Effects of Heroin on Prosocial Behavior in Rats and its Modulation by the Anterior Insula

by

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ABSTRACT

Opioid use rates and related deaths continue to be a public health crisis; while there are many contributing factors to opioid use disorders, criteria for diagnosis include problems related to social functioning. Previous research indicates that laboratory rats, which are frequently used as animal models of addiction-related behaviors, are capable of prosocial behavior. The following collection of studies were performed to determine the effects of heroin on prosocial behavior in rats, as well as the role of the insula in both self-administration of heroin and prosocial behaviors. All of the experiments were conducted utilizing an established model of prosocial behavior in rats in which a performing rat releases a cagemate from a restrainer. The occurrence of and latency to free the confined rat was recorded. After baseline rescuing behavior was established, rats were allowed to self-administer heroin (0.06 mg/kg/infusion i.v.), and subsequent experimental conditions were imposed.

Experimental conditions, in a series of different studies, included comparing heroin reinforcers with sucrose, chemogenetically modulating the insular cortex (both stimulatory and inhibitory processes) and administering excitotoxic lesions in the insula. There were significant differences in saving behaviors between heroin and sucrose groups demonstrating an opioid induced loss of prosocial behavior. Modulating the insula chemogenetically resulted in some restoration of these opioid related deficits, and insular lesions did not significantly impact prosocial behaviors, however, there were significant differences between rates of heroin intake in lesioned animals versus non-lesioned

controls. Taken together, these results demonstrate the deleterious effects of heroin on prosocial behaviors and offer further support for the role of the insula in both addiction and social constructs.

DEDICATION

Mom and Dad, Bear and Azalia.

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CHAPTER 1

GENERAL BACKGROUND AND SIGNIFICANCE

Opiates are a broad class of alkaloids that are widely used for their analgesic and anti-tussive properties. Naturally occurring opiates found in the latex of the opium poppy Papaver somniferum include codeine and morphine, the latter of which is acetylated to form the highly addictive opioid heroin (diacetylmorphine). Other opioid alkaloids, now manufactured almost entirely via synthetic routes, include oxycodone, hydromorphone, methadone, buprenorphine, and fentanyl, to name a few (Bond & Witton, 2017). The term "opiate" is often used in reference to codeine and morphine, as is the term "opioid", though some clinicians and researchers prefer to use the term opiate to refer to naturally derived opioid alkaloids and the term opioid to refer to endogenous opioid systems as well as both naturally occurring and synthetic drugs that bind to opioid receptors. Prior to reviewing information on the current opioid epidemic and rodent models of opioid addiction, we will first review the mammalian endogenous opioid systems.

Endogenous Opioid Signaling

The endogenous opioid system is implicated in numerous normal and pathophysiological processes, including addiction, nociception, consummatory behaviors, reproduction, mood regulation, thermoregulation, respiration, and immune and peripheral organ function (Bodnar, 2013). This system is comprised of several families of neuropeptides, including pro-opiomelanocortin (POMC)-derived endorphins, proenkephalin (PENK) and (PDYN)-derived peptides, each of which is enzymatically

cleaved to form multiple bioactive fragments. Additional members of the opioid peptide family include endomorphins, the precursor of which has remained elusive, nociceptin/orphanin FQ, nocistatin, morphiceptin, and many others. These neuropeptides are released from dense core vesicles and bind to one or more separate yet widely distributed G-protein coupled receptors including μ, δ, κ, and opioid receptor-like 1 (ORL1;Compton, Jones, & Baldwin, 2016; Maher, Martin, & Childers, 2005). Numerous variants of each of these receptors can arise from alternative splicing and posttranslational modifications (Stein, 2016). Other receptors such as sigma and ORL1 receptors have been proposed to bind opioid drugs, but their inclusion into the classical family of opioid receptors is debated due to structural differences and non-traditional binding profiles.

Opioid peptides as well as synthetic opiate analgesics such as morphine, hydromorphone, oxycodone, etc. exert their pharmacological effects primarily by activating one or more of the classic μ, δ, or κ receptors. These receptors are G-protein coupled receptors (GPCRs) that utilize inhibitory G proteins such as Gi/Go to inhibit cyclic adenosine monophosphate (cAMP) production and reduce neural activity (Al-Hasani & Bruchas, 2011). While opioid receptor activation produces a general reduction in cellular activity, this can also lead to increased firing of various neuronal populations (i.e., midbrain dopaminergic neurons; (Johnson & North, 1992) via disinhibition of local inhibitory GABAergic interneurons, resulting in increase of dopamine release in the shell of the nucleus accumbens (Compton, Jones, & Baldwin, 2016). While beyond the scope

of the present chapter, chronic opioid-induced adaptations in receptor function and intracellular signaling can result in the development of tolerance and physiological dependence, leading to higher doses needed to achieve the same desired effects.

Historical Aspects of Social Influences on Opiate Use

The use of opium dates back over 4000 years, as written accounts of its medical applications have been found in Asia Minor and surrounding areas, and many references to opium use can be found in the writing of the ancient Greeks as well (Kapoor, 1995). From these regions, it is believed that opium use subsequently spread westward into Europe and eastward toward India, China, and other parts of Southeast Asia. However, in the 13th century, opium use in Europe was drastically reduced as of the result of Inquisition-stoked associations of the drug with Eastern religions. Nevertheless, the use of opium for both its euphorigenic and medical properties continued to become a widespread societal issue in countries such as China. Numerous legislative efforts were made to suppress the use of opium in China, yet its use and trafficking became a source of international conflict that culminated in the First and Second Opium Wars between China and Britain (Goldberg & Latimer, 2014). Around the end of the Second Opium War in the 1860s, the United States was undergoing exponential growth as a result of industrialization, the lucrative allure of the California Gold Rush, and the expansion of the railroad industry. This rapid growth resulted in a significant labor shortage, and many impoverished Chinese citizens sought work and financial stability through the promise of

opportunity in the United States (Olive, 2017). Ultimately, however, Chinese immigrants were ostracized for many reasons including the low cost of their labor, and forced to form their own subcommunities within the U.S. This stigmatization of Chinese immigrants carried over to their use of opium, forcing them to confine their use in so-called "opium dens". While many factors likely played a role, it is plausible to argue that this demonization of Chinese immigrants and their opium use was one of the first widespread negative social influences on the use of these drugs. Ironically, however, it was these same opium dens that introduced opium to non-Chinese citizens (Goldberg & Latimer, 2014).

Other significant events in the development and spread of opioid use soon followed (Olive, 2017). One of the first was in 1805 when German pharmacist Friedrich Sertürner first isolated and characterized morphine as the primary active alkaloid component of opium latex. Subsequent work identified additional active alkaloids including codeine, thebaine, and papaverine. Not long after, the hypodermic syringe was developed which permitted easy administration of morphine and other opiates directly into the bloodstream or muscle. This invention came at a fortuitous time, as the American Civil War erupted the following decade, and morphine was found to be a potent pain reliever for wounded soldiers. Its widespread use as a battlefield analgesic resulted in a high incidence of morphine dependence in soldiers. Despite its widespread use in this exclusively male population, recreational opiate use also became popular among upper class women (Courtwright, 1982). The post-Civil War spike in morphine dependence led

scientists to explore the development of other analgesics, and it was soon discovered that boiling morphine in acetic acid produced diacetylmorphine. In the late 1800's German pharmaceutical company Bayer commenced marketing of diacetylmorphine under the brand name of Heroin as an anti-tussive and treatment for tuberculosis. In the early 1900's, the American Medical Association approved the use of prescription heroin and promoted its dispensing in lieu of morphine. The Controlled Substances Act of 1970, however, placed heroin into Schedule 1, a category that includes drugs with high potential for abuse and no medical uses.

The Current Opiate Epidemic

Currently, opioid use is at an all-time high (Rudd, 2016). Much of the current epidemic is being attributed to the dramatic increase in the prescribing of opioid analgesics (Ciccarone, Ondocsin, & Mars, 2017; Ostling et al., 2018). The Centers for Disease Control (CDC) reports that the number of issued prescriptions for opioids has quadrupled since 1999; however, there has not been a parallel increase in reports of incidences of chronic pain (Department of Health, Services, & for Disease Control, 2017). A European study, examining gender differences in opioid dependent adults reported no gender differences regarding the age of onset of regular opioid use; however, at up to 4 years of consistent use there are gender specific differences in the consequences of the development of opioid use disorder. Women were shown to exhibit more social issues and men experience more legal difficulties than women, specifically looking at history of arrests (Hölscher et al., 2010).

Heroin is one of the most commonly used and opioids, and can be administered in several different ways, including smoking, oral intake, insufflation, or most commonly intravenous injection (SAMSA, 2016). It is not uncommon for a new user to start out using heroin in a less invasive way such as smoking or insufflation, but begin injecting heroin to increase the rate of onset and intensity of the "high" (Bond & Witton, 2017). When consumed, heroin gives the user a feeling of intense euphoria. Initially users will feel warm and flushed, their limbs will feel heavy, and they will experience dry mouth, sometimes accompanied by nausea and vomiting. These initial experiences are sometimes followed by feelings of lethargy and impaired mental functioning (NIDA, 2014). Factors in the increase of heroin use and the associated mortality rate include the introduction of an abuse deterrent formulation of Oxycontin, and then fentanyl and its analogues, as well as the relatively low cost and availability of heroin (Ciccarone et al, 2017; Ostling et al, 2018; Rudd, 2016, Comer via edits, 2019).

Since the end of the 20th century, heroin was the drug of choice for low-income, Caucasian males between the ages of 18-25 (Jones, Logan, Gladden, & Bohm, 2015). However, the ongoing increase of heroin use is now showing record rates in both males and females, and spanning the entire socioeconomic spectrum (Cicero & Ellis, 2017; Compton et al., 2016; Jones, Campopiano, Baldwin, & McCance-Katz, 2015). In addition, the demographics in heroin use has transitioned from predominant use by low-income ethnic minority males in urban environments to use by both genders in suburban and rural areas (Cicero & Ellis, 2017). Like other abused substances, there is a cyclical

pattern that occurs with opioid use, in which the drug is consumed, euphoria and intoxication ensue, tolerance and physical dependence develop after repeated use, larger amounts of the drug are ingested, and symptoms of withdrawal emerge when drug is not used. These feelings of dysphoria and negative affect prompt drug seeking to stave off the physical and mental discomfort (Koob, 2008).

Opioid Addiction and Social Function

Opioid use disorder (OUD) is characterized in the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM-V) as meeting at least two or more criteria that include behaviors such as impaired control over drug use, compulsive use, continued use despite harm, craving, withdrawal symptoms, or persistent or recurrent social or interpersonal problems caused or made by the effects of opioids (APA, 2013). Many studies have aimed to evaluate intrapersonal problems and their role in substance use disorders; however, understanding the social influences on opioid addiction is complex because it incorporates a dynamic set of genetic, environmental, and social factors.

While it is widely accepted that a person's peer group or social network impacts their drug using behaviors (Buchanan & Latkin, 2008; Eitan, Emery, Bates, & Horrax, 2017; Luthar, Anton, Merikangas, & Rounsaville, 1992; Russell, Trudeau, & Leland, 2015), researchers continue to examine the directionality of social influence. It is unknown if someone with OUD self-selects into a peer group of people who use drugs, or if the peer group impacts the person's decision to begin using (Bohnert, Bradshaw, &

Latkin, 2009). Results from a self-report study investigating this phenomenon found evidence that both scenarios are at play, but that change in drug use over time was not due to a change in their friends' drug taking behavior, but a function of a changing social network (Bohnert, Bradshaw, & Latkin, 2009). This is supported by findings that psychosocial factors including peer-group relationships, family issues, employment and social support can be used as predictors of long-term opiate use (Scherbaum & Specka, 2008). Cicero and Ellis (2017) recently reported in a meta-analysis of studies of prescription opioid users, that prescription opioids are often used as a "safe" alternative to illicit opiates with less stringent legal ramifications for non-medical use and distribution. In addition, while "getting high" and alleviation of withdrawal are often the primary motivating factors in non-medical use of prescription opioids, other factors include coping with life stressors and self-medication of psychological and emotional issues (Cicero & Ellis, 2017).

Social networks and influences are contributing factors not only at first use and during development of opioid use disorder, but also during the escalation from non-injection to injection use (Koram et al., 2011). Koram, et al (2011) found that the transition from using heroin via the smoked or snorted routes to intravenous injection was associated with the users' perception of their peer's drug-related behaviors. Initial drug use is highly influenced by family and peers, and especially in young individuals, the transition to injection drug use is socially sanctioned and condoned by friends (Sherman,

Smith, Laney, & Strathdee, 2002). Moreover, the increased prevalence of heroin use has diminished its traditional social stigma (Cicero & Ellis, 2017).

Successful cessation of opiate use is also impacted by whether a recovering addict's social network consists of active drug users or drug-free persons (Buchanan & Latkin, 2008; Eitan et al., 2017). Individuals who successfully completed treatment and remained abstinent after treatment had reduced their drug-using network members significantly, compared to non-quitters (Buchanan & Latkin, 2008). Though directionality is an ongoing topic of study, neighborhood poverty is significantly correlated with current heroin use (Williams & Latkin, 2007), and an important aspect of maintaining or attenuating heroin addiction is the type and quality of a social relationship. While this social aspect can offer a benefit to reducing drug use, it does not buffer against the detriments associated with neighborhood poverty. Williams and Latkin (2007) found that social support of friends and family significantly impacted ongoing heroin use, but only if the friends and family were a positive support. If social networks were prone to drug use, then drug use was reinforced and persisted. Results of another study examining social influences on the escalation of opioid use in adolescents found that increased exposure to drug-using peers lead to a higher likelihood of initiation of drug use and greater use durations and frequency (Russell et al., 2015). In addition to interpersonal relations, psychosocial functioning is predictive of later drug use (Griffith, Knight, Joe, & Simpson, 1998a). Other significant risk factors include age of first drug experimentation,

sensation-seeking personality traits (Luthar et al., 1992), proclivity to depression, and low self-esteem (Griffith, Knight, Joe, & Simpson, 1998).

Drugs are often consumed in social settings, and much like the peer/network relationship and opioid use disorder, the directionality of the social context and drug effects is unclear. When considering occasional users, or the acute effects of drugs on the individual during the early stages of use, subjective effects of opioids may be heightened by the presence of peers, or the valence of social interaction might be mediated by opioids (de Wit & Sayette, 2018). In a review of the literature, de Wit (2018) examined the directionality of this phenomenon and found that opioids appeared to increase prosocial effects and reduce subjective responses to negative social perceptions. Little research is available on the effects of social context on mood responses to opiates; however, de Wit (2017) discussed a study in which men with a recent history of heroin use were allowed to self-administer heroin in a clinical setting. The men were initially were more social, but over the remaining 8 days of the study, they chose to spend more and more time alone. There is currently very little research in humans that has examined this exact construct in controlled settings; however, there are preclinical studies in which the rewarding effects of opiates in laboratory animals are modulated by social enrichment and or social interaction as will be discussed below.

Familial support and peer relationships are an important component of someone suffering from OUD having success in treatment and recovery (Griffith et al., 1998). Interestingly, individuals struggling with substance abuse have more contact with their

families than non-drug users, even if their familial situation is dysfunctional or stressful (Griffith et al., 1998b; Stanton & Shadish, 1997). Additionally, people with a history of dysfunctional family dynamics are significantly more likely to associate with deviant and/or drug using peers (Griffith et al., 1998). The proximity of the family to the drug using individual, and their influence on the development and maintenance of the drug dependence via modeling, enabling, or stressors, make social and familial involvement in treatment necessary. A comprehensive meta-analysis of treatment outcomes for drug-dependent individuals revealed that clients who participated in therapy that incorporated family involvement used significantly less drugs after treatment than those who received therapy that did not include family participation (Stanton & Shadish, 1997). Social and familial networks are useful in recovery, but the quality of support is highly influential. For example, the higher the number of substance users within the social network the higher the likelihood of relapse (Wasserman, Stewart, & Delucchi, 2001).

The most common methods of treatment for opioid dependence are maintenance pharmacotherapies (i.e. methadone, buprenorphine), or cognitive behavioral therapy, and most often, a combination of both psychotherapy and medication maintenance. Methadone, a full μ receptor agonist, has been widely used as a treatment for opioid use disorder for nearly 40 years (Donny et al, 2005). Buprenorphine is a partial μ opioid agonist, and while considered safer than methadone (lower risks of overdose and respiratory depression) it still has some abuse liability (Blaine., 1992). Methadone and

buprenorphine aid in treatment by activating the same opioid receptors in the brain as the used opioid, thus lessening cravings and attenuating withdrawal symptoms.

Standard rodent models of opioid dependence

Self-administration.

The animal self-administration paradigm is widely considered to have the most face and predictive validity as a model of human drug intake (Belin-Rauscent, Fouyssac, Bonci, & Belin, 2016; Panlilio & Goldberg, 2007). Laboratory models of self-administration allow researchers to observe and quantify physiological, behavioral, and cognitive aspects of drug effects (Jones & Comer, 2013). This method allows researchers to design and develop studies that are illustrative of the human condition, in that the animals voluntarily determine if and how much drug they self-administer. The self-administration model is rooted in operant conditioning, which contends that a drug serves as a reinforcer when the probability of an animal making a response to receive an infusion of drug increases (Dworkin, 2003). Laboratory animals can self-administer both drug (heroin, cocaine, alcohol) and non-drug (sucrose, food) reinforcers through a variety of clinically relevant routes, including orally, intracranially, or intravenously (Belin-rauscent & Belin, 2011). Often, delivery of the reinforcer is paired with a discrete cue such as a light, tone, or both. A major strength of the self-administration model is that it allows experimental subjects to consume opioids volitionally and intravenously; which is consistent with the most common method of heroin use (Panlilio & Goldberg, 2007). In order to allow laboratory animals to self-administer opioids, first they need to be

surgically implanted with an intravenous catheter. The catheter is usually implanted into a jugular vein and attached to a subcutaneous vascular port. A spring tether then connects the port to a computer-controlled syringe pump. The animal can initiate a drug infusion by pressing a lever or inserting their nose into a nosepoke aperture (Watterson et al., 2014).

Studies that employ the self-administration paradigm often use operant reinforcement schedules. A common schedule is a fixed ratio (FR) schedule, in which a specific number of responses are required in order for the animal to receive the drug reinforcer. For example, in an FR1 schedule, a single lever press results in one reinforcer (Dworkin, 2003). Progressive ratio (PR) is another schedule of reinforcement, in which the number of lever presses needed to receive each successive reinforcer progressively increases (often arithmetically or exponentially). In a PR schedule, the session ends when the animal fails to respond for a specified time, and the last ratio value completed is considered the "breakpoint". This schedule is often used to quantify how motivated the laboratory animal is to receive the drug, and is thus a measure of reinforcer strength or efficacy (Panlilio & Goldberg, 2007).

More complex behavioral paradigms can be developed to assess other aspects of maladaptive substance use such as cue-induced drug seeking and reinstatement (Dworkin, 2003). The reinstatement model is considered a useful method that is thought to be related to the phenomenon of relapse in drug dependent humans (Shaham, Shalev, Lu, De Wit, & Stewart, 2003). This model operates on the idea that after stable levels of

drug self-administration are achieved, extinction of operant responding is achieved by withholding the drug reinforcer, and subsequently drug-seeking behavior can be reinstated as the result of brief exposure to the drug (drug priming), stressors, or re-exposure to drug-paired cues (Belin-rauscent & Belin, 2011; Namba, Tomek, Olive, Beckmann, & Gipson, 2018).

Previous research has established that laboratory animals willingly intravenously self-administer opioids, and will continue to increase their intake if given unlimited access, while animals that have limited access (3 hours or less, daily) fail to develop an opioid physical dependence or increase intake (Chen et al., 2006). Chen et al (2006) proposed and tested an animal model of opioid dependence in which they observed escalating heroin self-administration in relation to sleep and food intake patterns. Their results indicated that if given the opportunity, rats will self-administer heroin to the point of physical dependence, here measured by tracking the reduced food and water intake in relation to the increase of heroin consumption. Interestingly, rats given the opportunity to self-administer heroin in long-access (>12 hour) sessions are more resistant to extinction when the drug is withheld (Badiani, Belin, Epstein, Calu, and Shaham, 2011). Rats that exhibit steady heroin intake show lower intracranial self-stimulation (ICSS) thresholds compared to escalating rats that show elevations in ICSS thresholds, indicative of a diminished impact of the rewarding effects of heroin and possibly reflective of opioid tolerance (Vendruscolo et al., 2011).

Conditioned place preference.

Another popular paradigm utilized in modeling opioid use disorder is the conditioned place preference (CPP) approach. While there are both biased and unbiased variations, a typical unbiased CPP experiment is conducted in a two-chambered apparatus featuring environmental cues that are unique to each chamber that do not produce any innate preference for either chamber (Beloate & Coolen, 2017). During conditioning, the animal is trained via passive drug injections to associate one set of contextual cues with the experience of a drug, and the contextual cues of the other compartment with a neutral substance such as saline. Following conditioning, animals are re-tested in a drug-free state, and if the animal chooses to spend more time in the drug-conditioned environment, then the drug is interpreted to have a rewarding value. Critics of the CPP paradigm argue that the ability of a drug to produce CPP does not always correlate to the human condition of drug use, as both natural rewards and drugs that have low shown abuse potential in humans produce CPP in rodents (Tzschentke, 2007). In addition, the number of drug exposures is relatively low in the CPP paradigm and is passively administered by an experimenter. Conversely, conditioned place aversion (CPA) occurs when an aversive drug experience, such as a very high drug dose or during withdrawal, drives the animal to spend less time in the drug or withdrawal associated environment.

Extensive research has been performed on opioids using the CPP paradigm, in species ranging from invertebrates to fish to mammals; however, the bulk of the literature has focused primarily on rodent species (i.e. mice, rats, and prairie voles) (Tzschentke,

2007). Morphine, heroin, buprenorphine, oxycodone, fentanyl are all opioid receptor agonists or partial agonists that induce CPP, with classical bell-shaped dose response relationships, with mid-range doses producing CPP and doses in either direction failing to do so (Rech, Mokler, & Briggs, 2012; Tzschentke, 2007).

Opioid antagonists such as naloxone are also capable of producing CPA, though some researchers have reported no effect of these treatments (Tzschentke, 2007). Interestingly, when animals conditioned with morphine receive naloxone injections before the CPP test session, they exhibit stronger CPP than the animals who received saline. Animals conditioned with morphine and naloxone simultaneously failed to develop CPP at all, suggesting that opioid antagonists block the acquisition but enhance the expression of morphine CPP (Bardo, Neisewander, & Pierce, 1989). Morphine CPP is also abolished by selectively blocking protein synthesis in the basolateral amygdala, hippocampus, or nucleus accumbens (Milekic, 2006; Pooriamehr, Sabahi, & Miladi-Gorji, 2017). When animals are prevented from synthesizing new proteins after conditioning with morphine and 24 hours prior to testing, they are unable to consolidate the contextual drug-related memories from the conditioning sessions.

