaine self-administration model, the number of synaptic AMPA receptors

is increased in protracted withdrawal (Loweth et al., 2014). A simplified short-access single session mode for cocaine self-administration also reports changes in AMPA/NMDA receptors, as well as altered mGlu1 mRNA levels in the nucleus accumbens (NAc) correlated with an increase in cue-elicited drug seeking behavior in protracted withdrawal (Halbout et al., 2014). Administration of an mGlu1 antagonist in protracted withdrawal increased drug-seeking behavior compared to control mice, implicating mGlu1 in attenuating drug-seeking behavior in protracted withdrawal (Halbout et al.,2014). Thus, providing support that a simple model of self-administration, like those used in this publication, is sufficient to study neurobiological changes associated with behavior in withdrawal. Furthermore, a study conducted by Lee and colleagues (2013) detected silent synapses in the projection from the basolateral amygdala to the NAc during early withdrawal. Results also showed that during late withdrawal time points, these silent synapses became unsilenced. To test the functional relevance of these silent synapses, optogenetic manipulations were conducted to re-silence these synapses in protracted withdrawal, and incubation of craving decreased. These findings indicate that silence synapse-based remodeling of the amygdala-NAc projection is critical for incubation of craving during cocaine withdrawal (Lee et al., 2013). Another study conducted by the Shaham laboratory found that systemic or central amygdala injections of an mGlu2/3 agonist attenuates enhanced extinction responding which is observed during late cocaine withdrawal (Lu et al., 2007). Thus, glutamate release within the central amygdala appears to be critical for increased drug-seeking during late withdrawal in cocaine-experienced animals. Literature also shows that in the NAc, alterations in dendritic spines occur during protracted withdrawal from cocaine self-administration (Christian et al., 2017). Additionally,

studies conducted in the Wolf laboratory show that CP-AMPARs are expressed at low levels in NAc synapses in drug-naïve rats (Conrad et al., 20018; Reimers et al., 2010), but expression of CP-AMPARs at NAc synapses increase along with cue-elicited incubation of cocaine craving (Conrad et al., 2008). The functional relevance of this increase in CP-AMPARs at NAc synapses was tested in studies conducted by Conrad and colleagues (2008), and results demonstrated that the new receptors formed in late withdrawal mediate incubation of cocaine craving. Additionally, dysregulated protein translation in the NAc has been demonstrated to be critical for incubation of craving (Werner, Stefanik, Milovanovik, Caccamise, and Wolf, 2018). Thus, there exist also a number of functionally relevant neurobiological changes within the NAc and amygdala that also accompany the incubation of cocaine-craving.

5.3 Prelimbic and Infralimbic Cortices

The ventral aspect of the PFC is subdivided into two regions: Infralimbic (IL) and Prelimbic (PL). Neuroanatomical evidence shows that the PL largely projects to the nucleus accumbens core while the IL projects to the nucleus accumbens shell; although, there is some overlap between their regions of innervation (Vertes et al., 2004). The PL and IL have been shown to opposingly regulate drug-seeking behavior. Traditionally, activation of the PL has been shown to potentiate drug seeking behavior (Pelloux, Murray, & Everitt, 2013) and the IL has been shown to attenuate drug seeking behavior (Peters, LaLumiere, & Kalivas, 2008). One specific study (Ma et al., 2014) showed that optogenetic reversal of silent synapse- based remodeling of IL-to-Nucleus Accumbens Shell projections potentiated cocaine-seeking behavior and remodeling of PL-to-Nucleus Accumbens Core projections

inhibited cocaine-seeking behavior at late withdrawal. This study shows mechanistic evidence suggesting silent synapse-based remodeling in the PL potentiates drug seeking behavior while the IL plays an opposing role.