Housing conditions, which dictate the level of baseline social enrichment available to each animal, have a significant effect on the sensitivity of rodents towards developing CPP for various drugs including opioids. Rats reared in group-housing conditions acquired CPP in response to a wide range of heroin doses tested, while animals reared in isolation developed CPP only in response to the highest dose (Schenk,

Hunt, Colle, & Amit, 1982). However, others have found that changes in the social climate between housing and conditioning contexts enhanced sensitivity to morphine CPP in adolescent mice (Kennedy, Panksepp, Runckel, & Lahvis, 2012). For example, mice reared in isolation but conditioned with peers developed CPP to lower doses of morphine compared to mice that were immersed in social groups for both housing and conditioning. These inconsistent findings may be due to differences between species or experimental methodology (i.e. different apparatus, social interactions within/outside drug context, etc.). It is also important to note these socially induced changes in opioid CPP sensitivity reflect only the relatively short period of CPP acquisition and do not necessarily generalize to other paradigms of long-term opioid use (Bozarth, Murray, & Wise, 1987; El Rawas, Thiriet, Lardeux, Jaber, & Solinas, 2009).

Interactions with drug-naïve animals have also been shown to slow the rate of acquisition of morphine CPP. Adolescent mice housed in peer groups that had previously been exposed to morphine acquired CPP to morphine more quickly and with lower doses than the animals housed with drug-naïve animals (Bates, Emery, Wellman, & Eitan, 2014a). The animals housed with drug-naïve conspecifics also extinguished CPP more rapidly, which reflects the ability of humans to have better success at sobriety when avoiding contact with other drug users (Neisewander, Peartree, & Pentkowski, 2012). The consideration of social enrichment is important for creating an accurate model of the human condition, as major contributing factors to opioid dependence in adolescence are

inadequate social support networks and social isolation (Bates, Emery, Wellman, & Eitan, 2014; Kennedy et al., 2012).

The CPP paradigm can also be used to model relapse. Following standard conditioning and testing for a place preference, animals undergo extinction either by additional conditioning with only saline in both compartments, or by repeated placement into the testing apparatus, which induces a steady reduction in preference for the drug-paired compartment in the absence of additional drug conditioning sessions. Following extinction, animals are exposed to either drug-related cues, stress or a priming injection of the original conditioning drug, which are sufficient to reinstate CPP, and thus serve as a model of relapse (Weiss, 2005). The CPP model of relapse has been adapted to incorporate social stressors, as these types of social interactions are a major contributor to the recidivism of recovering addicts in humans (Ribeiro Do Couto et al., 2006). Social stress in rodents can be induced via interaction with an aggressive male conspecific who is prone to fighting. When subjected to this stressful interaction after extinction training, CPP can be reinstated (Ribeiro Do Couto et al., 2006; Tzschentke, 2007). Social defeat can also prevent CPP acquisition when experienced a few days before testing, which suggests a decrease in sensitivity to the rewarding effects of morphine (Coventry, D'Aquila, Brain, & Willner, 1997). These data support the hypothesis that social stress can modulate morphine reward and promote relapse.

Summary of existing literature on social influences on maladaptive opioid use behaviors in rodents

Social and environmental enrichment.

In preclinical research, attempts to understand the effects of the environment on the development and maintenance of opioid use often incorporate the paradigm of environment enrichment. An enriched environment is one that contains objects in the home cage such as a running wheel, toys, tubes, and other additions as compared to the standard polycarbonate cage environment that a laboratory animal typically resides in (Xu, Hou, Gao, He, & Zhang, 2007). An environment can also be considered enriched if the laboratory animal subject is housed with other animals, as opposed to being housed in isolation. The benefits of an enriched environment appear to have protective effects from the consequences of opioid use, as discussed below.

An early study examined the role of social environment in the consumption of an oral morphine solution, where rats were raised either in social colonies versus in isolation (Alexander, Beyerstein, Hadaway, & Coambs, 1981). In addition to comparing the effects of initial environment (social vs isolated), this study was conducted in a counterbalanced fashion such that half of each group was placed in the opposite condition 65 days into the study. Results indicated that rats initially housed in an enriched environment consumed less morphine than isolated rats. Further, rats living in a colony at time of testing consumed less morphine than isolated rats regardless of their prior environmental conditions (Alexander et al., 1981). These findings are among the earliest to demonstrate

the influence of social housing conditions and environmental enrichment on voluntary opioid intake in rodents.

The impact of social enrichment was further tested in a study that examined how quickly isolated rats learned to intravenously self-administer heroin as well as the amount of heroin self-administered compared to group housed rats (Bozarth et al., 1987). In this experiment, rats were either housed individually in cages that precluded any visual or tactile contact with other rats, or in groups of 10 in a large cage that allowed for ample social interaction. Rats were allowed to self-administer heroin in 2-hour sessions for a total of 25 days (in five 5-day a week blocks). After each self-administration session, rats were returned to their respective isolated or group-housing conditions. Both groups of animals learned to self-administer heroin over the five 5-day blocks of 2 hr/day heroin access, yet single-housed rats self-administered significantly more heroin during the 2nd, 3rd, and 4th blocks of 5-day periods time than socially housed animals. By the end of the study there were no significant differences in total heroin intake, yet isolated rats acquired self-administration more quickly (Bozarth et al., 1987). These observations may be an indication that social enrichment affects the initial reinforcing effects of heroin and/or learning related to general operant conditioning.

The positive effects of social enrichment are further supported in a study where socially isolated rats (both male and female) were shown to ingest a higher volume of a morphine-containing water solution than their socially housed conspecifics (Raz & Berger, 2010). Another study that examined the potentially insulating effects of

enrichment on the rewarding properties of opiates also showed changes in intake rates between environmentally enriched rats and those housed in standard conditions (Abadi, Miladi-Gorji, & Bigdeli, 2016). In this study, subjects were randomly assigned into enriched or standard environments, passively made dependent on morphine with twice daily injections (10 mg/kg, 12 h intervals) of morphine for 14 days, and then underwent naloxone-precipitated withdrawal. During withdrawal, rats were given the option of consuming a morphine or sucrose-containing solution. Results showed that rats who had experienced environmental enrichment consumed significantly less morphine at a dose of 0.5 mg/ml morphine than rats housed under standard conditions.

In another study (El Rawas et al., 2009), mice were randomly assigned to either enriched or standard housing conditions.. Mice were then tested for the development of a heroin or sucrose CPP, assessed for opioid-stimulated locomotor activity, and also underwent in vivo microdialysis procedures to examine heroin-induced changes in extracellular dopamine in the nucleus accumbens. Results show that the rewarding effects of heroin were reduced in mice from the enriched environment condition, but sucrose-conditioned mice showed no effect of environment (El Rawas et al., 2009). However, no effects of housing condition were observed on the locomotor activating effects of heroin nor the ability of heroin to elevate extracellular dopamine levels in the nucleus accumbens. These data indicate that environmental enrichment offers some protective benefits in regard to heroin (i.e., diminished conditioned rewarding effects), although locomotor and mesolimbic dopaminergic effects of heroin appear to be unaltered.

These rodent studies show that an enriched environment has several beneficial outcomes with regards to opioid intake. There is also evidence that an enriched environment is potentially relevant for maintaining abstinence during treatment (Galaj, Manuszak, & Ranaldi, 2016; Peck, Galaj, Eshak, Newman, & Ranaldi, 2015). For example exposure of mice to environmental enrichment during withdrawal eliminates behavioral sensitization and conditioned place preference to cocaine (Solinas, Chauvet, Thiriet, El Rawas, & Jaber, 2008). Other studies have investigated the role of an enriched environment in drug-seeking despite aversive consequences as well as when heroin self-administration has been attenuated (Galaj, Manuszak, & Ranaldi, 2016; Peck, Galaj, Eshak, Newman, & Ranaldi, 2015). In one study (Peck et al., 2015), researchers utilized a conflict paradigm, which consists of administering a series of electric shocks to the floor of an operant conditioning chamber below the operandum (i.e., lever) immediately following response-contingent delivery of the drug, and increasing in intensity until the animal ceases all drug self-administration (Cooper, Barnea-Ygael, Levy, Shaham, & Zangen, 2007). Thus, in this model, subjects are presented with a conflict between self-administering a drug and receiving an electric shock, which is intended to mimic an aversive consequence of drug intake. Rats were first allowed to self-administer heroin for approximately 15 days before being randomly assigned to either an enriched or standard housing environment condition. After 2 days in their respective conditions, response-contingent shock delivery commenced with an escalating current until rats did not press the lever that previously resulted in drug delivery for 3 consecutive days. Results of this

study showed that even though rats from both the enriched and standard environment conditions achieved abstinence following introduction of the electric shock, enriched rats reached the criteria for maintaining abstinence (3 consecutive days of an absence of lever presses) significantly faster than rats housed in a standard environment (Peck et al., 2015). These data support the idea that an enriched environment is potentially a useful tool in reducing the motivation to self-administer opioids, even under conditions of punishment and negative consequences that result from drug intake.

The promising and possibly insulating abilities of an enriched environment are further supported in another study by Galaj and colleagues (Galaj et al., 2016). In this study, single-housed rats were allowed to self-administer heroin for 15 days in 3-hour daily sessions. Lever presses resulting in an infusion of heroin were paired with presentation of a light cue. After heroin acquisition, rats were randomly assigned into an enriched or standard housing condition. After 15 days in their new enriched environments, rats underwent extinction sessions for 30 days, in which they were put back into the self-administration apparatus, but lever presses did not result in drug infusion or light cue presentation. The day after the last extinction session, reinstatement sessions commenced where the light cue was non-contingently presented twice, and subsequent lever presses resulted in activation of the light cue and syringe pump but without an actual infusion of heroin. Results showed that rats in the enriched environment conditions pressed the active lever during reinstatement significantly less than rats in the

standard environment condition. Thus, cue-triggered heroin seeking can be diminished by environmental enrichment.

The benefits of environmental enrichment may extend into subsequent generations, as offspring of morphine dependent-rats consumed less morphine if their parents had been reared in an enriched environment as opposed to a standard environment (Pooriamehr, Sabahi, & Miladi-Gorji, 2017). Also, when pregnant morphine dependent mothers were allowed to exercise via swimming (Torabi, Pooriamehr, Bigdeli, & Miladi-Gorji, 2017) or wheel running (Haydari, Miladi-Gorji, Mokhtari, & Safari, 2014), their offspring consumed significantly less morphine than the offspring of the morphine-dependent sedentary mothers. Conversely, while social enrichment seems to be an insulating factor in some aspects of opiate addiction, there is also evidence that the loss of a social reward can result in a vulnerability to drug-seeking (Beloate & Coolen, 2017). This phenomenon was studied in the context of pair bonding and social and environmental enrichment. Beloate & Coolen (2017) attribute the resulting drug seeking behavior to changes in the mesocorticolimbic pathway.

Behavioral Sensitization

Another animal model of drug addiction is behavioral sensitization, which typically consists of measuring horizontal locomotor activity and is believed to reflect lasting brain changes that result from chronic drug exposure and can also model individual differences in drug sensitivity. Sensitization refers to a progressively increased or enhanced response (e.g., locomotor activity) to a stimulus (e.g., drug injection)

induced with repeated exposure to the same or related stimuli (Steketee & Kalivas, 2011). While the majority of research on behavioral sensitization has been performed in the context of psychostimulants, there have been some use of this model with opiates. In one study (Xu et al., 2007), standard-environment and enriched-environment-housed mice were placed in a novel environment, and it was shown that the enriched mice demonstrated reduced locomotor activity compared to the standard housed mice. Acquisition of behavioral sensitization was observed in mice under either housing conditions, yet the magnitude of behavioral sensitization in the enriched environment condition was less pronounced than that in the standard environment condition. These animals then received six additional daily injections of morphine followed by a challenge injection of morphine (10 mg/kg) after a 5-day drug-free interval. Mice in the environmentally enriched condition showed a significantly less robust behavioral response (i.e., expression of sensitization) than mice in the standard environment condition.

A similar study was performed with rats, in which adolescent and adult rats, either group-housed or singly housed, received repeated injections of morphine. The adolescent morphine enriched-environment rats did not exhibit the enhanced locomotor response as compared to the saline only and saline enriched-environment rats. This relationship was not found (to a statistically significant degree) in the adult rats. Demonstrating, in this case, that adolescent rats were vulnerable to social influences on morphine sensitization, but the adult rats were not (Hofford, Schul, Wellman, & Eitan, 2012). Another study

encompassed both environmental factors and stress to investigate the influence of social crowding upon morphine-induced locomotor behavior. Each condition had 4 rats per cage, but in the social crowding condition, the cages were 50% smaller than the size of the standard cages. Results showed that the social overcrowding procedure could produce morphine sensitization in rats with higher baseline motor activity (Xigeng et al., 2004). These differences were only significant under stress conditions, which provides a translational value to the study, as human addiction is often related to and impacted by various stressors.

Animal model of stress in addiction research

There is substantial evidence that stress plays a role in the development and maintenance of drug addiction in humans (Koob, 2008). Researchers utilize animal models of stress and addiction in order to investigate the neural and behavioral mechanisms involved. In order to induce stress in laboratory rodents, aversive conditions are employed such as footshock, restraint or tail pinch, or involve social or maternal deprivation (Conrad, Ortiz, & Judd, 2017; Lu, Shepard, Hall, & Shaham, 2003). Most stressors activate corticotrophin-releasing factor (CRF) containing systems in the brain (Koob, 2008b), which mediates the hypothalamic activation of the hypothalamic–pituitary–adrenal (HPA) axis, which in turn facilitates peripheral hormonal, autonomic, and behavioral responses (Varghese et al., 2015). It is theorized that CRF systems are relevant to all stages of the addiction cycle but plays the biggest role in the withdrawal/negative affect stage (Zorrilla, Logrip, & Koob, 2014). Chronic use of

dependence inducing drugs can potentially lead to a state of stress and negative affect (Zorrilla, Logrip, & Koob, 2014). Alteration in the typical functioning of CRF has been implicated in the drive to use substances and the susceptibility to relapse (Zorrilla, Logrip, & Koob, 2014). Maternal separation, as a stressor, is also implicated in over-activation of the HPA axis in adulthood, and evidence suggests it is associated with increased opiate self-administration in adulthood (Neisewander et al., 2012).

Another study similar to the conflict model described earlier examined the effects of stress on morphine self-administration via pairing of mild electric shocks with lever pressing for morphine. Each time a rat pressed a lever, it received a shock, followed by the infusion of morphine. The shock duration was either 0.2 or 0.02 seconds, and when the rats experienced the shock of a longer duration, they increased their lever pressing until they reached lethal levels of morphine consumption (Lu et al., 2003). Thus, these lethal effects could have been due to the analgesic effects of morphine, whereby rats could have increased their self-administration in order to alleviate the pain associated with the shock, which may have resulted in overdose. However, the ability of a stressor to increase opiate self-administration is not necessarily specific to electric shock stress, as another study that utilized food deprivation as a stressor and observed increased etonitazene self-administration only on days of food restriction (Carroll & Boe, 1982).

Newer rodent paradigms for assessing prosocial behavior in opiate addiction have recently been developed. In an attempt to provide insight into the neural mechanisms of impaired social function in opioid addiction, Tomek, Stegmann, & Olive, (2018) utilized

an established prosocial paradigm in which a rat will release or rescue a conspecific from a plastic restrainer instead of receiving food or other palatable rewards (Ben-Ami Bartal, Decety, & Mason, 2011) (discussed in detail in chapter 2). After baseline rescuing behaviors were measured, rats were allowed to self-administer sucrose pellets (orally) or heroin (intravenously) in long access (6 hr/day) sessions for two weeks. The day after the final day of heroin self-administration, rats were given the opportunity to choose between releasing their cage-mate from the restraint or continuing to self-administer heroin or sucrose. Results showed that rats with a history of sucrose self-administration continued to release their cagemate, whereas rats who had a history of heroin self-administration did not continue to release their cagemate and continued to self-administer heroin (Tomek et al., 2019). These results are consistent with a study in which 'rescuer' rats were given an injection of either the benzodiazepine midazolam, the beta-adrenergic antagonist nadolol, or saline (Ben-Ami Bartal et al., 2016). Rats administered nadolol continued to rescue their cagemate at the same rate as controls; however midazolam-administered rats released their conspecific less often, all the while still opening the restraint door for chocolate (Ben-Ami Bartal et al., 2016). The authors interpreted these results as a drug induced interruption or dysregulation of social affective processing that appears necessary to motivate the rat to open the restraint door for its cage-mate. Overall, these findings suggest that the rescue paradigm may represent a novel rodent model of impaired social function in opioid use disorders. Utilization of this model may lead to improved strategies in facilitating recovery and treatment.

Limitations of current models

While each of the animal models discussed above has various strengths, there are weaknesses as well. For example, in humans and rodents, stress can contribute to drug use and relapse; however, the types of stressor humans encounter are typically related to a relationship, socioeconomic status, major life event, or lack of social support, as opposed to the aversive physical stressors administered to rodents in a laboratory (Heilig, Epstein, Nader, & Shaham, 2016). These differences are necessary to reconcile if claims of translational effectiveness are being made, or goals of improving outcomes for drug-addicted individuals are going to be realized. In environmental enrichment studies, a major limitation is the control group, which most often consists of animals in isolation, which is also sometimes a methodology used as a stressor and therefore a potential confound, as stressors can impact drug-taking behaviors (Beloate & Coolen, 2017; Marcello Solinas, Thiriet, Chauvet, & Jaber, 2010). Limitations of CPP include inconsistent experimental results that may be due to differences between species or experimental methodology, such as utilizing different CPP apparati and social circumstances of the experimental subjects (Neisewander et al., 2012). It is also important to note these socially induced changes in opiate CPP sensitivity reflect only the relatively short period of CPP acquisition and do not necessarily generalize to other paradigms of long-term opiate use (Bozarth et al., 1987; El Rawas et al., 2009). Sensitization is also a useful and complex model; however, it is not only contingent upon ongoing exposure to drugs of abuse, it is affected by learning and environmental

circumstances (Robinson & Berridge, 2000), making it difficult to precisely identify the underlying mechanisms. While intravenous self-administration paradigms are considered the gold standard of rodent models of addiction and have the benefits of volitional drug intake and self-titration of desired amount ingested, they bear various limitations such as subject attrition due to loss of catheter patency, experience of post-surgical discomfort, and artificial drug-taking environments that usually lack the presence of conspecifics. There is thus a need for improved approaches with even more translational value.

Conclusions

The preclinical research models thus far have made significant technological strides over the last 40 years in their attempts to provide insight into the behavioral, physiological, and environmental basis of opiate addiction. These models have aided researchers in making significant progress in understanding of the neurobiological mechanisms of addictive drugs. These models are for the most part reliable, replicable, and accurately capture various aspects of addiction in humans. Research on social influences of addiction has demonstrated that enriched environments and social circumstances are beneficial in the reduction of drug taking and drug seeking behaviors in both humans and animals (Abadi et al., 2016; Hofford et al., 2012; Kennedy et al., 2012; Raz & Berger, 2010; Russell et al., 2015). However, despite the myriad of rodent models of opioid intake and/or dependence at the disposal of researchers worldwide, the goal remains to continue to further develop improvements within existing treatment modalities and discover more effective strategies for the prevention of OUD.

CHAPTER 2

EFFECTS OF HEROIN ON PROSOCIAL BEHAVIOR IN RATS

Abstract

Opioid use disorders are characterized in part by impairments in social functioning. Previous research indicates that laboratory rats, which are frequently used as animal models of addiction-related behaviors, are capable of prosocial behavior. For example, under normal conditions, when a "free" rat is placed in the vicinity of a rat trapped in a plastic restrainer, the rat will release or "rescue" the other rat from confinement. The present study was conducted to determine the effects of heroin on prosocial behavior in rats. For two weeks, rats were given the opportunity to rescue their cagemate from confinement, and the occurrence of and latency to free the confined rat was recorded. After baseline rescuing behavior was established, rats were randomly selected to self-administer heroin (0.06 mg/kg/infusion i.v.) or sucrose pellets (orally) for 14 days. Next, rats were retested for rescuing behavior once daily for 3 days, during which they were provided with a choice between freeing the trapped cagemate or continuing to self-administer their respective reinforcer. Our results indicate that rats self-administering sucrose continued to rescue their cagemate, whereas rats self-administering heroin chose to not rescue their cagemate. These findings suggest that rats with a history of heroin self-administration show deficits in prosocial behavior, consistent with specific diagnostic criteria for opioid use disorder. Behavioral paradigms providing a choice between engaging in prosocial behavior and continuing drug use may be useful in

modeling and investigating the neural basis of social functioning deficits in opioid addiction.

Introduction

Rates of opioid use have dramatically climbed over the last 15 years. Opioid overdose rates have also reached an all-time high, increasing threefold between 2010 and 2015, with 12,989 heroin-related deaths in 2015 alone (Rudd, 2016). A major factor that has led to increased heroin use is the over-prescription of opioid analgesics, which can lead to opioid dependence and transition to heroin use. Also contributing to this problem is the increasing amount of inexpensive and readily available heroin, which often contains fentanyl or other highly potent opioids (Ciccarone et al., 2017).

Opioid use disorder is characterized in the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (DSM-5) by symptoms and behaviors that include impaired control over drug use, continued drug use despite harm, craving for the drug, withdrawal symptoms during abstinence, and persistent or recurrent social or interpersonal problems caused by or resulting from opioid use (APA, 2013). These social and interpersonal problems are the focus of the current study. Many clinical studies have aimed to evaluate interpersonal problems and their role in substance use disorders; however, this has proved to be difficult due to the fact that such problems in the context of drug dependence are dynamic and underpinned by a conglomerate of physiological, psychosocial, environmental, and motivational dysfunctions. Despite this, it has been shown that among heroin-dependent individuals,

undergraduate marijuana users, psychiatric patients, incarcerated inmates, and police officers, empathy and socialization are particularly deficient in heroin-dependent individuals (Kurtines, Hogan, & Weiss, 1975). Therefore, loss of prosocial function may be an important feature of heroin addiction.

Previous research indicates that laboratory rats, which are frequently used as animal models of addiction and related behaviors, are capable of pro-social or empathy-like behaviors. Under normal conditions, when a "free" rat is placed in the vicinity of a "trapped" rat in a plastic restrainer (see Fig. 1), the rat will release the other rat from confinement, even if it has to give up food to do so (Ben-Ami Bartal et al., 2011). Further, the free rat will share its food with the trapped rat after it has been released (Ben-Ami Bartal et al., 2011). A subsequent study found that rats will continue to rescue "stranger" rats (i.e., non-cagemates), but not "stranger" rats of a different strain. However, once rescuer rats were acclimated to the different strain, they release it from confinement (Ben-Ami Bartal, Rodgers, Bernardez Sarria, Decety, & Mason, 2014). Ultimately, this indicates that previous social experience is a factor in pro-social behavior in rats. In another study, Sato, Tan, Tate, & Okada (2015) used a water-based paradigm, whereby a rescuer rat was allowed to release a soaked conspecific, and showed that rats previously soaked in water learned to rescue the soaked rat more quickly than those who had not been previously soaked.