Recent literature suggests that the IL could also play a role in potentiating incubated drug-seeking behavior (Shin et al., 2017). Shin et al., (2017) and colleagues manipulated extracellular glutamate levels in both the PL and IL for the analysis of behavioral effects at early and late withdrawal time-points. This study was conducted based on prior literature demonstrating that incubated cocaine-seeking is associated with a withdrawal dependent increase in cue-elicited glutamate release within the ventromedial PFC (Shin et al., 2016). Based on the results of the Ma et al. (2014) study discussed above, they hypothesized that lowering extracellular glutamate levels in the PL would decrease drug seeking behavior in late withdrawal and lowering extracellular glutamate levels in the IL would have opposing behavioral effects (Shin et al., 2017). Lowering extracellular glutamate levels in the PL robustly decreased drug-seeking behavior as expected. However, lowering extracellular glutamate levels in the IL also decreased drug seeking behavior (to a lesser agree than observed in the PL). This study suggests a novel role for the IL in potentiating drug seeking behavior rather than participating in attenuating drug seeking behavior alone. These novel findings for the manipulation of extracellular glutamate, arouse our curiosity in IL and PL differences for other biochemical correlates in protracted cocaine withdrawal such as: Homer1b/c, pERK, and Homer 2a/b, PI3K and PKCε. As such, a future direction for this study is to conduct western blotting procedures to determine protein difference in IL and PL

regions during cocaine withdrawal as it relates to the manifestation of incubated cue-elicited cocaine-seeking.

5.4 Other Frontal Cortical Regions: Orbital Frontal Cortex, Insula, Anterior Cingulate Cortex

A second future direction of this study is to expand this PFC analysis to other frontal cortical brain regions that show changes in biochemical markers as craving incubates during cocaine withdrawal. FMRI studies have identified cue-elicited activation of the Orbital Frontal Cortex (OFC), Insular Cortex, and Anterior Cingulate Cortex (ACC) during cocaine withdrawal (Childress et al., 1999; Maas et al., 1998), leading to the choice of these brain regions as other targets for this study. To the best of my knowledge, the OFC, Insula, and ACC have not been studied in the context of incubated responding. However, these brain regions have been studied for other aspects of cocaine addiction in both animals models and humans. This literature is sufficient formulate hypotheses about the activity of these brain regions at early and late cocaine withdrawal time-points.

5.4.1 Orbital Frontal Cortex & Insula

Functional and neuroanatomical literature evidence implicates the insula and OFC as relevant brain regions to target for a biochemical analysis of markers during incubation of cocaine craving. Human fMRI studies have shown activation of both the OFC and Insula in cocaine withdrawal (Childress et al., 1999; Maas et al., 1998; Goldstein and Volkow, 2002). One specific study in rats shows that optogenetic inhibition of projections from the lateral OFC to basolateral amygdala inhibit cue induced reinstatement of cocaine-seeking behavior

using an extinction/reinstatement model (Arguello et al., 2017). This study argues a critical role for the OFC in modulating behavioral effects of cocaine withdrawal. Additionally, neuroanatomical evidence (Koob & Volkow, 2017) shows that the insula is interconnected with the PFC, OFC, extended amygdala and habenula. Prior literature suggests that these brain regions are implicated for various aspects of addiction such as: drug-craving, stress, binging, and intoxication. Insular connectivity with the various brain regions mentioned suggest its possible activation during late cocaine withdrawal. Based on this literature, I predict to see an increase in biochemical indices of activity in both the OFC and Insula in late withdrawal.

5.4.2 Anterior Cingulate Cortex

Literature shows activation of the anterior cingulate cortex (ACC) in cocaine addicts in cocaine withdrawal upon cocaine cue exposure (Childress et al 1999; Ray et al 2015). However, prior literature shows no significant time-dependent changes in GluN2B (Szumlinski et al., 2016), Homer 2a/b (Gould et al., 2013), mGlu5 (Miller et al.,2017), PKCe (Miller et al., 2017) and pERK (Miller et al., 2017) in the more dorsal aspects of the PFC (ACC + dorsal aspect of the PL) as craving incubates during cocaine withdrawal. Additionally, Koya et al., 2009 reports only modest time-dependent increases in pERK in the dorsomedial PFC, compared to robust increases observed in the more ventromedial aspect of this brain region. Based on this, I do not expect to see many changes in biochemical indices of activity from early to late withdrawal in this brain region.

5.5 Summary

In summary, the future directions of this present study serve to identify changes in neurobiological markers in protracted cocaine withdrawal using a simplified model for cocaine self-administration. As it is known that there are functional differences between the PL and IL, these future directions also aim to analyze sub-region differences for biochemical changes previously observed in the ventromedial PFC. Furthermore, as current research focuses on these more ventral PFC sub-regions, these studies aim to expand our search for biochemical correlates of incubation to the ACC, OFC and Insula. Additionally, identification of target proteins will be followed up by neuropharmacological studies to determine the relevance of each protein for the incubation of cocaine-seeking behavior.

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