Figure 1. Example of rat releasing a trapped rat from a plastic restraint. (Photo credit: Elisha Fornwalt).

The current study was designed to evaluate the effects of heroin on prosocial behavior in rats. Specifically, we examined whether a history of heroin self-administration would affect rats' willingness to aid a conspecific by releasing it from confinement. Unlike previous research using passive administration of a particular drug (Ben-Ami Bartal et al., 2016); in the present study rats were given the choice of rescuing their cage mate from the restrainer and/or self-administering either a drug (heroin) or non-drug (sucrose) reinforcer. We hypothesized that rats with a history of heroin self-administration would choose to continue to self-administer the drug instead of freeing the

trapped rat, whereas rats with a history of sucrose self-administration would continue to rescue their cagemate.

Methods

All experimental procedures were conducted with the approval of the Institutional Animal Care and Use Committee at Arizona State University and in accordance with the Guide for Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

Animals.

Sixty-four male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing ~250 g, were pair-housed upon arrival on a 12-hour reversed light–dark cycle (lights off at 0700 hr), and given ad libitum access to food and water during all experimental procedures except during behavioral testing. All experimental sessions took place during the dark phase of the light-dark cycle. Upon arrival, one rat from each pair was randomly selected and its tail was marked with a permanent marker. This rat was designated the rescuer rat while the other rat was designated the trapped rat. Thus, a total of n=32 rats were randomly selected to be rescuer rats, and n=32 cagemates were designated to be trapped rats. Animals were handled and weighed individually for 5 minutes daily for three weeks to allow them to acclimate to handling procedures (Fig. 2A).

Assessment of baseline prosocial behavior.

Following handling, animals underwent a baseline assessment of prosocial behavior. Testing occurred at the same time of day every day for 2 weeks (Fig. 2A). In this procedure, each designated trapped rat was placed in a plastic restrainer (Harvard Apparatus, Holliston, MA; modified to 13.3 cm x 8.0 cm x 8.9 cm;) with a removable door, and the restrainer was placed in an operant conditioning chamber (Med Associates, St. Albans, VT; model ENV-008, custom modified to 43.2 cm x 20.3 cm x 22.9 cm). All self-administration chambers were located inside sound attenuating cubicles equipped with a house light and exhaust fan designed to mask external noise and odors and were interfaced to a PC computer. Chambers were equipped with two nosepoke response holes on one wall, and a 4.2 × 5 cm pellet receptacle equipped with head entry detector between the response holes. Each response hole was located approximately 7 cm above a stainless-steel grid floor and positioned above each was a 2.5-cm diameter white stimulus light. Located near the top of the self-administration chambers was a Sonalert speaker that provided an auditory stimulus during reinforcer delivery. Located outside each chamber were a computer-interfaced syringe pump and pellet dispenser. For rats assigned to self-administer heroin, the syringe pump delivered the drug solution via a single-channel liquid swivel mounted atop the chamber via polyethylene tubing. For rats assigned to self-administer sucrose, the pellet dispenser delivered sucrose pellets into the receptacle. All rescuing and self-administration sessions were video recorded. Following placement of the trapped rat inside the restrainer into the operant chamber, the rescuer rat was then

placed in the chamber, followed by illumination of a house light. The occurrence of and latency to rescue the trapped rat was recorded for each session. The maximum amount of time allowed in the operant chamber for each session was initially 1 hr, which was then reduced to 45 min and ultimately 30 min over the course of the 2 weeks of testing. In order to reduce the possibility that removal from the testing apparatus was a motivating factor for rescuing behavior, upon freeing of the trapped rat, rats remained in the chambers for the duration of the session. Rats failing to release the trapped rat across the two weeks of baseline testing were removed from the study.

Surgical procedures.

After establishment of baseline rescuing behavior, rescuer rats were randomly assigned to self-administer heroin or sucrose as a non-drug reinforcer. Animals assigned to the heroin group were then surgically implanted with intravenous catheters into the jugular vein according to our previously published procedures (Watterson et al., 2014). Rats were anesthetized with isoflurane (2% v/v) vaporized oxygen at a flow rate of 2 l/min. Rats received pre-incision injections of buprenorphine (0.05 mg/kg, s.c., Henry Schein Animal Health, Dublin, OH) and meloxicam (1 mg/kg, s.c., Henry Schein Animal Health,). Surgical sites were shaved and disinfected with 1% povidone-iodine. A 2 cm incision was made in order to locate and isolate the right or left jugular vein. A sterile silastic catheter filled with 100 U/ml heparin was inserted 2.5 cm into the vein. The catheter was secured to the surrounding tissue with silk sutures, and the opposite end of

the catheter was tunneled subcutaneously to the dorsum where it exited the skin between the scapulae. The catheter was secured to a vascular access port (Plastics One, Roanoke, VA or Instech Laboratories, Plymouth Meeting, MA – see note below), then sutured to the surrounding tissue. The wound was then closed with Ethicon nylon sutures and topically treated with topical lidocaine and triple antibiotic ointment. The access port was covered with an aluminum cap to prevent damage from cagemate chewing. All rats were allowed to recover from surgery for 5 days prior to the initiation of self-administration procedures (Fig. 2A). During this time, rats were single housed and received daily intravenous infusions of Timentin (66 mg/ml, dissolved in sterile saline containing 70 U/ml heparin, 0.1 ml volume) to maintain catheter patency and protect against infection. Meloxicam (2.5 mg/kg, s.c.) was administered once daily for 5 days following surgical procedures to provide additional relief of post-surgical discomfort, and buprenorphine (0.03 mg/kg s.c. was administered once daily for 3 days. To minimize any potential influence of peri- and post-operative analgesics (i.e., buprenorphine) on prosocial behavior, rats assigned to the sucrose control group did not undergo sham surgical procedures but were separated from their cagemate during this time to maintain the same social conditions across groups. After recovery from surgery, all rats were returned to their respective pair-housing conditions.

Self-Administration Procedures

Following post-surgical recovery, rats underwent 6-hr daily self-administration sessions (Fig. 2A). For animals assigned to self-administer heroin, nosepoke entries into

the designated active response hole resulted in delivery of heroin (Cayman Chemical, Ann Arbor, MI) at a dose of 0.06 mg/kg per infusion. This dose of heroin was selected as it is a relatively low dose that produces robust operant responding with a reduced incidence of somatic withdrawal symptoms which might confound interpretation of motivation factors that contribute to prosocial behaviors as compared to higher doses, and also produces moderate escalation of intake when access periods are 6 hr in length as employed in the present study (Dai et al, 1989; Vendruscolo et al, 2011). Heroin was dissolved in sterile saline and delivered in a volume of 0.06 ml over a 2 sec period. For rats assigned to self-administer sucrose, nosepoke entries into the designated active response hole resulted in delivery of a 45 mg sucrose pellet (BioServ, Frenchtown, NJ). Self-administration was conducted on a fixed ratio 1 (FR1) schedule of reinforcement. Each heroin infusion or sucrose pellet delivery was followed by a 20-sec timeout period, during which additional active nosepokes were recorded but produced no drug infusions. Each reinforcer delivery was accompanied by concurrent illumination of a stimulus light located directly above the active response hole, and simultaneous presentation of an auditory tone (2900 Hz, ~65 dB) for 2 sec. Nosepokes into a separate inactive hole had no programmed consequences at any time during the experiment. Self-administration sessions were conducted 7 days per week for 14 consecutive days. To verify catheter patency, rats were periodically administered sodium methohexital (10 mg/ml i.v., 0.2 ml volume) and observed for brief periods of immobility. Rats failing to demonstrate

catheter patency continued to undergo testing throughout all experimental phases to serve as a non-patent reference group.

Since larger, customized operant conditioning chambers were required to accommodate the restrainer while allowing simultaneous self-administration behavior, extended length stainless steel tethers were utilized for intravenous infusions. However, in initial pilot studies, we found that extended length tethers increased tension on the vascular access ports and compromised catheter patency in many of the first cohort of animals. As a result, tethers made of a lighter material and vascular access ports with lower vertical profiles were used for all subsequent cohorts of animals. All other conditions detailed above were identical to those described above, and data from the rats that lost catheter patency during the experiment were included in the analyses as a non-patent surgerized control group. Analysis of video recordings (see below) revealed that neither the tether nor vascular access port type hindered the rats' ability to open the restrainer door.

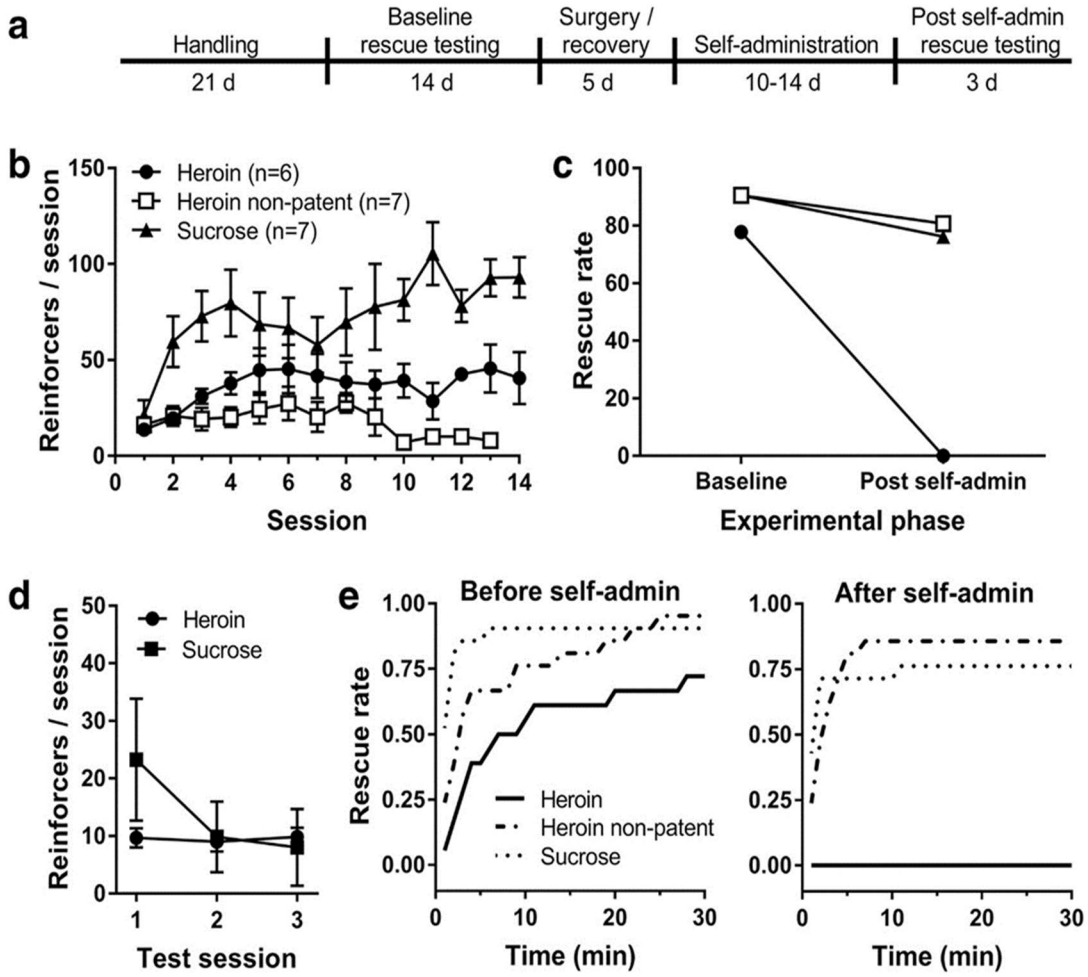

Figure 2. (A) Timeline of different phases of the current study. (B) Number of reinforcers earned during the self-administration phase across the three experimental groups (heroin, n=6), heroin non-patent (n=7), and sucrose (n=7). (C) Rescue rate (defined as the proportion of sessions where rescuing behavior was observed) in each experimental group during the final 3 test sessions days prior to commencement of self-administration (baseline), and the final 3 tests sessions following the self-administration phase (post self-admin). (D) Number of reinforcers earned by rats in the heroin (n=6) and sucrose (n=7) groups across the 3 test sessions in which animals had simultaneous access to both their respective reinforcer and the trapped rat. E) Total proportion of rescues across the 30 min in the test chamber for each of the reinforcer conditions (n=6 heroin, n=7 heroin non-patent, and n=7 sucrose) before and after exposure to self-administration acquisition within the experiment. Rats had concurrent access to applicable reinforcers during the pre and post phases.

Re-assessment of prosocial behaviors.

Starting on the day following the last day of heroin or sucrose self-administration, the rescue paradigm was repeated as described above, with the exception that rats in the heroin group were attached to the infusion tethers as during the self-administration phase. Animals were tested for prosocial behaviors in 30-min sessions for 3 consecutive days, while being allowed concurrent access to their respective reinforcer (i.v. heroin or sucrose pellets, Fig. 2A). All post self-administration test sessions were video recorded.

Assessment of Opioid-Induced Behaviors

To address the possibility that heroin intoxication might interfere with the ability to perform the rescuing task, video recordings of the last three rescue test sessions in heroin self-administering rats were analyzed by three investigators blind to experimental condition. Opioid-induced behaviors were quantified using a rating scale developed by Seip and colleagues (2012), where stupor, pica, stereotypy, aberrant grooming, hyperactivity, and non-specific swallowing behaviors were rated on a scale of 0 to 4, with 0 representing an absence of the behavior and 4 representing severe or high frequency of occurrence. Scores for each measure were then averaged across all observers. Percent agreement between raters was as follows: stupor 83%, pica 100%, stereotypy 92%, grooming 100%, hyperactivity 92%, swallowing 100%.

Data analyses.

Primary dependent measures were observation of rescuing the trapped rat, latency to rescue, number of active/inactive nose-pokes emitted, and number of heroin or sucrose reinforcers delivered. Observation of rescuing behavior on at least 3 days was required in order to establish baseline behavior and progress to the self-administration phase. Following heroin or sucrose self-administration, rescuing behavior was again measured three times in order to determine post self-administration rates of rescue, which was defined as the proportion of rats completing the rescue task across the test session in 1-minute intervals. Self-administration data (number of infusions earned) were analyzed using a two-way ANOVA, with self-administration session and number of reinforcers delivered in each group as main factors. However, for repeated measures analysis of rescuing behavior in combination with heroin or sucrose self-administration, a multilevel model was used, where individual observations were nested within each individual rat. In order to account for observation of rescuing behavior as well as the latency to rescue, we conducted a survival analysis using the accelerated failure time (AFT) model in order to model the rate of rescue as predicted by the treatment condition (sucrose vs. heroin), the stage in the study (pre vs. post self-administration), and the interaction between these two variables (for details on survival analysis and the AFT model, see Allison, 2010). The primary measure of interest was a potential interaction between reinforcer group (heroin or sucrose) the stage of the study (pre or post self-administration), which would indicate whether the change of rate in rescue from the pre- to post self-administration stages

differed across reinforcer type. Due to the lack of variability of the rescue rate in the heroin group after the self-administration, in which none of the rats rescued their cagemates, we imposed a penalty in order to obtain conservative but stable parameter estimates (Gart, 1966, adapted for survival analysis; see Agresti, 2002 for methods regarding handling of monotonic responses). All analyses were conducted in SAS Version 9.4. Statistical significance was considered to be reached for p-values < 0.05.

Results

A total of n=7 rats were excluded from the study due to failure to demonstrate baseline rescuing behavior, and n=2 were excluded for failure to acquire heroin self-administration (>10 infusions per session). An additional n=3 animals were removed from the study due to surgical complications. Therefore, the final sample size consisted of n=13 rats (n=6 in the heroin group and n=7 in the sucrose group). An additional n=7 animals lost catheter patency during the experiment as demonstrated by lack of behavioral response to sodium methohexital. However, data from these animals were included in the analyses to control for the possibility that catheter implantation procedures and/or limited heroin intake influenced prosocial behavior.

During the self-administration phase (Fig. 2B), we observed a significant effect of self-administration session [$F_{13,198} = 6.69$, $P < 0.001$] and reinforcer group [$F_{2,198} = 7.68$, $P < 0.001$, EE = -1.09], with the number of reinforcers earned being increased as compared to the first self-administration session, and the number of sucrose reinforcers

obtained being greater than that of the number of heroin reinforcers. In addition, the number of heroin reinforcers obtained was greater in the patent vs. non-patent groups (Fig 2B).

Table 1 shows the descriptive statistics for each condition including the proportion of rescues, the median latency to rescue, and the mean and standard deviation of the time until rescue among rats that demonstrated rescuing behavior for each reinforcer group and phase of the experiment.

Table 1
Proportion of rescues, median latency (in sec) to rescue, mean and standard deviation of latency to rescue (in sec) for each reinforcer group and experimental phase.

Treatment condition	Stage Pre or post self admin	Proportion of rescues	Median latency to rescue (in sec)	Mean (SD) of latency to rescue (in sec)
Sucrose	Pre	90.5%	60	78.83 (69.02)
	Post	76.2%	74	88.88 (153.20)
Heroin	Pre	77.8%	344	430.57 (464.91)
	Post	0%	N/A	N/A
Heroin non-patent	Pre	90.5%	146	362.11 (451.73)
	Post	80.7%	123	129.00 (96.85)

N/A, not applicable due to failure to demonstrate rescuing behavior. SD, standard deviation

On average, prior to self-administration of heroin or sucrose, rescue rates were 78-91% (Fig. 2C). After the self-administration phase, rats self-administering sucrose rescued

76% of the time, and non-patent heroin rats rescued 81% of the time, whereas the rats receiving heroin stopped rescuing completely (0% rescue rate, Fig. 2C).

The main interest of the study was to determine whether the reinforcer condition had a significant effect on the change in rescuing behavior from pre- to post- self-administration phases, and it was found that this effect was significant. Table 2 shows the maximum likelihood parameter estimates of the multilevel survival analysis for the reinforcer condition, phase of the study, and their interaction, as well as the confidence intervals for each parameter estimate.

Table 2

Analysis of maximum likelihood estimates for each variable for the multilevel survival analysis comparing heroin and sucrose rats

Variable	*df*	Parameter Estimate Mean (SD)	*t* Statistic	*p*-value	95% C.I. Lower Bound	95% C.I. Upper Bound
Reinforcer condition	12	1.71 (0.81)	2.1	0.06	-0.06	3.47
Phase	12	0.41 (0.43)	0.94	0.37	-0.54	1.36
Treatment by Phase interaction	12	3.26 (0.91)	3.57	<0.005	1.27	5.26

The main parameter of interest was the treatment by stage interaction, which was statistically significant ($t12 = 3.57$, $P < 0.005$). These results indicate that after self-administration, rats receiving heroin significantly reduced their rescuing rate in comparison to rats receiving sucrose. These results are also reflected on Figs. 2C and 2D,

which show that rats in the sucrose group consistently rescued before and after self-administration.

We further compared data from the group of n=7 rats that lost patency during the experiment to the sucrose group (Figure 2C) in order to rule out the possibility that the catheter implantation (rather than self-administration of heroin) was the reason for the change in rescue rates. A multilevel survival analysis was conducted comparing the non-patent and the sucrose rats. The results indicated that none of the parameter estimates were significantly different from 0 (see Table 3 for statistical values), indicating that the rats in the sucrose and non-patent heroin groups did not differ from each other in their rescuing rate across reinforcer conditions or phase of the experiment.

Table 3
Analysis of maximum likelihood estimates for each variable for the multilevel survival analysis comparing sucrose rats and the rats that lost catheter patency during the experiment.

Variable	*df*	Parameter Estimate Mean (SD)	*t* Statistic	*p*-value	95% C.I. Lower Bound	95% C.I. Upper Bound
Reinforcer condition	13	1.02 (0.52)	1.96	>0.05	-0.10	2.14
Phase	13	0.40 (0.52)	0.75	0.45	-0.72	1.52
Reinforcer by stage interaction	13	-0.38 (0.37)	-1.02	0.31	-1.18	0.42

Figure 2D shows the number of sucrose or heroin reinforcers obtained during each of the three sessions when concurrent self-administration and rescuing behavior was

assessed. We observed neither an effect of session ($F_{2,24} = 2.24$, $P = 0.13$] nor reinforcer group [$F_{1,24} = 0.32$, $P = 0.58$, EE=1.19], indicating no differences in reinforcers obtained across the three sessions. Due to the loss of catheter patency, reinforcers obtained by rats in the non-patent heroin group were excluded from this analysis. Fig. 2E shows the total proportion of rescues during the 30-min (1800 sec) rescue sessions for each reinforcer condition before and after the self-administration phase. The line representing heroin self-administration is flat line at y=0, indicating that no rescues were observed.

To control for the possibility that the lack of observation of rescuing behavior in the heroin group was due to drug intoxication, we performed an assessment of opioid-induced behaviors during each of the final three test sessions using a rating scale develop by Seip and colleagues (2012). On a scale of 0 to 4, with 0 representing an absence of the behavior and 4 representing high levels of the behavior, the following scores were obtained (mean±SEM): stupor (0.99±0.21), pica (0.22±0.03), stereotypy (1.53±0.18), aberrant grooming (0.15±0.15), hyperactivity (0.58±0.22), and non-specific swallowing (0.44±0.44). These values are lower than those observed by Seip and colleagues (2012) following non-contingent heroin exposure, where values of 2-4 were typically observed for these behaviors. In addition, although not quantified, such behaviors were not generally observed in animals in the non-patent of sucrose groups. These observations suggest that a lack of performance of the rescuing task in heroin self-administering rats was not likely a result of inability to do so due to heroin intoxication.

Discussion

The current study confirms previous findings that rats will exhibit prosocial behavior by releasing their cagemate from a plastic restrainer (Ben-Ami Bartal et al., 2011; Sato et al., 2015). We demonstrate that rats with a history of sucrose self-administration continue to rescue their cagemate, while rats with a history of heroin self-administration choose to continue heroin intake and do not rescue their cagemate. Further, rats that lost catheter patency during the experiment, and thus had a history of heroin intake but were unable to receive heroin during the final test, continued to rescue their cagemates. Thus, heroin intake appears to reduce the likelihood of prosocial behavior, particularly in the continued presence of drug access, which is reflective of the loss of social motivation and functioning in opioid-dependent individuals (APA, 2013).

One possible interpretation of our findings is that the intoxicating effects of heroin might have inhibited the ability of rats to perform the rescuing task when provided simultaneous access. However, we assert that this is unlikely for several reasons. First, using an opioid-induced behavioral rating scale developed by Seip and colleagues (2012), we did not observe evidence of significant heroin-induced stupor or other opioid-induced behaviors during these test sessions. In addition, the amount of heroin intake during these sessions (~9-10 infusions per 30 min session) were much lower than the amount of intake during longer access self-administration sessions (40-50 infusions per 6 hr session). Thirdly, a recent study by Ben-Ami Bartal et al. (2016) using a similar paradigm showed that rats receiving an acute administration of the benzodiazepine midazolam were still

able to open the restrainer to obtain a palatable reward. Thus, heroin intoxication was not likely a factor in inhibiting rescuing behavior in the present study, although it is of interest for future studies to determine what level of heroin intake reduces the ability to perform the rescue task.

Some critics of this animal models of prosocial behavior, particularly the notion that it represents an expression of "empathy"-like behavior, argue that the willingness to release the trapped rat is primarily motivated by seeking social contact and interaction (Schwartz, Silberberg, Casey, Kearns, & Slotnick, 2017; Silberberg et al., 2014). In an attempt to address this issue, Ben-Ami Bartal and colleagues (2014) utilized a novel paradigm to demonstrate that rats would continue to release their cagemate from a restrainer even when the trapped rat was released into a separate chamber away from the rescuer rat, thus removing any subsequent social contact. Still, there is no universal agreement as to whether these behaviors are expression of empathy-like or social contact motivated behaviors. Regardless, we assert that such behaviors can be viewed as representative of prosocial functioning.

Prior research examining social behavior of rats with a history of opioid exposure has shown that rats maintained in isolation vs. group housing more rapidly acquire heroin self-administration; however, over time, isolated rats do not self-administer more heroin overall than rats maintained in group housing conditions (Bozarth et al., 1987). Another study showed that group housed rats showed increased conditioned rewarding effects of heroin (0, 20, 40, and 80 μg/kg, s.,c.) as compared to isolated rats (Schenk et al., 1982).

Together these results suggest that housing conditions and social interaction may influence the rewarding and reinforcing effects of opioids.

The precise mechanisms and brain regions underlying heroin-induced deficits in social functioning and motivation are unknown. Likely substrates are cortico-limbic circuits that involve the dorsal anterior cingulate cortex, anterior mid-cingulate cortex, supplementary motor area, and bilateral insula, which can be considered core neural networks involved in social information processing and empathy-related behaviors (Fan, Duncan, de Greck, & Northoff, 2011; Namkung, Kim, & Sawa, 2017). In addition, a relationship between substance abuse and the insula was supported by a recent fMRI study that identified a positive association between activity in the posterior insula, substance abuse, and general disinhibition (Abram et al., 2015). In rodents, inhibition of protein synthesis in the insula disrupts drug-related contextual memories (Contreras et al., 2012), and this region also plays a role in drug seeking when alternative goals or competing contingencies are available (Naqvi, Gaznick, Tranel, & Bechara, 2014). Given the role of the insula in both drug-seeking and prosocial behaviors, future studies are needed to attempt to restore prosocial behavior following heroin intake using chemogenetic activation or other neuromodulatory approaches in one or more of these brain regions.

A potential limitation of the present study is the attrition of some subjects due to loss of catheter patency. However, rats that lost catheter patency during the experiment, and therefore had more limited heroin intake, continued to demonstrate prosocial

behavior, suggesting that impaired social function may be a function of amount of prior drug intake. Additional studies are needed to examine other factors such as heroin dose, length of self-administration history, repeated cycles of intoxication and withdrawal, acute vs. protracted stage of abstinence, and whether these findings extend to other abused opioids (e.g., oxycodone or fentanyl) or other drugs of abuse (e.g., psychostimulants). Finally, it should also be noted that despite engaging in rescuing behavior following the self-administration phase, rats in the sucrose group also continued to self-administer sucrose (see Fig. 2D). These observations suggest that rats are capable of both prosocial and appetitive behaviors when both are available simultaneously and are therefore not mutually exclusive of each other. It is therefore of interest to further explore potential influences of the presence of a trapped conspecific on the acquisition of heroin self-administration, since trapped cagemates were not present during this portion of the present study.

Overall, our findings also suggest that the rescue paradigm may represent a novel rodent model of impaired social function in opioid use disorders. Utilization of this model may give insight into the neural mechanisms of impaired social function in opioid addiction and may lead to improved target strategies in facilitating recovery and treatment. As mentioned previously, a diagnostic criterion for opioid use disorder is persistent use despite interpersonal problems due to opioid use. Thus, if prosocial behavior can be restored in individuals with opioid use disorder via a specifically developed therapy and/or targeted medications, this may allow successful rebuilding of

social support systems and relationships affected by previous drug use, with the ultimate goal of having greater chances of reaching and maintaining sobriety. Finally, as mentioned previously, heroin use is often initiated due to prior dependence on prescription opioids as a result of pain management, so future studies could explore the propensity to disengage from prosocial behaviors as a result of opioid self-medication to alleviate pain.

CHAPTER 3

RESTORATION OF PROSOCIAL BEHAVIOR IN RATS AFTER HEROIN SELF-ADMINISTRATION VIA CHEMOGENETIC ACTIVATION OF THE ANTERIOR INSULA

Abstract

The anterior insular cortex (AIC) has recently emerged as a brain region of interest in addiction research, as it mediates social and emotional processing as well as the interoceptive effects of abused drugs. Previous studies examining prosocial behavior in rodents have demonstrated that rats will release a cagemate trapped in a plastic restrainer in place of receiving food and will continue to open the restrainer door even if subsequent social interaction is prevented. We recently replicated and extended these findings by showing a disruption of prosocial behavior following heroin self-administration, which we hypothesize to be a result of heroin-induced changes in brain regions mediating these behaviors, such as the AIC. In the present study, we performed two experiments, one in which we utilized chemogenetics to selectively stimulate excitatory AIC pyramidal neurons, and a second experiment in which we inhibited AIC pyramidal neurons, to examine effects on heroin-induced social deficits. After baseline rescuing behavior was established, rats received bilateral infusions of viral vectors encoding either a control virus (CaMKIIα-GFP), stimulatory DREADD (AAV-CaMKIIα-hM3Dq-mCherry) (Experiment 1), or inhibitory DREADD (AAV-CaMKIIα-hM4Di-mCherry) (Experiment 2), into the AIC. Following recovery, rats were allowed to

self-administer heroin (0.06 mg/kg/infusion) 6 hr/day for 2 weeks. Prior to re-assessment of rescuing behavior, all animals were administered clozapine-N-oxide (1.5 mg/kg, i.p.) to assess effects of chemogenetic activation or inhibition of the AIC. Our results demonstrate that relative to control virus infused animals, chemogenetic activation of the AIC reversed prosocial deficits induced by heroin self-administration, whereas chemogenetic inhibition of the AIC had no effect. Thus, stimulatory neuromodulation of the AIC may be a novel approach to restoring opioid-induced deficits in prosocial behavior.

Introduction

Heroin abuse and overdose-related deaths have reached an all-time high in the United States, reaching epidemic status (Jalal et al. 2018). The feelings of euphoria elicited by heroin use are largely resultant of increased dopamine release throughout the mesolimbic reward circuitry in the brain (Johnson and North 1992; Compton and Volkow 2006; Corre et al. 2018). Chronic drug use alters brain structure and chemical neurotransmission via depletions in both gray and white matter volume, as well as disrupts connectivity in regions important for decision-making, impulse-control and executive function (Upadhyay et al. 2010; Wollman et al. 2017). One brain region gaining traction for its critical role in driving addiction is the insular cortex (Naqvi et al. 2014; Droutman et al. 2015; Kroll et al. 2018) which has been shown to be important for integrating the interoceptive constructs of motivation and emotion, as well as those produced by drugs of abuse, into conscious feelings of craving (Koob and Volkow 2016).

In humans, activity in the insular cortex is correlated with both addiction-related behaviors and important abilities needed for social interaction (Heilig et al. 2016). fMRI studies examining brain regions associated with empathy found that the insula was consistently activated (Fan et al. 2011). Interestingly, individuals with a history of opioid-use demonstrate lower levels of empathetic ability. It has been shown that when comparing heroin-dependent individuals with marijuana users, psychiatric patients, prison inmates, and police officers, heroin-dependent participants scored the lowest in measures of empathy and sociability (Kurtines et al. 1975). The loss of prosocial

behaviors following heroin intake is likely a result of opioid-induced changes in brain mechanisms mediating these behaviors. Most recently, Kroll and colleagues (Kroll et al. 2018) further investigated the neural substrates of empathy and the impact of opioids by administering a battery of neuropsychological tests to participants with a history of non-medical prescription opioid use compared to opioid-naïve controls. Results of this study demonstrated opioid-related deficits in the ability to recognize emotions in facial expressions, prosody of voices, and other emotion recognition tasks, and that these deficits are dose-dependent. These data lend further support to the notions that opioids can induce social impairments.

In an attempt to give insight into the neural mechanisms of impaired social function in opioid addiction, we previously utilized an established paradigm in rodents developed by Ben-Ami Bartal and colleagues (Ben-Ami Bartal et al. 2011) in which a rat will release or rescue a conspecific from a plastic restrainer instead of receiving food or other palatable rewards. Our results showed that rats with a history of sucrose self-administration continued to release their cagemate, whereas rats who had a history of heroin self-administration did not continue to release their cagemate and continued to self-administer heroin (Tomek, Stegmann, & Olive, 2019). These results are consistent with a study in which 'rescuer' rats administered the benzodiazepine midazolam, but not the beta-adrenergic antagonist nadolol, released their conspecific less often, while still opening the restraint door for chocolate (Ben-Ami Bartal et al. 2016). The authors interpreted these results as a drug-induced interruption or dysregulation social affective

processing that appears necessary to motivate the rat to open the restraint door for its cagemate.

In order to further examine the role of the insula in both heroin addiction-related and prosocial behaviors, the current study used the rodent prosocial paradigm as previously published (Tomek et al. 2019), in conjunction with chemogenetic approaches to selectively activate or inhibit the excitatory pyramidal neurons within the insula. In this study, we specifically targeted the anterior insular cortex (AIC), due its prominent role in both opioid addiction- and prosocial-related behaviors (Naqvi et al. 2014; Droutman et al. 2015; Wollman et al. 2017). Chemogenetics harnesses the utility of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), which are genetically engineered muscarinic acetylcholine receptors (hM3Dq and hM4Di) that are not activated by endogenous neurotransmitters but are only by an exogenous otherwise inert ligand, such as clozapine-N-oxide (CNO). Here, we refer to the hM3Dq as a "stimulatory DREADD", and hM4Di as an "inhibitory DREADD". They are considered excitatory and inhibitory because they function via the Gq and Gi pathway, respectively. Plasmids encoding DREADDS were packaged into an adeno-associated viral vector (AAV), along with cell-type specific promoter (calcium/calmodulin-dependent protein kinase II alpha, CaMKIIα) which allows for selective expression in cortical excitatory neurons (Liu and Jones 1996), and a fluorescent reporter protein (mCherry). A viral vector lacking the DREADD transgene and encoding green fluorescent protein (GFP) was used as a control. In vitro slice electrophysiology was used to verify DREADD functionality. Based on the existing

literature reviewed above, we hypothesized that chemogenetic stimulation of the AIC would improve deficits in prosocial behavior induced by heroin self-administration, whereas chemogenetic inhibition of this region would exacerbate heroin-induced social deficits.

Methods

Animals

Sixty-nine male Sprague Dawley rats (Charles River Laboratories) were used for these studies. Sixty-six were used for Experiments 1 and 2 (one half of this number were the rats inside the restrainer), and an additional n=3 were used for electrophysiological validation of DREADD function. Rats weighed approximately 250 g upon arrival and were pair-housed upon arrival on a 12-hour reversed light–dark cycle (lights on at 0700 hr) and given ad libitum access to food and water during all experimental procedures except during behavioral testing. All experimental sessions took place during the dark phase of the light-dark cycle. Upon arrival, one rat of each pair was randomly selected and its tail was marked with a permanent marker. These rats were designated the rescuer rats while the other rat in the pair was designated the trapped rat. Rats were individually handled and weighed for 5 min daily for two weeks, post-arrival, to allow them to acclimate to experimental procedures.

Assessment of baseline prosocial behavior

Following habituation to handling, animals underwent baseline assessment of prosocial behavior. Testing occurred at the same time every day for 3 weeks. In this

procedure, each designated trapped rat was placed in a plastic restrainer (Harvard Apparatus, Holliston, MA; modified to 13.3 cm x 8.0 cm x 8.9 cm;) with a removable door, and the restrainer was placed in an operant conditioning chamber (Med Associates, St. Albans, VT; model ENV-008, modified to 43.2 cm x 20.3 cm x 22.9 cm). All rescuing sessions were video recorded. Following placement of the trapped rat into the operant chamber, the rescuer rat was then placed in the chamber, followed by illumination of a house light. The occurrence of and latency to rescue the trapped rat was recorded for each session. The maximum amount of time allowed in the operant chamber for each session was initially 1 hr, but then reduced to 45 min and ultimately 30 min over the course of the 3 weeks of testing. The session time was only reduced if the rat began rescuing immediately. In order to reduce the possibility that removal from the testing apparatus was a motivating factor for rescuing behavior, upon freeing of the trapped rat, rats remained in the chambers for the duration of the session. After the first week, if the rat had not shown rescuing behavior at least one time, the rat was assigned to be the trapped rat and its cagemate was assigned to be the rescuer rat. Previous research indicates that a rat who experiences stressful stimuli will learn to rescue its cagemate more quickly than a naïve rat (Sato et al. 2015). Rescuer rats were allowed to undergo 2 full weeks of baseline rescuing behavior. Rats failing to release the trapped rat across the last two weeks of baseline saving were removed from the study.

Surgical procedures

After 2 weeks of rescue testing in the operant chambers, all of the "rescuer" animals were surgically implanted with intravenous catheters into the jugular vein according to our previously published procedures (Tomek et al. 2019). Briefly, rats were anesthetized with isoflurane (2% v/v) vaporized in oxygen at a flow rate of 2 l/min. Rats received pre-incision, subcutaneous injections of buprenorphine (0.05 mg/kg, s.c., Henry Schein Animal Health, Dublin, OH) and meloxicam (1 mg/kg, s.c., Henry Schein Animal Health,). Surgical sites were shaved and disinfected with 1% iodine. An approximately 2 cm incision was made in order to locate and isolate the right or left jugular vein. A sterile silastic catheter filled with 100 U/ml heparin was inserted 2.5 cm into the vein. The catheter was secured to the surrounding tissue with silk sutures, and the opposite end of the catheter was tunneled subcutaneously to the dorsum where it exited the skin between the scapulae. The catheter was secured to the vascular access port subcutaneously, then sutured into place. The wound was then closed with Ethicon nylon sutures and topically treated with topical lidocaine and triple antibiotic ointment. A 2.5-cm incision was made between the scapulae for implantation of a threaded vascular access port (Instech Laboratories, Plymouth Meeting, MA). The port was covered with an aluminum cap to prevent damage from cagemate chewing.

Virus infusion procedures were conducted during the same surgical procedure as catheter implantation. Prior to surgery, half of the rescuer rats were randomly assigned to receive either the control virus (AAV8-CaMKIIα-EGFP, Addgene #50469), stimulatory

DREADD virus (AAV8-CaMKIIα-hM3Dq, Addgene #50476), or inhibitory DREADD virus (AAV8-CaMKIIα-hM4Di, Addgene #50477) by use of a random number generator. The skin overlying the skull was shaved and scrubbed with betadine, and an incision was made to expose the skull surface. After appropriately placed holes were drilled into the skull, a stereotaxic microinjector (Kopf Instruments) was lowered into the anterior insular cortex (AP: +2.8, ML: +/- 3.5, DV: -6 mm) from skull surface and bregma according to a stereotaxic atlas (Paxinos and Watson 2014). A total of 0.5 µl of the appropriate virus (stimulatory DREADD [titer: $\geq 3x10^{12}$ vg/ml], inhibitory DREADD [titer: $\geq 2x10^{12}$ vg/ml], or control [titer: $\geq 1x10^{12}$ vg/ml]) was infused into each hemisphere. Cranial holes were covered with bone wax dental cement and the wound was sutured closed. The wound was then treated with 2% bacitracin/polymyxin B/neomycin and 5% xylocaine, and sutured closed with 3-0 Vicryl sutures. Animals received meloxicam (10 mg/kg s.c.) once daily for 5 days to minimize post-surgical pain and discomfort. All rats were allowed to recover from surgery for 5 days prior to the initiation of drug self-administration. During this time, the animals received daily intravenous infusions of 70 U/ml heparinized Timentin (66 mg/ml, dissolved in sterile saline containing 70 U/ml heparin, 0.1 ml volume) to maintain catheter patency and protect against infection. Meloxicam (2.5 mg/ml, s.c.) was also administered for 5 days following surgical procedures to provide additional relief of post-surgical discomfort. Rats were single housed during recovery. After 6 days of post-operative care to recover from surgery, all rats were returned to their respective pair-housing conditions.

Self-administration procedures

Following recovery from surgery, rats underwent 6 hr daily heroin self-administration sessions. All self-administration chambers were located inside sound-attenuating cubicles equipped with a house light and exhaust fan designed to mask external noise and odors and were interfaced to a personal computer (PC). Rats performed the rescuing paradigm in the same chamber as self-administration to avoid any potential environmental or context confounds. Chambers were equipped with two nose-poke holes on one wall with a 4.2 × 5.0 cm food pellet receptacle equipped with head entry detector and placed between the nose-poke holes. Each response hole was located approximately 7 cm above a stainless-steel grid floor, and positioned above each lever was a 2.5-cm diameter white stimulus light. Located near the top of the self-administration chambers was a Sonalert speaker that provided an auditory stimulus during reinforcer delivery. Located outside each chamber was a syringe pump interfaced to the computer. When attached to the tether for heroin, the syringe pump delivered the drug solution via a single-channel liquid swivel mounted atop the chamber via polyethylene tubing. In each session, nosepokes into the designated active hole resulted in delivery of heroin (Cayman Chemical, Ann Arbor, MI) at a dose of 0.06 mg/kg per infusion. Heroin was dissolved in sterile saline and delivered in a volume of 0.06 ml over a 2 sec period. Self-administration was conducted on a fixed ratio 1 (FR1) schedule of reinforcement. Each heroin infusion was followed by a 20-sec timeout period, during which additional active nosepokes were recorded but produced no drug infusions. Each

reinforcer delivery was accompanied by concurrent illumination of a stimulus light located directly above the active hole, and simultaneous presentation of an auditory tone (2900 Hz, ~65 dB) for 2 sec. Nosepokes into a separate inactive hole had no programmed consequences at any time during the experiment. Six-hour self-administration sessions were conducted 7 days per week for 14 consecutive days. To verify catheter patency in the heroin group, rats were periodically administered sodium methohexital (10 mg/ml i.v., 0.2 ml volume) and observed for brief periods of immobility.

Re-assessment of prosocial behaviors

On the day following the last day of heroin self-administration, the rescue paradigm was repeated as described above, with the exception of rats being attached to the infusion tethers as during the self-administration phase. The animals were tested for prosocial behaviors in one-hour sessions for 3 consecutive days, while being allowed concurrent access to heroin. Twenty minutes prior to being placed into the operant chambers, rats were administered clozapine-N-oxide (CNO) (1.5 mg/kg i.p., dissolved in sterile saline). One half of the group was randomly assigned to receive CNO and placed into the operant chamber and tested for rescuing behavior for 3 consecutive days (1 hr /day rescuing, 6 hr/day heroin self-administration); the other half of the group received CNO and only access to heroin without assessing rescuing behavior for 3 consecutive days (6 hr/day). These procedures were conducted in a counterbalanced designed, such that following the first 3 days of testing, the opposite procedure was performed on the next 3 days, resulting in 6 total days of heroin self-administration, three of which

assessed rescuing behavior. During sessions in which rescuing behavior was assessed, after one hour of access to rescue, the rat in the restrainer was removed from the chamber (unless it was released by the experimental animal prior to the one hour elapsing), and the other rat was allowed to remain in the chamber to continue heroin self-administration for another 5 hr to avoid the potential influence of drug withdrawal throughout the reassessment of the prosocial behavior. The latency of rescue was timed and recorded, as well as the number of infusions each rat received the first hour in the chamber.

Electrophysiological Recordings

For verification of DREADD functionality, whole-cell patch clamp electrophysiology was performed in brain tissue slices prepared from experimentally naïve rats infused with one of the three DREADD constructs as described above (stimulatory, inhibitory or control). Following viral infusion, animals were given at least 3 weeks of postsurgical recovery to allow optimal virus expression. Animals were then anesthetized with CO_2 and rapidly decapitated. Brains were rapidly removed and submerged in ice-cold carbogen (95% O_2 / 5% CO_2) saturated cutting solution (cutting artificial cerebrospinal fluid, aCSF) containing (in mmol/L): NaCl, 120; $NaHCO_3$, 25; Dextrose, 10; KCl, 3.3; NaH_2PO_4, 1.23; $CaCl_2$, 1.8; $MgCl_2$, 2.4. Solution osmolarity was adjusted to 295±5 mOsm and pH adjusted to 7.40±0.03. Brains were then transferred to a cutting chamber of a vibrating tissue slicer (Leica, VT1000S) and 300 µm thick coronal slices containing the insular cortex were prepared in ice-cold cutting aCSF. Slices were then placed in a holding chamber filled with recording aCSF solution containing (in

mmol/L): NaCl, 120; $NaHCO_3$, 25; KCl, 3.3; NaH_2PO_4, 1.23; $CaCl_2$, 0.9; $MgCl_2$, 2.0; dextrose, 10, osmolarity adjusted to 295±5 mOsm and pH adjusted to 7.40±0.03. The holding chamber aCSF was continuously bubbled with carbogen and incubated at 34°C for 45 minutes and then allowed to cool to room temperature before recording. Prior to recording, slices were transferred to a recording chamber where they were perfused continuously at a flow rate of 1-2 mls/min with filtered, carbogen-saturated recording aCSF solution.

DREADD-expressing pyramidal cells within the insula were visually identified using infrared DIC microscopy with an Olympus BX51WI microscope. Fluorescence (mCherry or GFP) was visualized using light emitted from a collimated LED (ThorLabs). Whole-cell recordings were made from the soma of identified virus-expressing pyramidal neurons after establishing a seal (resistance range: 1-10 GΩ). Recording pipettes (<20 mΩ), made from thin-walled capillary tubes were filled with an intracellular solution containing (in mmol/L): K-gluconate, 135; NaCl, 12; K-EGTA, 1; HEPES, 10; Mg-ATP, 2 and tris-GTP, 0.38. Osmolarity was adjusted to 285±5 mOsm and pH adjusted to 7.30±0.01. All recordings were conducted using Axograph software. Responses were digitized at 10kHz and saved on a disk using a digidata interface (Axon Instruments) and analyzed offline using Axograph.

Upon membrane rupture, cells were allowed to equilibrate for a minimum of 5 min prior to recording. During equilibration time, resting membrane potential, capacitance and membrane resistance were continually monitored. All recordings were

conducted in current clamp, where cell membrane potentials for labeled cells were maintained at either hyperpolarizing potentials (-80 mV) for hM3Dq, spiking threshold potentials (-45 mV) for hM4Di, or resting potentials (-65 mV) for GFP controls. After membrane potential stabilization was achieved, the DREADD agonist clozapine-N-oxide (CNO) was bath applied at 10 μM for a minimum of 5 min. Changes in membrane potential and spontaneous activity were observed.

Data analyses

The dependent variables for heroin self-administration data were the number of reinforcers obtained per session prior to acquisition of heroin self-administration, as well as during the first hour of sessions where animals were provided simultaneous access to heroin and the trapped cagemate. The rationale for analysis of only the first hour of the 6-hr session following acquisition of heroin self-administration was that rescuing was only measured for the first hour of this session, and the primary objective of these sessions was to measure heroin intake in the presence of a cagemate. Self-administration data were analyzed by multilevel ANOVA, with virus infused as a between-subjects factor and session as a within-subjects factor. However, due to differences in baseline rescuing behavior exhibited prior to the initiation of heroin self-administration in rats in Experiment 1 (see Results), it was necessary to separate animals into two different cohorts, and cohort was therefore analyzed as a factor for this experiment.

For rescuing behavior, dependent measures were observation of freeing the trapped rat and latency to rescue. Given the structure of the data in which each rat was

measured multiple times in each treatment condition and stage of the study, a multilevel model was used nesting observations within rats. In order to account for observation of rescuing behavior, as well as the latency to rescue, a survival analysis using the accelerated failure time (AFT) model was used. This allows us to model the rate of rescue as predicted by the treatment condition (stimulatory or inhibitory DREADDs, or control virus), the stage in the study (before and after acquisition of heroin self-administration), and an interaction between these two variables. We have previously used similar analyses as described elsewhere (Tomek et al. 2019); also see (Allison 2010) for details on survival analysis and the AFT model. The primarily measure of interest was the interaction between the treatment condition and the stage of the study, as this would indicate whether the change of rate in rescue from the pre-acquisition to the post-acquisition stages differed across DREADD virus versus control conditions. In the analyses, we controlled for the rats' cohort membership in the stimulatory DREADD vs. control groups, as two cohorts were used in this part of the study. All analyses were conducted in SAS Version 9.4.

Results

Electrophysiological validation of DREADD function:

All DREADD expression (hM3Dq, hM4Di and control) was readily identifiable within the AI of recorded slices. A representative image DREADD expression within the AI as well as individual cells is shown in Fig. 3A. As shown in Fig. 3B, AI pyramidal neuron activity can be modulated via chemogenetic approaches, where we observed

expected neuronal activation, inhibition, and no effects in hM3Dq, hM4Di, or control virus expressing cells, respectively. For cells expressing hM3Dq, CNO bath application readily induced cell depolarization and elicited action potentials, whereas CNO bath application inhibited action potential firing in cells expressing hM4Di. Furthermore, CNO bath application had no effect on membrane potential and did not elicit action potentials in neurons expressing the control DREADD vector. Representative electrophysiological traces are shown in Fig. 3B.

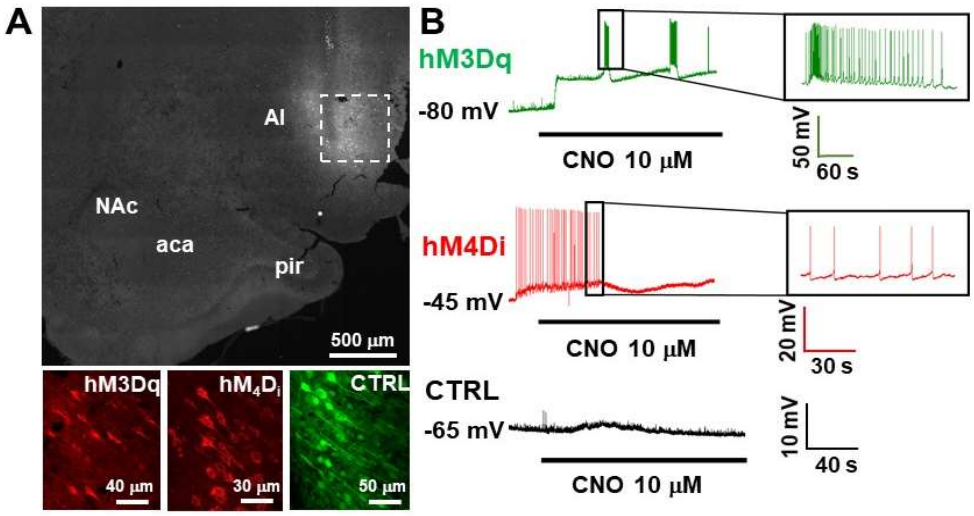

Figure 3. *DREADD function validated using whole cell electrophysiology. A) DREADD expression within the AI (dashed box) was observed in all recorded animals. Representative images showing cell bodies containing each vector is shown (bottom). B) Representative electrophysiological traces for hM3Dq (green; top), hM4Di (red; middle) and control (black; bottom) are shown. Black line below traces represents CNO bath application.*

Experiment 1 - Effects of chemogenetic activation of the anterior insula on prosocial behavior

A total of n=15 animals were used in the study examining the effects of the stimulatory DREADD. Two rats (and their cage-mates) were excluded from the analysis due to loss of catheter patency. Therefore, a total of n=13 animals were used for the analysis. Two separate cohorts of animals were used for this part of the experiment; cohorts are displayed separately due to their initial differences in baseline rescuing, which was controlled for in additional analyses. In the first cohort, n=3 rats were infused with the stimulatory DREADD virus and n=2 were infused with the control virus. In the second cohort, n=5 were infused with the stimulatory DREADD virus and n=3 were infused with the control virus. Thus, the total number of animals receiving the stimulatory DREADD were n=8 and the total number of animals receiving the control virus were n=5.

Figure 4A shows heroin intake across the initial 14 sessions of self-administration for the first cohort, and Figure 4B shows heroin intake during this stage for the second cohort. No effects of either virus infused (control or stimulatory DREADD; $p>0.05$) or cohort ($p>0.05$) were observed. However, for all groups, heroin intake increased gradually across all sessions ($F(1,168)=34.86$, $p<0.0001$), indicating acquisition of heroin self-administration. Figures 4C and 4D show heroin intake for cohorts 1 and 2, respectively, during the first hour of the three post-acquisition self-administration session

during which cagemates located inside the restrainer was also present. No significant differences were observed when heroin intake data were analyzed as a function of virus infused, cohort, or session (all p-values >0.05).

Each animal's rescuing behavior was measured 14 times before self-administration and 3 times during self-administration where the behavior of freeing the trapped rat and the latency to rescue were recorded. Figures 4E and 4F show the average proportion of rescues per rat across the two treatment conditions and the two stages of study for the first and second cohorts.

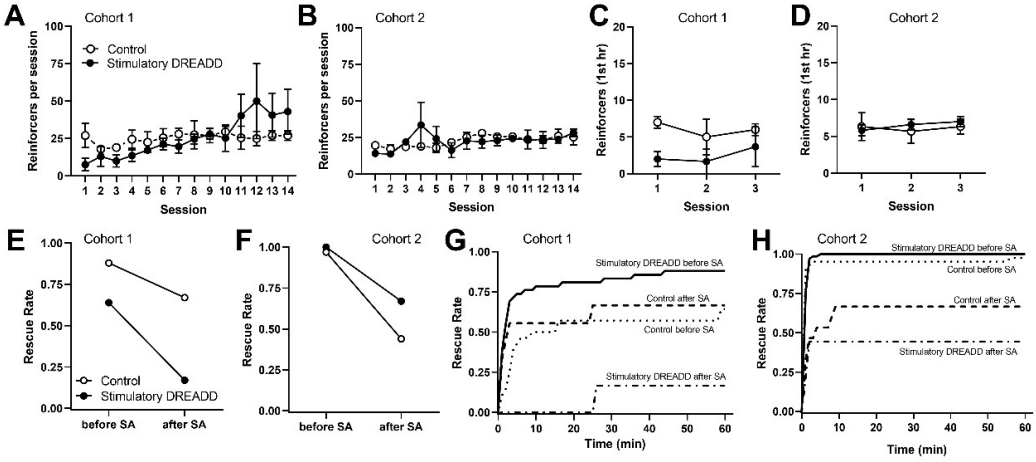

Figure 4. *A) Average heroin intake per session during the initial 14 sessions where they received heroin but did not need to rescue a rat for the first cohort. B) Average heroin intake per session during the initial 14 sessions where they received heroin but did not need to rescue a rat for the second cohort. C)Average heroin intake in the first hour during the 3 test sessions where they were given the choice between receiving heroin and rescuing its cage-mate for the first cohort. D) Average heroin intake in the first hour during the 3 test sessions where they were given the choice between receiving heroin and rescuing its cage-mate for the second cohort. E) Average proportion of rescues during each self-administration phase for each treatment condition in the first cohort. F) Average proportion of rescues during each self-administration phase for each treatment condition in the second cohort. G) Total proportion of rescues across the 60 minutes in the test chamber for each of the virus conditions before and after the self-administration*

phases of the experiment for the first cohort. H) Total proportion of rescues across the 60 minutes in the test chamber for each virus condition before and after the self-administration phases of the experiment for the second cohort.

Figures 4E and 4F show the average proportion of rescues in both cohorts of animals prior to and after acquisition of heroin self-administration. In the first cohort of animals, prior to heroin self-administration, the average proportions of rescues were 88% and 64% for rats receiving the stimulatory DREADD or control virus, respectively, and following acquisition of heroin self-administration, these proportions decreased to 67% and 17%, respectively. In the second cohort of animals, prior to acquisition of heroin self-administration, the average proportions of rescues were 100% and 97% for rats receiving the stimulatory DREADD or control virus respectively, and following acquisition of heroin self-administration, these rates dropped to 67% and 44%, respectively. Table 4 shows the descriptive statistics for each treatment condition and stage of the study, including the average proportion of rescues per rat, and the mean, median, and standard deviation for the latency in rescue (in seconds) for the rats that rescued in both cohorts of rats.

Table 4. *Average proportion of rescues per rat across the treatment conditions and self-administration phase, and median and mean in latency among the rats that rescued for the two cohorts*

Cohort	Treatment Condition	Stage Before or After Self-Administration	Proportion of Rescues (percent)	Median Latency to Rescue (in seconds)	Mean (SD) of latency (in seconds)
First	Stimulatory DREADD	Before	88	106	320 (596)
		After	67	76	316 (584)
	Control Virus	Before	64	226	608 (924)
		After	17	1546	1546 (0)
Second	Stimulatory DREADD	Before	100	35.5	48 (43)
		After	67	115	307 (525)
	Control Virus	Before	97	28.5	32 (18)
		After	44	49	55 (35)

As previously described, a multilevel survival analysis was conducted controlling for the two cohorts. The effect of interest was the interaction effect between the treatment condition and self-administration stage, as this would indicate whether rats in the two treatment conditions responded differently to the self-administration procedures with regard to rescuing behaviors. This interaction effect was found to be significant (t_{12} = -2.32, $p < 0.05$, EE= -1.26), indicating that across the two cohorts, animals in the two treatment conditions reacted differently to the self-administration stage, although all rats decreased their rescuing behavior following acquisition of heroin self-administration (t_{12} = 6.9, $p < 0.05$, EE= -1.19). Figures 4G and 4H show the total proportion of rescues during the 60-min period for each treatment condition before and after acquisition of heroin self-administration for the first and second cohorts.

Experiment 2 - Effects of chemogenetic inhibition of the anterior insula on prosocial behaviors

A total of n = 18 animals were used in this study. Seven animals received the active inhibitory DREADD virus and n = 11 received the control virus. Figure 5A shows heroin intake across the initial 14 sessions of self-administration. No effects of virus infused (control or inhibitory DREADD; $F(1,89)=0.06$, $p=0.81$) was observed. However, for both groups, heroin intake increased gradually across all sessions ($F(1,89)=18.71$, $p<0.0001$), indicating acquisition of heroin self-administration. Figure 5B shows heroin intake during the first hour of the three post-acquisition self-administration session during which cagemates located inside the restrainer were also present. No significant differences were observed when heroin intake data were analyzed as a function of virus infused or session (all p-values >0.05).

For rescuing behavior, a multilevel survival analysis was conducted using the AFT model to account for observation of rescuing behavior as well as the latency to rescue, modeling the rate of rescue as predicted by the treatment condition (control virus or inhibitory DREADD), the stage in the study (before and after/during heroin acquisition via self-administration), and the interaction between these two variables. The primary measure of interest was the interaction between the treatment condition and the stage of the study, as this would indicate whether the change of rate in rescue from the pre self-administration to the post self-administration stages differed across virus condition. Table 5 shows the descriptive statistics for each treatment condition and stage of the study,

including the average proportion of rescues per rat, and the mean, median, and standard deviation for the latency in rescue (in seconds) for the rats that rescued.

Table 5. Average proportion of rescues per rat across the treatment conditions and self-administration phase, and median and mean in latency among the rats that rescued.

Treatment Condition	Stage Before or After Self-Administration	Proportion of Rescues (percent)	Median Latency to Rescue (in seconds)	Mean (SD) of latency (in seconds)
Inhibitory DREADD	Before	99	53	186 (402)
	After	45	102	118 (63)
Control Virus	Before	95	39	118 (336)
	After	33	80	204 (272)

As shown in Figure 5C, prior to heroin self-administration, the average proportion of rescues per rat was 99% and 95% for rats receiving the inhibitory DREADD or control virus respectively, and following acquisition of self-administration, these rates decreased to 45% and 33%, respectively. An interaction between self-administration stage and treatment group was not observed ($t_{17} = 0.20$, n.s.), indicating that the rats from the two treatment conditions (inhibitory DREADD or control virus) did not react differently to the self-administration stage. All rats decreased their rescuing behavior after acquisition of heroin self-administration ($t_{17} = 11.03$, $p < 0.05$, EE= -.83) as compared to before self-administration, regardless of whether they received the inhibitory DREADD or control virus. Figure 5D shows the total proportion of rescues during the 60-min period for each treatment condition before and after acquisition of heroin self-administration.

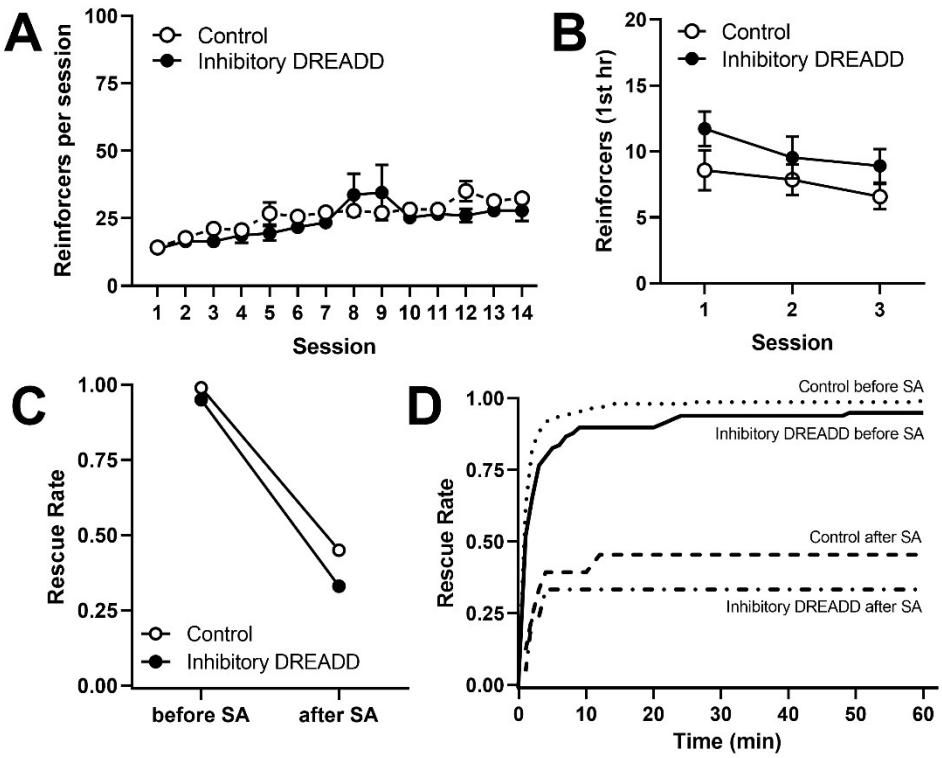

Figure 5. *A) Average heroin intake per session during the initial 14 sessions. B) Average heroin intake during the first hour of the 3 test sessions where rats were given the choice between receiving heroin and rescuing its cagemate. C) Average proportion of rescues during each self-administration phase for each treatment condition. D) Total proportion of rescues across the 60 minutes in the test chamber for each of the virus conditions before and after the self-administration phases of the experiment.*

Discussion

The current study confirms and extends previous findings that a history of heroin self-administration decreases prosocial behaviors in rats (Tomek et al. 2019). Further, these data demonstrate that utilizing chemogenetics to selectively stimulate the anterior insula can restore heroin-induced deficits in prosocial behaviors. These findings shed new

light on mechanisms and brain regions underlying heroin-induced impairments in prosocial functioning and lend support of the role of the insula in these social behaviors. Endogenous opioids, specifically through activation of the mu opioid receptor (MOR), are known to play a role in prosocial functioning (Heilig et al. 2016). For example, MOR knockout mice are unable to form normal attachments with their own mothers (Moles et al. 2004). Conversely, systemic treatment with MOR agonists reduce signs of stress from socially isolated rat pups (Carden et al. 1991). In humans, endogenous opioids peptides acting through MOR in the insula appear to mediate social attachment and bonding (Hsu et al. 2013; Nummenmaa et al. 2015). The AIC has reciprocal connections to limbic regions such as the amygdala, the anterior cingulate cortex, ventral striatum, and the dorsolateral prefrontal cortex (Gogolla 2017). These regions play a role in motivational, emotional, and cognitive functions, and initiating chemogenetic activation of neurons in the insula may, to some degree, restore some of the heroin impaired connectivity between these regions, motivating the rats to resume opening the restrainer door for their trapped cagemate.

Chemogenetic inhibition of the insula did not produce any significant differences in heroin intake or prosocial behaviors in active virus compared to control virus animals. This may be due to fact that inhibitory DREADDs have been found to suppress only about ~60% of firing rates in affected cells after CNO i.p. injections, resulting in a reduction of activity, not a complete cessation (Chang et al. 2015; Smith et al. 2016). Alternatively, and more likely, the heroin-induced deficits in prosocial behavior were

already at low levels and inhibiting the insula did not produce changes due to floor effects.

A limitation of the current study includes the difference in baseline rescuing between the two cohorts of rats in the stimulatory DREADD study (Experiment 1). The two cohorts used were found to have different pre-test recuing baselines; however, the baseline differences were controlled for statistically, and the results exhibited the same significant differences between experimental and control animals, and in the same direction.

Another limitation to this study was the potential biological effects of the DREADD agonist CNO. While initially believed to be physiologically inert, various studies have emerged demonstrating that at some doses and in some species, CNO is reverse metabolized to clozapine (Chen et al. 2015; MacLaren et al. 2016; Thompson et al. 2018), which can exert physiological effects and could potentially confound behavioral experiments. Taking this into consideration along with the recommended efficacy doses of CNO being between 0.1 to 3 mg/kg (Smith et al. 2016), our study was conservatively designed by including a dose of CNO within this recommended range (1.5 mg/kg), and the use of control virus lacking the coding sequence for either DREADD.

In the current study, we show that chemogenetic activation of the insula restored prosocial behaviors following heroin-intake; however, there was no significant difference in heroin intake between the active and control virus animals, and chemogenetic inhibition of the insula had no effect on prosocial behaviors or heroin intake. As a result,

future studies should investigate alternative ways to modulate the insula in an attempt to further refine and elucidate its role in addiction and related behaviors. Overall, the results of these experiments help narrow the quest for more anatomically targeted strategies in the attenuation and treatment of opioid use disorders.

CHAPTER 4

EFFECTS OF INSULAR CORTEX LESIONS ON HEROIN SELF-ADMINISTRATION AND PROSOCIAL BEHAVIOR.

Abstract

Increasing evidence suggests that the anterior insular cortex (AIC) plays a major role in substance use disorders as well as social functioning. However, how, and to what extent the insula contributes to the development and maintenance of substance use disorder remains unknown, thereby limiting our understanding of its causal role in addiction. As a result, we investigated whether bilateral excitotoxic lesions of the AIC differentially influenced the escalation of heroin self-administration (SA) during extended access to the drug. We also investigated the influence of AIC lesions on prosocial behavior, as measured by using an established animal model of prosocial behavior in which a rat releases its cagemate from a restraint. In this experiment, after baseline saving was established, and after 11 days of heroin self-administration (6 hours a day, on an FR1 schedule), Sprague Dawley rats received bilateral lesions of the AIC. After microinjections were administered, rats continued heroin self-administration for an additional 9 days which included 3 days of lesion formation, 3 days of SA with terminal lesions, and 3 days of post-lesion SA. During post-test SA, rats had concurrent access to their trapped cagemate in order to measure the occurrence and latency of rescuing behavior as compared to baseline rescuing rates. Results, in contrast to established lesion literature, demonstrated an increase in heroin use compared to controls. Rats who had

received AIC lesions did not significantly differ in saving behavior from controls, but both groups rescued less than before heroin self-administration. While not what expected, these results offer another piece of the puzzle in understanding the role of the insula and how heroin-associated interoceptive mechanisms change over the course of escalation and may represent an important component of opioid use disorder.

Introduction

Problematic opioid use continues to be a national health crisis with number of opioid users and overdoses occurring at an alarming rate. While the last couple years have seen a welcome decrease in opioid overdoses (Gladden, O'Donnell, Mattson, & Seth, 2019) the overall rates are still startlingly higher than they were as recent as a decade ago (CDC, 2018). With 1.7 million people suffering from opioid use disorder in a given year (CBHSQ, 2018), continued investigation into viable prevention and treatment solutions is imperative. There are many genetic, social, economic, and contextual factors that contribute to the development and maintenance of opioid use disorder, however, there is also evidence that positive social interactions and social support is useful in reaching and maintaining sobriety (Buchanan & Latkin, 2008; Eitan et al., 2017; Luthar et al., 1992; Russell et al., 2015). Criteria for Opioid Use Disorder diagnosis include continued opioid use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of opioids. Important social, occupational or recreational activities often are given up or reduced because of opioid use (DSM V). Individuals with a history of opioid use demonstrate lower levels of prosocial or empathetic ability, perhaps as a result of their opioid use (Kurtines et al., 1975; Kroll et al., 2018). Restoration of these pro-social behaviors can potentially contribute to their success in recovery.

The neural circuitry underlying Opioid Use Disorder is not well understood, and recent evidence in the drug dependence literature point to the importance of regions that

mediate social and emotional aspects of behavior (Franklin et al., 2002; Heilig, Epstein, Nader, & Shaham, 2016b; Mackey & Paulus, 2013; Naqvi, Gaznick, Tranel, & Bechara, 2014). Along with the anterior cingulate cortex and the orbitofrontal cortex, the insula has been shown to be activated in functional imaging studies when participants are presented with drug-related cues (Naqvi, Rudrauf, Damasio, & Bechara, 2007). The insula plays an important role in both the craving associated with opioid use disorder, but also in the processing of social interactions (Heilig et al., 2016). Interestingly, individuals with smaller than normal insula have been found to have higher scores on depression measures (Sliz & Hayley, 2012) and a higher prevalence of substance use disorders than healthy controls with neurotypical insulae (Ansell, Rando, Tuit, Guarnaccia, & Sinha, 2012; Droutman, Read, & Bechara, 2015). Cocaine users have been found to have decreases in insular gray matter concentrations, with ranges in reductions between 5-11%, compared to healthy controls (Franklin et al., 2002; Naqvi et al., 2014). Counterintuitive to this correlation, in humans who have suffered a stroke or lesion in the anterior insula, existing nicotine dependence is seemingly effortlessly attenuated (Naqvi et al., 2007).

Rodent studies have also supported a role of the insula in addiction-related behaviors. In one study, researchers used a conditioned place preference paradigm to show that lidocaine-induced inactivation of the insula reduced preference for an amphetamine-paired context, followed by resurgence of this preference after inactivation had subsided (Contreras et al., 2012). Additionally, rats with lesions of the anterior

insula induced after escalation of cocaine self-administration were able to restore control of intake, whereas rats that were lesioned prior to cocaine exposure increased their cocaine intake (Rotge et al., 2017). These data demonstrate the loss of control of cocaine intake can be differentially impacted by lesioning the AIC, this offers support that drug - associated interoceptive functioning of the AIC potentially changes over the course of escalation of intake and may represent an important component of substance use disorder.

In order to further investigate the role of the insula in prosocial behavior and opioid use disorder we previously used an established rodent model of empathy (Ben-Ami Bartal, 2011) adapted to self-administration procedures in order to identify heroin induced social deficits in rats (Tomek et al., 2019). We observed that chemogenetic activation of glutamatergic neurons in the anterior insula resulted in recovery of prosocial functioning following heroin intake (Chapter 3). However, chemogenetic inactivation of these neurons in the anterior insula did not produce any effects on heroin-induced prosocial deficits.

While these results suggest that chemogenetic inactivation of the anterior insula does not alter prosocial behaviors in animals with a history of opioid intake, an alternative explanation is that chemogenetically inhibiting the anterior insula does not produce complete silencing of cortical glutamatergic neurons (Chang et al. 2015; Smith et al. 2016). Further, chemogenetic inhibition only produces transient inactivation of neurons and requires repeated injections of CNO, as opposed to more permanent effects of chemical lesions, such as those produced by quinolinic acid (QA). Therefore, the

present experiment aimed to address these potential shortcomings by incorporating excitotoxic lesioning of the anterior insula. In line with Rotge's (2017) work, in which lesions after cocaine acquisition resulted in rats regaining control of intake, we performed lesions after rats had acquired stable patterns of heroin self-administration. Based on the outcomes from the chemogenetic studies, we hypothesized that there would be a decrease in prosocial behavior; and also, rats with anterior insula lesions would reduce their level of heroin intake.

Methods

All experimental procedures were conducted with the approval of the Institutional Animal Care and Use Committee at Arizona State University and in accordance with the Guide for Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

Animals

Male Sprague Dawley rats (Charles River Laboratories), weighing approximately 250 g, were pair-housed upon arrival on a 12-hour reversed light–dark cycle (lights on at 0700 hr), and given *ad libitum* access to food and water during all experimental procedures except during behavioral testing. All experimental sessions took place during the dark phase of the light-dark cycle. Upon arrival, one rat of each pair was randomly selected, and their tails marked with a permanent marker; these rats were designated the rescuer rats while the other rat in the pair was designated the trapped rat. Rats were

handled and weighed daily for two weeks to allow them to acclimate to handling procedures.

Assessment of Baseline Prosocial Behavior

Following habituation to handling, animals underwent a baseline assessment of prosocial behavior. Testing occurred at the same time every day for 3 weeks. In this procedure, each designated trapped rat was placed in a plastic restrainer (Harvard Apparatus, Holliston, MA; modified to 13.3 cm x 8.0 cm x 8.9 cm) with a removable door, and the restrainer was placed in an operant conditioning chamber (Med Associates, St. Albans, VT; model ENV-008, modified to 43.2 cm x 20.3 cm x 22.9 cm). All self-administration chambers were located inside sound-attenuating cubicles equipped with a house light and exhaust fan designed to mask external noise and odors and were interfaced to a personal computer (PC). Chambers were equipped with two nose-poke holes on one wall with a 4.2 × 5 cm food pellet receptacle complete with head entry detector and placed between the nose-poke holes. Each response hole was located approximately 7 cm above a stainless-steel grid floor and positioned above each hole was a 2.5-cm diameter white stimulus light. Located near the top of the self-administration chambers is a Sonalert speaker that provided an auditory stimulus during reinforcer delivery. Located outside each chamber was a syringe pump interfaced to the computer. Rats performed the saving paradigm in the same chamber as self-administration to avoid any environmental or contextual confounds. When attached to the tether for heroin, the syringe pump delivered the drug solution via a single-channel liquid swivel mounted atop

the chamber via polyethylene tubing. All rescuing and self-administration while rescuing sessions were video recorded. Following placement of the trapped rat into the operant chamber, the rescuer rat was then placed in the chamber, followed by illumination of a house light. The occurrence of and latency to rescue the trapped rat was recorded for each session. The maximum amount of time allowed in the operant chamber for each session was initially 1 hr. In order to reduce the possibility that removal from the testing apparatus is a motivating factor for rescuing behavior, upon freeing of the trapped rat, the rescuer rats remained in the chambers for the duration of the session. The rat inside the restraint was removed from the operant chamber immediately upon release in order to avoid the released rat initiating nosepokes that would result in the rescuing rats being infused with heroin that was not self-administered. After the first week, if the rat had not demonstrated rescuing behavior at least one time, the rat was switched with the restrained rat. Previous research indicates that a rat who experiences the stressful restraint will learn to rescue more quickly than a naïve rat (Sato, 2015). The switch only occurred after the first 7 days of the original rescuing rat not opening the restrainer door. The rescuer rats were given 2 full weeks of baseline rescuing.

Surgical Procedures

After 2 weeks of rescue testing in the operant chambers, half of the rats were randomly assigned to either the quinolinic acid lesion group or the control (PBS) injection group by use of a random number generator. All of the animals underwent surgery to implant intravenous catheters into the jugular vein according to our previously

published procedures (Watterson et al., 2014). During the surgery to implant the jugular catheter, rats were also provided intracranial cannula aimed at the insular cortex. Rats were anesthetized with isoflurane (2% v/v) vaporized oxygen at a flow rate of 2 l/min. Rats received pre-incision, subcutaneous, injections of buprenorphine (0.05 mg/kg, s.c., Henry Schein Animal Health, Dublin, OH) and meloxicam (1 mg/kg, s.c., Henry Schein Animal Health,). Surgical sites were shaved and disinfected with 1% betadine. An approximately 2 cm incision was made in order to locate and isolate the right or left jugular vein. A sterile silastic catheter filled with 100 U/ml heparin was inserted 2.5 cm into the vein. The catheter was secured to the surrounding tissue with silk sutures, and the opposite end of the catheter was tunneled subcutaneously to the dorsum where it exited the skin between the scapulae. The catheter was secured to the vascular access port (Instech Laboratories, Plymouth Meeting, MA) subcutaneously, then sutured into place. The wound was closed with Ethicon nylon sutures and topically treated with topical lidocaine and triple antibiotic ointment. The port was covered with an aluminum cap to prevent damage from cagemate chewing.

For the stereotaxic procedure, the skin overlying the skull was shaved and scrubbed with betadine, and an incision was made to expose the skull surface. After appropriately placed holes were drilled in the skull, guide cannulae (33 ga outer diameter, 26 ga inner diameter) were bilaterally aimed at the anterior insular cortex (AP: +2.8, ML: +/- 3.5, DV: -6 mm) from skull surface and bregma (according to the atlas of Paxinos and Watson, 2007). The cannulae were secured with dental cement and the skin stitched

closed. The wound was then treated with 2% bacitracin/polymyxin B/neomycin and 5% xylocaine, and sutured closed with 3-0 Vicryl sutures. Animals received meloxicam (10 mg/kg s.c.) once daily for 5 days to minimize post-surgical pain and discomfort. All rats were allowed to recover from surgery for 5 days prior to the initiation of drug self-administration. During this time, the animals received daily intravenous infusions of 70 U/ml heparinized Timentin (66 mg/ml, dissolved in sterile saline containing 70 U/ml heparin, 0.1 ml volume) to maintain catheter patency and protect against infection. Rats were single housed during recovery. After recovery from surgery, all rats were returned to their respective pair-housing conditions.

Self-administration Procedures

Following recovery from surgery, rats underwent 6-hr daily heroin self-administration sessions. In each session nosepokes into the designated active hole resulted in delivery of heroin (Cayman Chemical, Ann Arbor, MI) at a dose of 0.06 mg/kg per infusion. Heroin was dissolved in sterile saline and delivered in a volume of 0.06 ml over a 2-sec period. Self-administration was conducted on a fixed ratio 1 (FR1) schedule of reinforcement. Each heroin infusion was followed by a 20-sec timeout period, during which additional active nosepokes were recorded but produced no drug infusions. Each reinforcer delivery was accompanied by concurrent illumination of a stimulus light located directly above the active hole, and simultaneous presentation of an auditory tone (2900 Hz, ~65 dB) for 2 sec. Nosepokes into a separate inactive hole had no programmed consequences at any time during the experiment. Six-hour self-

administration sessions were conducted 7 days per week for 17 consecutive days. To verify catheter patency, rats were periodically administered sodium methohexital (10 mg/ml i.v., 0.2 ml volume) and observed for brief periods of immobility. Six days prior to the reassessment of prosocial behaviors, rats were briefly anesthetized with isoflurane as described above and underwent microinjections into the anterior insula. Initially, n=4 animals received infusions of vehicle (phosphate buffered saline, PBS) at a rate of 1 μl/min for 1 min. However, in a pilot group of animals from whom data were excluded, it was determined that this flow rate was not compatible for producing accurate lesions with quinolinic acid. Therefore, for the remainder of animals included in the present study, either quinolinic acid (40 μg/μl, pH=7.4, n=6) or phosphate buffered saline was infused at a rate of 0.35 μl/min over the course of 1 min (n=7). Herein, we therefore refer to these groups as "lesioned" and "control" rats, respectively. Microinjectors were left in place following the injection in order to ensure the infusate was adequately dispersed.

Re-assessment of Prosocial Behaviors

On the day following the last day of heroin self-administration, the rescue paradigm was repeated as described above, with the exception of rats being attached to the infusion tethers as during the self-administration phase. The animals were tested for prosocial behaviors in one-hour sessions for 3 consecutive days, while being allowed concurrent access to heroin. One half of the group was randomly assigned to either be allowed to self-administer heroin and/or rescue their cagemate for 3 days; the other half of the group was attached to the self-administration tether and only had access to heroin

without the opportunity to rescue for 3 days. Then the groups were switched. The purpose of this experimental design was to make sure the administration of the CNO did not impact the rewarding effects of the heroin. We wanted the rats to just have CNO and heroin without access to the saving in order to be able to identify any changes in self-administration behavior. After one hour of access to rescue, the rat in the restrainer was removed from the chamber (unless it was released by experimental animal prior to the one hour elapsing) and the heroin rat was allowed to remain in the chamber to continue self-administering heroin for another 5 hours to avoid withdrawals throughout the reassessment of the prosocial behavior. The occurrence and latency of rescue was recorded, as well as the number of heroin infusions each rat received the first hour in the chamber and total number of infusions across the 6-hour session.

Assessment of lesions

Approximately 24-48 hr following the last behavioral test session, animals were deeply anesthetized with Somnasol (150 mg/kg sodium pentobarbital, i.p.) and transcardially perfused with 0.1 M phosphate-buffered saline containing 0.1% heparin, pH 7.4, followed by 4% paraformaldehyde (pH=7.4). Brains were removed, post-fixed for 48 hr, cryoprotected in 30% w/v sucrose for at least 48 hr, and coronal sections through the anterior insula were obtained at 40 μm thickness on a Leica CM1900 cryostat (Leica Microsystems, Buffalo Grove, IL, USA) and mounted onto gelatin-coated microscope slides. Slides were heated overnight at 50°C prior to staining with Fluorojade C (FJC) (EMD Millipore, Billerica, MA, USA), a fluorescent marker of degenerating

neurons. (Schmued et al., 2005; Sewalia et al. 2018). Briefly, slides were immersed sequentially in the following solutions: 80% ethanol/1% NaOH for 5 min, 70% ethanol for 2 min, dH_2O for 2 min, 0.06% w/v $KMnO_4$ for 15 min, dH_2O for 2 min, 0.0001% w/v FluoroJade C (FJC) + 0.1% acetic acid for 10 min, and d_H2O 3 × 1 min. Slides were then dried on a slide warmer at 50°C for 30 min, immersed in xylene for 2 min, coverslipped using DePeX mounting media (Electron Microscopy Sciences, Hatfield, PA, USA), and stored in darkness until microscopic analysis . Sections were viewed under brightfield microscopy at 10x magnification and images obtained via digital camera. Lesion accuracy was assessed by approximation of the amount of anterior insula showing FJC-positive staining per coronal section in at least 4 sections from each animal. Data from animals showing damage to <10% of the total insula per section or with damage outside the anterior insula were excluded from analyses.

Data analyses

The dependent variables for heroin self-administration data were the number of reinforcers obtained per session after heroin self-administration was acquired, three days of self-administration after the lesion was terminally formed, as well as during the first hour of sessions where animals were provided simultaneous access to heroin and the trapped cagemate. The analyses were performed for both first hour of the six hour session, as well as the entire 6 hour session to compare times for when the rat had concurrent access to save their conspecific. Self-administration data were analyzed by

multilevel ANOVA, with acid lesion as a between-subjects factor and session nested as a within-subjects factor.

For rescuing behavior, dependent measures were observation of freeing the trapped rat, (meaning yes or no, whether or not the lesioned rat opened the door for the trapped rat) and latency to rescue (how long, in seconds, it took the lesioned rat to open the door for the trapped rat). Given the structure of the data in which each rat was measured multiple times in each treatment condition and stage of the study, a multilevel model was used nesting observations within rats. In order to account for observation of rescuing behavior, as well as the latency to rescue, a survival analysis using the accelerated failure time (AFT) model was used. This allows us to model the rate of rescue as predicted by the treatment condition (acid lesion or control rat), the stage in the study (heroin intake before and after insular lesion), and an interaction between these two variables. We have previously used similar analyses as described elsewhere (Tomek et al. 2019); also see (Allison 2010) for details on survival analysis and the AFT model. The primarily measure of interest was the interaction between the treatment condition and the stage of the study, as this would indicate whether the change of rate in rescue from the pre-acquisition to the post-acquisition stages differed across acid lesion versus control conditions. All analyses were conducted in SAS Version 9.4.

Results

A total of n=46 animals were used in the study (23 of which were cagemate rats placed in the restraint, and 23 underwent catheter implantation and self-administration procedures to examine the effects of insular lesions), 9 pair (n=18) were control animals from an initial execution of the study aka "first lesion cohort" in which the lesioned animals could not be included due to the lesion extending past the insula, but the control group data was satisfactory and able to be included. Data from n=one rat were excluded from the analysis due to the lesion extending past the insula (and cagemate subsequently subtracted from total n). Five pairs from the first lesion cohort of control animals were excluded because they never acquired baseline saving behavior. Therefore, a total of n=13 animals were used for the analysis, where n=6 rats were intracranially infused with quinolinic acid (see fig 6 for placement) and n=7 were infused with phosphate buffered saline as a control with 4 of those 7 animals being from the first lesion cohort, 3 from the second. For the first part of the analysis, we evaluated how heroin intake was affected by the treatment group and treatment stage.

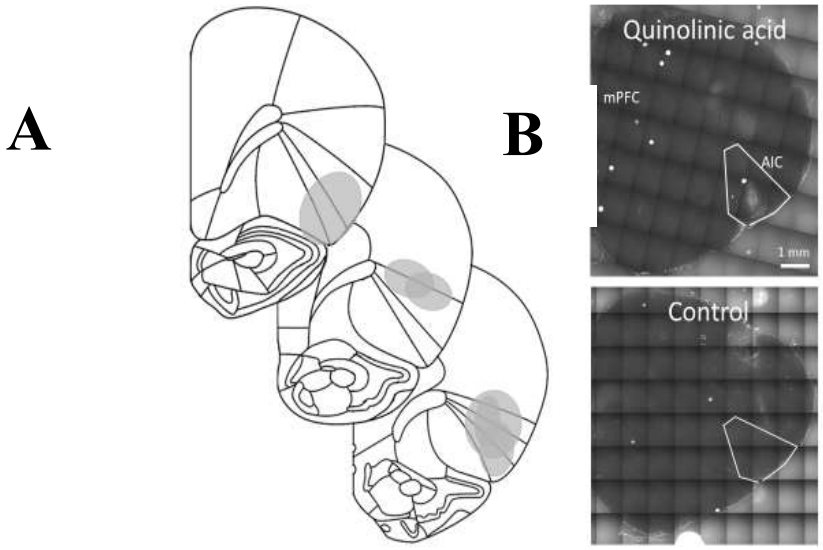

Figure 6: *A. Placement of quinolinic acid lesion in each subject, as identified by shaded gray areas. B. Histology photographs of the insular region of an animal from the acid lesion condition and the control condition: demonstrating the lesioned cells in the insula in the acid lesioned animal, and the absence of a lesion in the control animal.*

Figure 7 shows heroin intake across the initial 11 sessions of self-administration (acquisition), infusion of quinolinic acid or phosphate buffered saline for controls occurred between sessions 11 and 12, a 3-day period to allow for lesion formation, and an additional 3 days of self-administration after the lesion was formed, for a total of 17 self-administration days. There were no significant differences between rats receiving quinolinic acid and vehicle control rats with regards to average heroin intake during the first 17 sessions (1 hr $t_{25} = -1.50$, p-value = .15; 6 hr $t_{25} = -1.74$, p-value = .09), and there were no significant differences across the post acid lesion saving sessions in terms

of the average heroin intake (1 hr $t_{25} = -1.95$, p-value = .06; 6 hr $t_{25} = 1.32$, p-value = .26). We performed post hoc multilevel t-tests comparing just the first 11 days, there was not a significant difference between control and lesion group in the first hour of their 6 hour sessions; however, there was a significant difference when entire 6 hour session was analyzed (6 hr $t_{25} = -2.62$, p-value = .009, EE= 1.17)

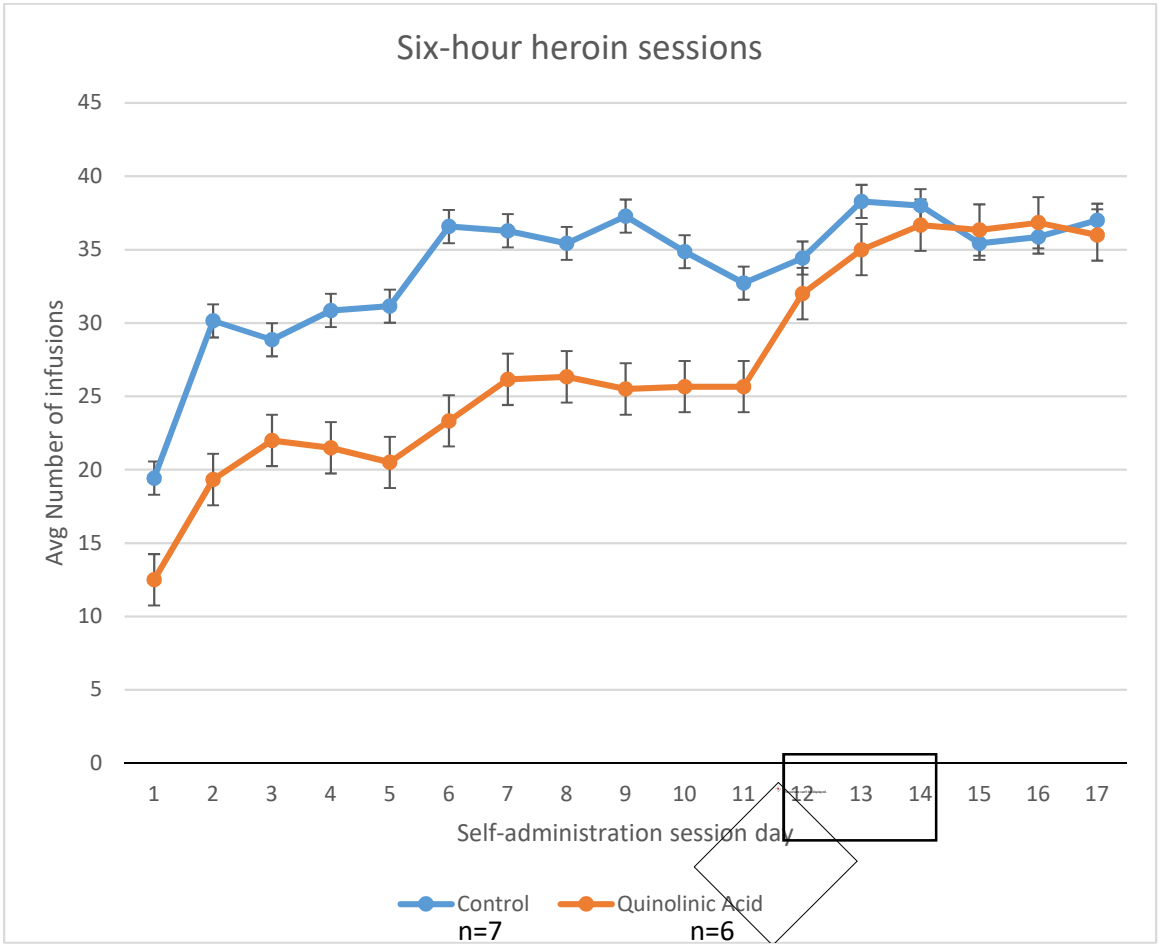

Figure 7: *Heroin intake across the initial 17 sessions of self-administration, lesion placement denoted by arrow. Lesion formation days denoted by the box outline.*

Despite the significant interaction when considering the treatment condition and treatment stage; the visual differences in average intake between groups, when graphed, prompted further investigation. In an attempt to get a better understanding of the impact of the lesion, if any at all, the analyses were performed again omitting the two lowest pressing animals. Results indicate that the interaction was still significant ($t_{86} = 4.44$, p-value < .0001, EE = .81); however, what was driving the difference was still unclear. Graphing the number of daily heroin infusions of each individual rat (see fig 8), offered an informative visual that showed that two particular lesioned animals (subject number 10 and number 19, with 90% and 50% lesion sizes, respectively) had a sharp incline in self-administration rates after the infusion of quinolinic acid. After this dramatic increase, one subject appeared to return to their previous intake trajectory, while the other appeared to continue to increase its heroin intake. These two animals may be driving the difference between the groups and the reason for the sharp incline may be due to the lesion, or more likely a confounding motivation such as pain. With such a small number of subjects, in this case, identifying the effects of the lesions is unclear.

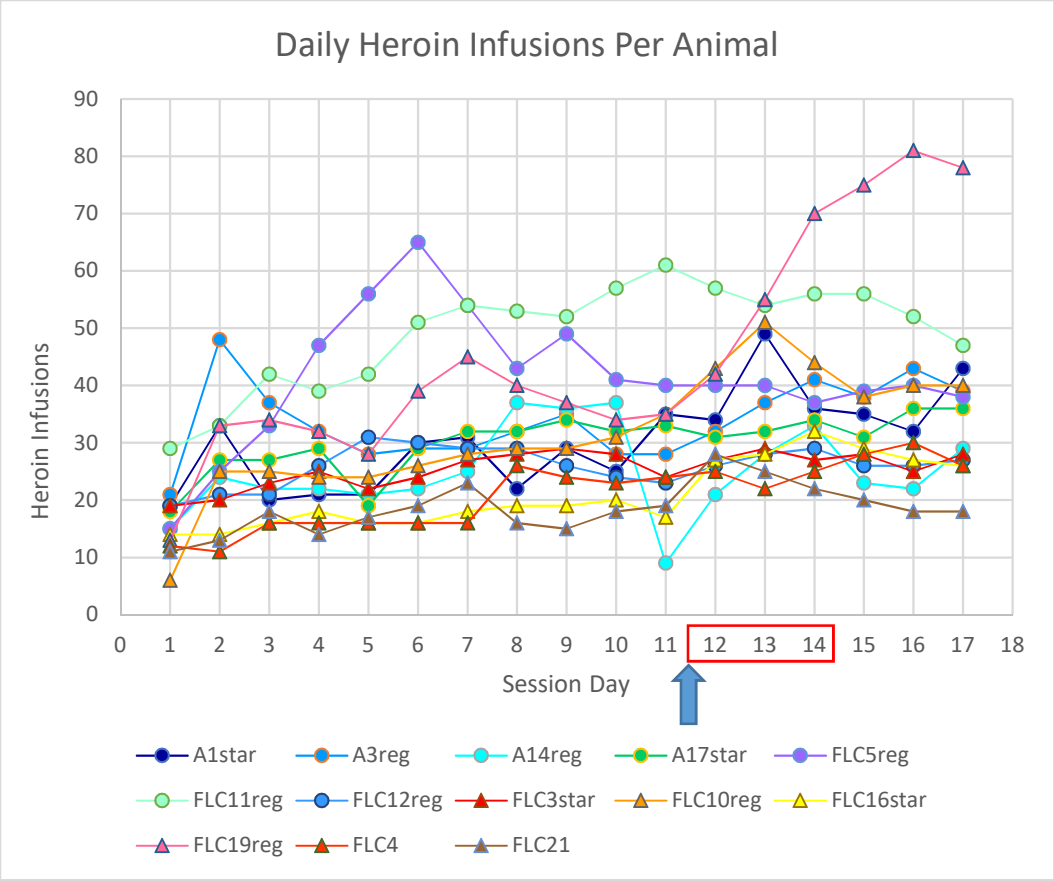

Figure 8: *Daily heroin infusions for each rat for 17 days of self- administration. Quinolinic acid was infused between sessions 11 and 12, as indicated by the arrow. The red box indicates quinolinic acid lesion formation days*

During the first treatment stage before the lesions were performed, the control rats obtained an average of 7.95 infusions per hour and 34.95 infusions per 6 hours; the lesioned rats used an average of 6.00 infusions per hour and 25.61 infusions per 6 hours. After lesioning, the control rats obtained 7.88 infusions per hour and 35.76 infusions per 6 hours; the lesioned lesion rats increased heroin intake from 6 infusions to 7.69 infusions per hour and from 25.61 infusions to 37.69 infusions per 6 hours. To test whether these

differences in change in heroin intake across the two groups were significant, a multilevel model was used, in which the interaction between the treatment condition and the treatment stage was tested for significance. The interaction was significant both for the 1-hour infusion ($t_{102} = 2.76$, p-value < .01, EE= 1.35) and the 6-hour infusion data ($t_{102} = 4.29$, p-value < .01). Figures 9 and 10 show the average heroin intake for each treatment group at each treatment stage for 1-hour and 6-hour sessions respectively. These figures illustrate that although the rats receiving control infusions were initially self-administering more heroin than rats receiving quinolinic acid, their heroin use changed very little, while the lesioned rats started off using less heroin than the control rats but increased their heroin intake significantly more than their prelesion self-administration intake compared to the control rats.

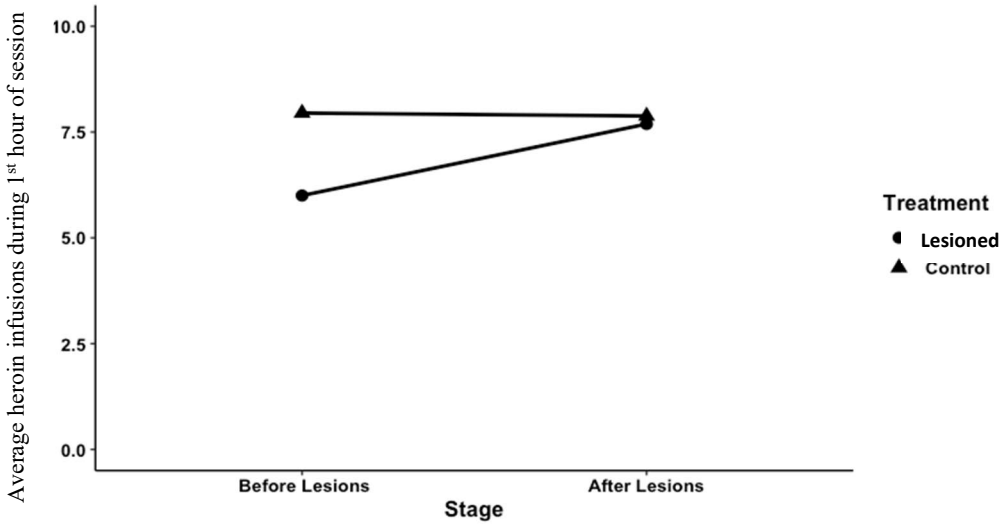

Figure 9: *Average number of heroin infusions (.06 mg/kg) by treatment group and experimental stage for first hour of session*

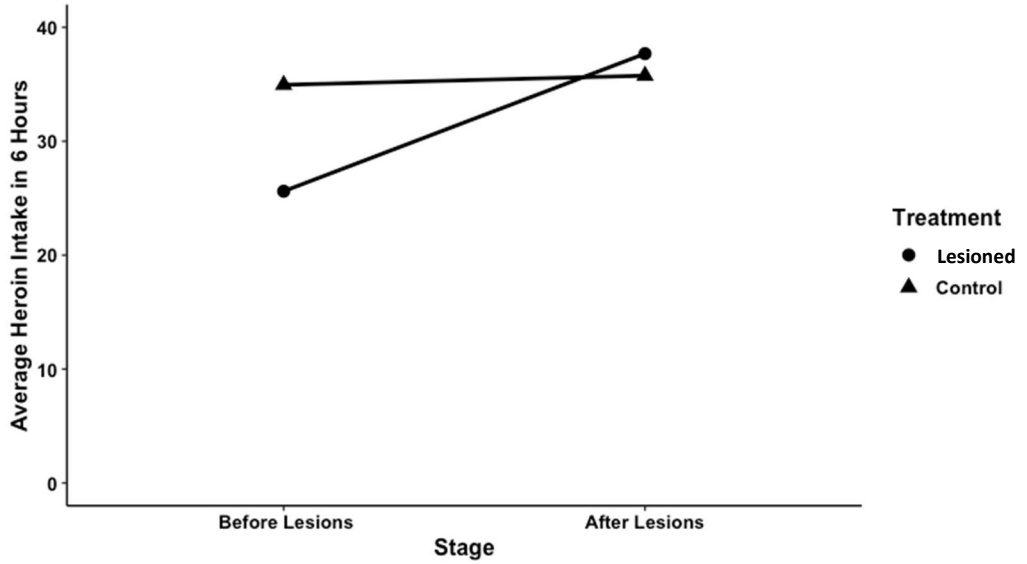

Figure 10: *Average number of heroin infusions (.06 mg/kg) by treatment group and experimental stage for six-hour session*

For the second part of the analysis, we evaluated how rescuing behavior was affected by treatment (quinolinic acid or vehicle control). Each animal's rescuing behavior was measured 14 times before self-administration and 3 times after the initial 17 days of self-administration where animal had concurrent access to heroin or rescuing its cagemate. Figure 11 shows the average proportion of rescues per rat prior to and after acquisition of heroin self-administration.

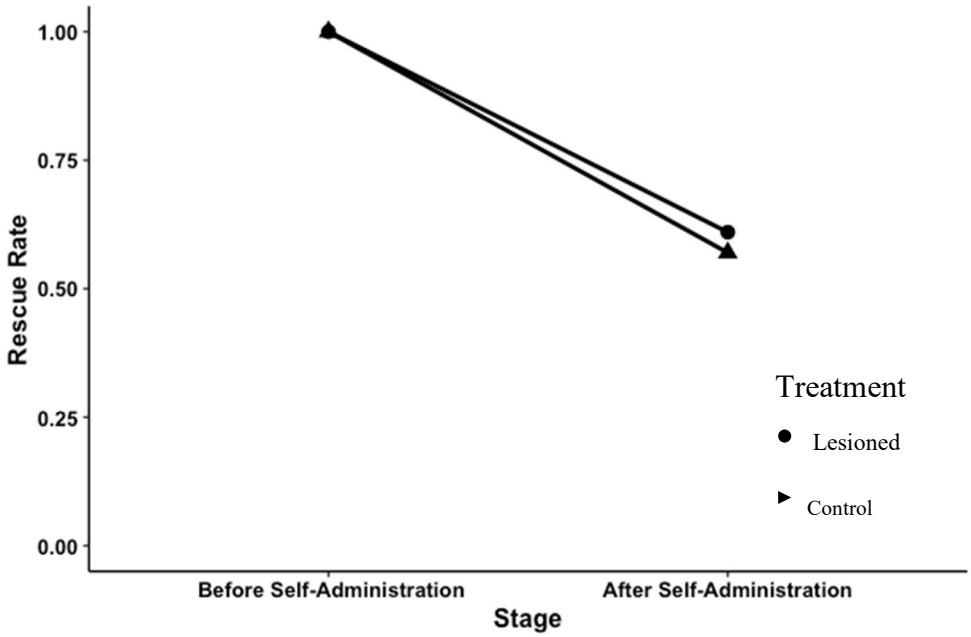

Figure 11: *Average proportion of rescues per rat by treatment group and treatment stage*.

Prior to heroin self-administration, the average proportions of rescues was 100% for the control and acid rats, and following acquisition of heroin self-administration, these proportions decreased to 57% and 61%, respectively. A multilevel survival analysis was conducted. The effect of interest was the interaction effect between the treatment condition and self-administration stage, as this would indicate whether rats in the two treatment conditions responded differently to the self-administration procedures with regard to rescuing behaviors. This interaction effect was found to be non-significant ($t_{12} = -1.6$, p-value = .14), indicating that animals in the two treatment conditions did

not differ across self-administration stage, although all rats decreased their rescuing behavior following acquisition of heroin self-administration ($t_{12} = 7.34$, $p < 0.01$).

Discussion

In the present study, we hypothesized that lesions of the AIC would produce deficits in prosocial behavior, as well as reduce the number of heroin self-administration infusions as compared to control animals. The current study confirms previous findings that a history of heroin self-administration decreases the proportion of rats willing to open a restrainer door in order to release a cagemate (Tomek et al., 2019). Even though there was no significant difference in rescuing behavior between the lesion and control groups, both conditions rescued less after heroin self-administration as compared to before self-administrations. Further, rats with lesioned insulae increased their heroin intake compared to controls. These data are in contrast to findings in humans that insular damage reduces cigarette smoking (Naqvi et al, 2014), and studies in animals examining effects of insular lesions on cocaine self-administration (Rotge et al, 2017). The reasons for these discrepancies are unknown but may be due to the smaller than anticipated lesions executed in this study. In initial pilot studies we completed utilizing this design, rats with lesions that extended beyond the insula showed reduced heroin intake compared to controls (data not shown), consistent with Rotge's work (2017) in which rats self-administering cocaine lesioned after acquisition showed reduced drug intake compared to control animals that had received cannula surgeries, but did not have any substances

infused into the insula. Thus, if smaller insular lesions increase use, and larger lesions in the insula decrease use, this would be consistent with the current literature in which the smaller the lesion the more likely to maintain nicotine use (Ansell et al., 2012; Droutman et al., 2015).

A limitation of the current study is the variability in lesion size and placement between animals. While damaging the insula has resulted in differences between groups, the amount of quinolinic acid and size of the lesion may have had a dichotomous effect on the outcome as it relates to heroin intake. Our results also may differ from the existing literature in that we infused phosphate buffered saline (PBS) into the anterior insula as a control, potentially causing unknown confounding damage to the insula. The Rotge (2017) experiments surgically implanted cannulae but did not inject any substances into their control group. Even though we did not see dead cells upon evaluation of the coronal brain slices of the control animals, this difference may potentially play a role in our findings. Future studies should aim to operationalize the process with more precision, perhaps by changing the amount of neurotoxin infused or flow rate of infusion. Additionally, another limitation in this particular experiment is evidenced by graphing individual rat data. The individual self-administration data seem to indicate that the increase in heroin self-administration after the lesion could be driven by a small portion of the lesioned cohort (i.e., 1 or 2 animals), raising questions regarding potential confounding effect of factors such as differences in lesion size on heroin self-administration, or individual differences in pre-lesion levels of heroin intake. In order to

parse these differences, analyses were run comparing different criterion days (the first 11 days) than the criterion determined in an a priori analysis (the first 17) showing that there were not significant differences in the first hour of the session, however there were significant differences between control and lesioned animals for the entire 6 hour self-administration session in the first 11 days.

Another potential limitation is that, quinolinic acid is an excitotoxin that destroys neuronal cell bodies but leaves axons including fibers of passage intact (Lugo-Huitrón et al, 2013), which differs from the human literature in which stroke damages the entire region, regardless of cell type or connections, with other varying unknown downstream effects. This may have contributed to the differences between our results and those in established literature. Future studies should consider electrolytic lesions to damage the insula in a way that will more closely mirror stroke damage and perhaps result in more similar outcomes to those demonstrated in the human literature. The insula acts as a hub of interoceptive cues, and with reciprocal connections to the prefrontal cortex, striatum and amygdala, those motivational and executive controls are being affected. The presence or absence of connections being maintained to these areas will change that relationship and function.

An important consideration when evaluating the results of this study as they compare to existing literature is the type and timing of drug administered in the experiments. The human literature, in regard to insular damage and drug intake, largely focuses on nicotine (Naqvi et al, 2007), while Rotge (2017) and colleagues examined

effects of lesions on cocaine intake, and our experiment investigated the effects of insular lesions on heroin intake. It is possible that the differences in the pharmacodynamics of each substance could result in very different lesion-induced consequences. Additionally, the timing of lesions can impact the outcome. As discussed previously, Rotge (2017) found that rats who were lesioned prior to cocaine intake escalation did not slow in intake, while rats who were lesioned after they acquired consistent intake were able to regain control of cocaine self-administration compared to controls. This timing aspect is also important in the human stroke literature, as the patients smoked cigarettes prior to insular stroke damage, and then seemingly effortlessly stopped smoking after insular damage occurred. We chose to lesion the insula after heroin acquisition had been established, based on the literature; however, the aforementioned pharmacodynamic differences based on drug class could potentially change the most effective timing as it impacts heroin intake trajectories. In future studies lesions can be introduced prior to heroin exposure to determine the most effective timing to prevent the acquisition of self-administration compared to controls.

While heroin intoxication could potentially be a factor in reducing rescuing behavior, we previously addressed this possibility in the initial experiment, detailed in chapter 2 of this dissertation, in which we utilized an established measure of opioid intoxication in rats developed by Seip et al (2012) to observe and report coded behaviors. Rats at the same dose (0.06 mg/kg) and session time (6 hours) as the current experiment were not found to exhibit behaviors consistent with opioid intoxication. Further, research

investigating the intoxicating effects of heroin in rodents found that rats would exhibit behavioral effects of heroin intoxication after self-administration of heroin nearly eight times the number of infusions (when considering equivalent doses) the rats in these experiments were self-administering during the reassessment of prosocial behavior sessions (Seip-Cammack et al, 2013).

The differences in heroin intake between the quinolinic acid-lesioned and control animals, regardless of lesion size or type, offers evidence that at least in some subjects the insula plays an important role in addictive behaviors. However, our results are too preliminary in terms of sample size and consistency of lesion extent to make any firm conclusions. Many other factors not examined here may influence the ability of insula neuromodulation to alter opioid use-related outcomes. Such factors include pre-lesion duration and level of heroin intake, which likely influence the development of tolerance, the type of insula lesion induced (i.e., axon-sparing, excitotoxic, neuron-selective, etc.), as well as other variables such as prior social history and individual differences in the interoceptive effects of opiates, both of which are mediated by the insula. Regardless, collectively, the studies outlined here suggest that the insula may be a potential target for further study and potentially targeted medications or alternative modulatory techniques in the quest for more effective opioid use disorder interventions.

CHAPTER 5

SUMMARY

The studies outlined in this dissertation were designed to establish a rodent model of prosocial deficits in the context of heroin addiction, and to examine the role of the anterior insula in modulating these deficits. We hypothesized that heroin self-administration would reduce prosocial behavior in rats, as measured by opening a restrainer door, releasing a trapped cagemate. We further hypothesized that chemogenetic activation of the anterior insula would restore prosocial behavior and inhibiting the insula would not impact prosocial behavior but reduce heroin intake after stable heroin use had been acquired. We concluded our set of experiments by investigating the effects of excitotoxic lesions in the anterior insula, we hypothesized that there would be a reduction of prosocial behavior, and that heroin intake, after acquisition, would be reduced.

Data presented in chapter 2 supported our hypothesis that heroin self-administration resulted in a reduction of prosocial behavior, rats who self-administered sucrose continued to release their cagemate, whereas rats who self-administered heroin ceased to open the restrainer door. Data presented in chapter 3 partially supported our hypotheses, results demonstrated that chemogenetic activation of the anterior insula restored the heroin induced loss of prosocial behavior in the excitatory DREADD group as compared to controls, however, we expected to see a reduction in heroin intake after chemogenetically inhibiting the anterior insula and there was not a difference in heroin

intake or prosocial behavior, between inhibitory DREADD rats and controls. Lastly, in regard to excitotoxic insular lesion data presented in chapter 4, our results were inconclusive. The initial study was confounded with quinolinic acid lesions extending past the anterior insula, so even though lesioned rats self-administered less heroin than controls and demonstrated reduced saving behaviors compared to controls, we were unable to attribute it to the lesion. A replication of the study also resulted in variable lesion sizes, the data showed lesioned animals had a more dramatic increase in heroin intake compared to controls, and no difference from controls in saving behavior, both results were the opposite of what we hypothesized; however, the difference seemed to be driven by a small subset of the sample and calls into question the role of the lesion in the increase in heroin intake, therefore we are unable to confidently ascertain the experimental catalyst for the differences between groups.

Opioids are a class of drugs that has been consumed for several millennia. Characterized by their mechanism of action, opioids bind to opioid receptors (μ, δ, or κ), which are normally activated by endogenous opioid peptides such as endorphins, enkephalins, and dynorphins. While opioids have served many purposes over the years, ranging from cough suppression to antinociception, they also provide the user with feelings of euphoria, making him or her susceptible to developing opioid use disorder. Despite their excellent analgesic properties, the propensity to overdose and/or develop opioid use disorder results in huge societal costs. While effective treatments for opioid

use disorder are available, collectively there is a long way to go in understanding the complex dynamic of factors contributing to the development and maintenance of OUD.

One of the criteria considered when diagnosing OUD, is when an individual continues opioid use despite having persistent or recurrent social or interpersonal problems caused or made worse by the effects of opioids. A plethora of research, going back decades, has examined the effects of drugs on the brain and how these effects impact social interactions and the environment. Themes that have persisted throughout the years are that drugs, even of different classes, impact decision making, impulsivity, and emotional functioning. A study by Kurtines et al in 1975 examined social functioning, including empathy, in a set of participants that included psychiatric in-patients, police officers, marijuana users, heroin users, and adolescents. There were significant differences in social emotional functioning between groups, with the heroin group scoring the lowest in established empathy measures. More recently, Kroll et al (2018) examined how prescription opioids impact empathy and found that individuals, who were chronic non-medical prescription opioid users, were less able to accurately categorize facial expressions into appropriate emotional categories and had other empathy-related deficits compared to opioid naïve controls. Further, along these lines, the types and quality of interpersonal relationships can impact the onset or maintenance of drug ingestion and addiction. Social functioning is important in recovery as well, individuals that have familial or peer support are more successful in completing treatment and maintaining abstinence than individuals without social support. These data, taken

together, show a real need to fully understand and be able to restore prosocial functioning if and when it is compromised by drug use.

Up until recently, a major criticism of rodent self-administration is that it fails to capture other aspects of the human condition that contribute to acquisition of drug use, such as social-emotional components. With this in mind, an exciting new avenue of research emerged when an animal model of empathy/prosocial behavior was described in 2011. Here, Peggy Mason and colleagues developed a clever paradigm in which a rat is trapped in a plastic restraint, and the trapped rat's cagemate is tasked with releasing the trapped rat. The rat will rescue its cagemate in place of obtaining food or some other palatable reward. The occurrence and latency of the rescue is observed and recorded. At first glance, this isn't necessarily convincing as a model of prosocial behavior, but Mason's lab went on to perform a series of experiments that ruled out many confounds and offered consistency with behaviors in human literature. Results of subsequent studies demonstrated that rats will release another rat they are familiar with versus an unfamiliar rat, and a rat of the same strain versus a rat of a different strain. These data are in line with in-group/out-group studies and bystander effect work that indicate humans are more likely to help someone they are familiar with versus a stranger and help someone who "looks" like them compared to someone who does not look like them. They also answered critics who claimed the rats were only rescuing the trapped rat for social interaction by designing a study in which rats were released into a distal chamber after being rescued, thus preventing any immediate reward of social interaction.

After discovery of this model, we decided to attempt to use Mason's rescuing paradigm as a metric for determining the effects of heroin on prosocial behavior (Tomek, et al, 2019). In our study, discussed in Chapter 2, rats established baseline rescuing behaviors, self-administered either heroin or sucrose, then were re-tested on their rescuing behavior while having concurrent access to their particular reinforcer. An important aspect of our design is that our rats were given the opportunity to rescue their cagemate in the same chamber where they self-administer heroin (in the next phase of the study). This not only allows the rat to have a dark, sound-attenuated area to either rescue (or not) their cagemate, it omits any potential contextual or environmental confounds for when they self-administer heroin. Results showed that rats who had self-administered heroin stopped saving their cagemate, whereas the sucrose rats continued to open the restraint door for their cagemate. We also utilized a sham surgery group whose catheter patency was compromised, and this group continued to rescue their cagemate at the same proportion of time as controls. These results may be evidence of heroin induced social deficits and a novel contribution to the field of addiction research. In order to confirm this, further investigation was necessary, with the addition of identifying potential neural substrates. Due to its role in both drug craving and social behaviors (Naqvi et al, 2007; Heilig et al, 2016; Franklin et al, 2002) the insula was selected as a potential region of interest.

The insula is a brain region located deep in the lateral sulcus, separating the frontal and parietal lobes from the temporal lobe. The insula has historically been studied

for its role in food aversion, homeostasis, and emotion, but recently, in large part due to seminal work by Nasir Naqvi at Columbia University; the insula has been an emerging brain region of interest in addiction research. In 2007, Naqvi et al published a paper describing the attenuation of craving and addiction to nicotine following an insular stroke. The insula is part of the limbic system and has reciprocal connectivity with brain regions that process emotional responses, decision making, and reward, as well as motor cortices. In recent years the insula has been the focus of many neuroimaging studies, identifying a host of different contexts in which the insula becomes activated. These studies include tasks like asking a participant to respond to facial expressions, vignettes, and other emotional stimuli in order to measure the occurrence of and brain regions involved in an empathic or prosocial response.

Considering established research demonstrating the importance of social interactions, the deleterious effects of opioids on social functioning (Kroll et al, 2018; Kurtines et al, 1975), and the implication of the insula in both behavioral phenomena, modulation of insula function and observing the outcomes was a natural progression. While study of stroke victims allows researchers to analyze years of data and numerous imaging studies, differences in data collection, analyses, and subjects results in sometimes contradictory results, but more importantly most of the results are correlational. In order to examine causal relationships between changes to the insula and prosocial behavior as they relate to drug intake, continuing with an animal model was necessary.

The studies discussed in Chapters 3 and 4 utilizing the rescuing paradigm described above used chemogenetics and excitotoxic lesions in order to examine the role of the insula in prosocial behaviors and opioid intake. First, we aimed to modulate the anterior insula with a state-of-the-art technique that uses DREADD's (designer receptors exclusively active by designer drugs) to either inhibit or excite pyramidal neurons in the insula. This process employs mutant stimulatory or inhibitory G-protein coupled receptors expressed by a viral vector, along with a specific promoter to provide cell type specificity as well as a fluorescent reporter protein. Once DREADD expression was achieved, we systemically administered an otherwise physiologically inert metabolite of clozapine called clozapine n-oxide (CNO) which causes increased or decreased activity of neurons expressing either an excitatory or inhibitory DREADD.

Much like the original heroin/sucrose social deficit study (Chapter 2), we established a baseline of rescuing behavior prior to any pharmacological interventions. After 21 days of rescuing (7 days of acquisition and 14 days of experimental criterion days for analyses), rats were allowed to self-administer heroin for 6 hours a day, 7 days a week, for 2 weeks. Prior to re-testing rescuing behavior, concurrent with heroin self-administration, rats were given IP injections of CNO 20 minutes before being placed into their operant chambers. In the first study examining effects of insula activation, all rats rescued less than they did prior to heroin intake, however, the rats infused with the virus expressing the excitatory DREADD rats showed rescuing behavior significantly more than controls. These results indicate a restoration of heroin-induced prosocial deficits. We

did not observe any significant differences between rats expressing the inhibitory DREADD and control animals with respect to heroin intake or rescuing behaviors.

As a result of our negative findings with the inhibitory DREADD, we decided to attempt a different technique to inhibit the insula in order to investigate whether we could disrupt heroin intake or heroin-induced social deficits. For this process, we opted to utilize quinolinic acid to lesion the AIC. Rotge (2017) recently published interesting (and promising) results in an AIC lesion study. These investigators lesioned rats with quinolinic acid before cocaine acquisition in one group, and after rats had already escalated cocaine intake in another group. Their findings showed that lesions induced prior to cocaine acquisition did not alter cocaine self-administration, but lesioning the rats after they had escalated cocaine self-administration slowed cocaine intake.

Our bilateral AIC lesion experiment (Chapter 4) investigated effects on heroin intake. In addition to examining overall intake of heroin during self-administration, we were also interested in how lesions would impact the rescuing behavior in the social deficit paradigm discussed in Chapters 2 and 3. We administered intercranial injections of phosphate buffered saline as a control group. While not expected, we observed interesting differences between our lesioned and control animals. In a pilot study, in which lesions inadvertently expanded past the AIC, results were similar to Rotge's work, in that lesioned rats slowed their heroin intake compared to controls. A replication of the study using less quinolinic acid resulted in smaller lesions and produced the opposite effect, where lesioned animals increased heroin self-administration compared to controls.

There were no differences between groups in prosocial rescuing behavior in either study. Even though our drug intake results were not consistent with the existing literature (Rotge, 2017, Naqvi, 2007) these findings still offer information regarding the role of the insula as a gatekeeper in drug-dependent behaviors, particular with respect to opioids versus psychostimulants.

As with all experimental designs, there are limitations to our research. In addition to some initial catheter patency issues and cohort differences in baseline rescuing behavior, the fine-tuning of the lesions offered a set of challenges that resulted in different lesion sizes. Utilizing excitotoxic lesions is an effective technique that allows a researcher to purposefully cause neuronal cell death, but when considering a cause and effect relationship, being able to keep a more precise size and locale in the desired brain region may increase likelihood that yielded results are accurate and interpretable.. Chemogenetics may be a strategy that allows us to separate these differences in a more controlled way, However, chemogenetics offered its own technical challenges with an unclear degree of cessation of neuronal activity, or induction of neural activity that is physiologically relevant. Although previous research indicated CNO to be physiologically inert, CNO might potentially reverse metabolize into clozapine (Chen et al. 2015; MacLaren et al. 2016; Thompson et al. 2018), and result in behavior changes independent of DREADD expression, which is an important limitation to acknowledge.

Despite these limitations, the observation that heroin impacted rescuing behavior in each of our three studies, and that manipulating the insula resulted in some behavior

effects, merits some avenues for future research. At the very least, these observations demonstrate the need to employ different experimental strategies when modulating the insula. Strategies should aim to identify how and what proportion of the AIC needs to be activated or inhibited in order to result in altered drug intake. Once that mechanism is better understood, targeted medications or other neuromodulatory approaches can potentially be developed to help individuals control drug intake. Other potential approaches include deep brain stimulation, recently being used in Europe and Asia as an addiction treatment. In these studies, researchers examine the effects of implanting a microchip into the nucleus accumbens (Wang et al, 2018; Qu et al, 2019; Ho et al, 2018). However, given the studies described herein future studies should consider extending this to include portions of the insula. And lastly, future directions should use these paradigms to investigate the long-term changes in prosociability after heroin use. Investigating the effects of withdrawal, opioid replacement treatments (e.g., buprenorphine), and even just the passage of time on restoration of prosocial behaviors would provide insight into the type and quality of changes that may be occurring in the brain, as well as elucidate other advances in designing treatment in the context of these findings.

REFERENCES

Abadi, A. H. A., Miladi-Gorji, H., & Bigdeli, I. (2016). Effect of environmental enrichment on physical and psychological dependence signs and voluntary morphine consumption in morphine-dependent and morphine-withdrawn rats. *Behavioural Pharmacology*, *27*(2–3), 270–278.

Abram, S. V., Wisner, K. M., Grazioplene, R. G., Krueger, R. F., MacDonald, A. W., & DeYoung, C. G. (2015). Functional coherence of insula networks is associated with externalizing behavior. *Journal of Abnormal Psychology*, *124*(4), 1079–1091.

Al-Hasani, R., & Bruchas, M. R. (2011). Molecular Mechanisms of Opioid Receptor-Dependent Signalling and Behaviour. *Anesthesiology*, *115*(6), 1363–1381.

Alexander, B. K., Beyerstein, B. L., Hadaway, P. F., & Coambs, R. B. (1981). Effect of early and later colony housing on oral ingestion of morphine in rats. *Pharmacology, Biochemistry and Behavior*, *15*(4), 571–576.

Ansell, E. B., Rando, K., Tuit, K., Guarnaccia, J., & Sinha, R. (2012). Cumulative Adversity and Smaller Gray Matter Volume in Medial Prefrontal , Anterior Cingulate , and Insula. *BPS*, *72*(1), 57–64.

Badiani, A., Belin, D., Epstein, D., Calu, D., and Shaham, D. (2011). Opiate versus psychostimulant addiction: the differences do matter.

Bardo, M. T., Neisewander, J. L., & Pierce, R. C. (1989). Novelty-induced place preference behavior in rats: Effects of opiate and dopaminergic drugs. *Pharmacology, Biochemistry and Behavior*, *32*(3), 683–689.

Bates, M. L. S., Emery, M. A., Wellman, P. J., & Eitan, S. (2014). Social housing conditions influence morphine dependence and the extinction of morphine place preference in adolescent mice. *Drug and Alcohol Dependence*, *142*, 283–289.

Bates, M. L. S., Emery, M. A., Wellman, P. J., & Eitan, S. (2014). Social housing conditions influence morphine dependence and the extinction of morphine place preference in adolescent mice. *Drug and Alcohol Dependence*, *142*, 283–289.

Belin-Rauscent, A., Belin, A. (2011). Animal Models of Drug Addiction. *Addictions - From Pathophysiology to Treatment*, *53*, 21–63.

Belin-Rauscent, A., Fouyssac, M., Bonci, A., & Belin, D. (2016). How preclinical models evolved to resemble the diagnostic criteria of drug addiction. *Biological Psychiatry*, *79*(1), 39–46

Beloate, L. N., & Coolen, L. M. (2017). Influences of social reward experience on behavioral responses to drugs of abuse: Review of shared and divergent neural plasticity mechanisms for sexual reward and drugs of abuse. *Neuroscience and Biobehavioral Reviews*, *83*(October), 356–372.

Ben-Ami Bartal, I., Decety, J., & Mason, P. (2011). Empathy and Pro-Social Behavior in Rats. *Source: Science, New Series*, *334*(60619), 1427–1430.

Ben-Ami Bartal, I., Rodgers, D. A., Bernardez Sarria, M. S., Decety, J., & Mason, P. (2014). Pro-social behavior in rats is modulated by social experience. *eLife*, *3*.

Ben-Ami Bartal, I., Shan, H., Molasky, N. M. R., Murray, T. M., Williams, J. Z., Decety, J., & Mason, P. (2016). Anxiolytic Treatment Impairs Helping Behavior in Rats. *Frontiers in Psychology*, *7*, 850.

Blaine, J. D., National Institute on Drug Abuse. (1992). *Buprenorphine, an alternative treatment for opioid dependence.* Rockville, MD (5600 Fishers Lane, Rockville 20857): U.S. Dept. of Health and Human Services, Public Health Service, Alcohol, Drug Abuse, and Mental Health Administration, National Institute on Drug Abuse .

Bodnar, R. J. (2013). Peptides Endogenous opiates and behavior : 2012. *Peptides*, *50*(January), 55–95.

Bohnert, A. S. B., Bradshaw, C. P., & Latkin, C. A. (2009). A social network perspective on heroin and cocaine use among adults: evidence of bidirectional influences. *Addiction*, *104*(7), 1210–1218.

Bohnert, A. S. B., Bradshaw, C. P., & Latkin, C. A. (2009). A social network perspective on heroin and cocaine use among adults: evidence of bidirectional influences. *Addiction*, *104*(7), 1210–1218.

Bond, A. J., & Witton, J. (2017). Perspectives on the Pharmacological Treatment of Heroin Addiction. *Clinical Medicine Insights: Psychiatry*, *8*,

Bozarth, M. A., Murray, A., & Wise, R. A. (1987). Influence of Housing Conditions on the Acquisition of Intravenous Heroin and Cocaine Self-Administration in Rats. *Pharmacology Biochemistry & Behavior*, *33*, 903–907.

Buchanan, A. S., & Latkin, C. A. (2008). Drug use in the social networks of heroin and cocaine users before and after drug cessation. *Drug and Alcohol Dependence*, *96*(3), 286–289.

Carroll, M. E., & Boe, I. N. (1982). Increased intravenous drug self-administration during deprivation of other reinforcers. *Pharmacology, Biochemistry and Behavior*, *17*(3), 563–567.

Chen, S. A., O'Dell, L. E., Hoefer, M. E., Greenwell, T. N., Zorrilla, E. P., & Koob, G. F. (2006). Unlimited access to heroin self-administration: Independent motivational markers of opiate dependence. *Neuropsychopharmacology*, *31*(12), 2692–2707.

Ciccarone, D., Ondocsin, J., & Mars, S. G. (2017). Heroin uncertainties: Exploring users' perceptions of fentanyl-adulterated and -substituted "heroin." *International Journal of Drug Policy*, *46*, 146–155.

Cicero, T. J., & Ellis, M. S. (2017). The prescription opioid epidemic: A review of qualitative studies on the progression from initial use to abuse. *Dialogues in Clinical Neuroscience*, *19*(3), 259–269.

Compton, W. M., Jones, C. M., & Baldwin, G. T. (2016). Relationship between Nonmedical Prescription-Opioid Use and Heroin Use. *New England Journal of Medicine*, *374*(2), 154–163.

Conrad, C. D., Ortiz, J. B., & Judd, J. M. (2017). Chronic stress and hippocampal dendritic complexity: Methodological and functional considerations. *Physiology and Behavior*, *178*, 66–81.

Contreras, M., Billeke, P., Vicencio, S., Madrid, C., Perdomo, G., González, M., & Torrealba, F. (2012a). A Role for the Insular Cortex in Long-Term Memory for Context-Evoked Drug Craving in Rats. *Neuropsychopharmacology*, *37*(9), 2101–2108.

Cooper, A., Barnea-Ygael, N., Levy, D., Shaham, Y., & Zangen, A. (2007). A conflict rat model of cue-induced relapse to cocaine seeking. *Psychopharmacology*, *194*(1), 117–125.

Cortices, T., Franklin, T. R., Acton, P. D., Maldjian, J. A., Gray, J. D., Croft, J. R., … Childress, A. R. (2002). Decreased Gray Matter Concentration in the Insular , Cocaine Patients. *Biological Psychiatry*, *51*(1), 134–142.

Courtwright, D. T. (1982). *Dark paradise: A history of opiate addiction in America.* Harvard University Press.

Coventry, T. L., D'Aquila, P. S., Brain, P., & Willner, P. (1997). Social influences on morphine conditioned place preference. *Behavioural Pharmacology*, *8*(6), 575–584.

Dai S, Corrigall WA, Coen KM. (1989) Heroin self-administration by rats: influence of dose and physical dependence. *Pharmacol Biochem Behav*;32:1009-15.

de Wit, H., & Sayette, M. (2018). Considering the context: social factors in responses to drugs in humans. *Psychopharmacology*, 1–11.

Department of Health, U., Services, H., & for Disease Control, C. (2017). *Annual Surveillance Report Of Drug-related Risks And Outcomes*. Retrieved from https://www.cdc.gov/drugoverdose/pdf/pubs/2017-cdc-drug-surveillance-report.pdf

Donny, E. C., Brasser, S. M., Bigelow, G. E., Stitzer, M. L., & Walsh, S. L. (2005). Methadone doses of 100 mg or greater are more effective than lower doses at suppressing heroin self-administration in opioid-dependent volunteers. *Addiction*, *100*(10), 1496–1509.

Droutman, V., Read, S. J., & Bechara, A. (2015). *Revisiting the role of the insula in addiction*.

Eitan, S., Emery, M. A., Bates, M. L. S., & Horrax, C. (2017). Opioid addiction: Who are your real friends? *Neuroscience and Biobehavioral Reviews*, *83*(July 2016), 697–712.

El Rawas, R., Thiriet, N., Lardeux, V., Jaber, M., & Solinas, M. (2009). Environmental enrichment decreases the rewarding but not the activating effects of heroin. *Psychopharmacology*, *203*(3), 561–570.

Fan, Y., Duncan, N. W., de Greck, M., & Northoff, G. (2011). Is there a core neural network in empathy? An fMRI based quantitative meta-analysis. *Neuroscience and Biobehavioral Reviews*.

Galaj, E., Manuszak, M., & Ranaldi, R. (2016). Environmental enrichment as a potential intervention for heroin seeking. *Drug and Alcohol Dependence*, *163*, 195–201.

Gladden, R. M., O'Donnell, J., Mattson, C. L., & Seth, P. (2019). Changes in Opioid-Involved Overdose Deaths by Opioid Type and Presence of Benzodiazepines, Cocaine, and Methamphetamine — 25 States, July–December 2017 to January–June 2018. *MMWR. Morbidity and Mortality Weekly Report*, *68*(34), 737–744.

Goldberg J, L. D. (2014). *Flowers in the blood: the story of opium.* New York: Skyhorse Publishing.

Griffith, J., Knight, D., Joe, G., & Simpson, D. (1998). Implications of Family and Peer Relations for Treatment Engagement and Follow-Up Outcomes: An Integrative Model. *Psychol Addict Behav*, *12*(2), 113–126.

Haydari, S., Miladi-Gorji, H., Mokhtari, A., & Safari, M. (2014). Effects of voluntary exercise on anxiety-like behavior and voluntary morphine consumption in rat pups borne from morphine-dependent mothers during pregnancy. *Neuroscience Letters*, *578*, 50–54.

Heilig, M., Epstein, D. H., Nader, M. A., & Shaham, Y. (2016a). Time to connect: bringing social context into addiction neuroscience. *Nature Publishing Group*, *17*.

Hofford, R. S., Schul, D. L., Wellman, P. J., & Eitan, S. (2012). Social influences on morphine sensitization in adolescent rats. *Addiction Biology*, *17*(3), 547–556.

Hölscher, F., Reissner, V., Di Furia, L., Room, R., Schifano, F., Stohler, R., … Scherbaum, N. (2010). Differences between men and women in the course of opiate dependence: Is there a telescoping effect? *European Archives of Psychiatry and Clinical Neuroscience*, *260*(3), 235–241.

Johnson, S. W., & North, R. A. (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *12*(2), 483–488.

Jones, C. M., Campopiano, M., Baldwin, G., & McCance-Katz, E. (2015). National and State Treatment Need and Capacity for Opioid Agonist Medication-Assisted Treatment. *American Journal of Public Health*, *105*(8), e55–e63.

Jones, C. M., Logan, J., Gladden, R. M., & Bohm, M. K. (2015). Vital Signs: Demographic and Substance Use Trends Among Heroin Users - United States, 2002-2013. *MMWR. Morbidity and Mortality Weekly Report*, *64*(26), 719–725.

Jones, J. D., & Comer, S. D. (2013). A review of human drug self-administration procedures. *Behavioural Pharmacology*, *24*(5–6), 384–395.

Kapoor, L. D. (1995). *Opium poppy: botany, chemistry and pharmacology*. Binghampton, New York: Haworth Press.

Kennedy, B. C., Panksepp, J. B., Runckel, P. A., & Lahvis, G. P. (2012). Social influences on morphine-conditioned place preference in adolescent BALB/cJ and C57BL/6J mice. *Psychopharmacology*, *219*(3), 923–932.

Koob, G. F. (2008a). A role for brain stress systems in addiction. *Neuron*, *59*(1), 11–34.

Koram, N., Liu, H., Li, J., Li, J., Luo, J., & Nield, J. (2011). Role of social network dimensions in the transition to injection drug use: Actions speak louder than words. *AIDS and Behavior*, *15*(7), 1579–1588.

Kroll, S., Nikolic, E., Bieri, F., Soyka, M., Baumgardner, M., Quednow, B. (2018). Cognitive and socio-cognitive functioning of chronic non-medical prescription opioid users. *Psychopharmacology*, *235*(12), 3451–3464.

Kurtines, W., Hogan, R., & Weiss, D. (1975). Personality Dynamics of Heroin Use. *Journal of Abnormal Psychology*, *84*(1), 87–89.

Lu, L., Shepard, J. D., Hall, F. S., & Shaham, Y. (2003). Effect of environmental stressors on opiate and psychostimulant reinforcement, reinstatement and discrimination in rats: A review. *Neuroscience and Biobehavioral Reviews*, *27*(5), 457–491.

Luthar, S. S., Anton, S. F., Merikangas, K. R., & Rounsaville, B. J. (1992). Vulnerability to drug abuse among opioid addicts' siblings: Individual, familial, and peer influences. *Comprehensive Psychiatry*, *33*(3), 190–196.

Mackey, S., & Paulus, M. (2013). Are there volumetric brain differences associated with the use of cocaine and amphetamine-type stimulants? *Neuroscience and Biobehavioral Reviews*, *37*(3), 300–316.

Maher, C. E., Martin, T. J., & Childers, S. R. (2005). Mechanisms of mu opioid receptor/G-protein desensitization in brain by chronic heroin administration. *Life Sciences*, *77*(10), 1140–1154.

Milekic, M. H. (2006). Persistent Disruption of an Established Morphine Conditioned Place Preference. *Journal of Neuroscience*, *26*(11), 3010–3020.

Namba, M. D., Tomek, S. E., Olive, M. F., Beckmann, J. S., & Gipson, C. D. (2018). The winding road to relapse: Forging a new understanding of cue-induced reinstatement models and their associated neural mechanisms. *Frontiers in Behavioral Neuroscience*, *12*.

Namkung, H., Kim, S.-H., & Sawa, A. (2017). The Insula: An Underestimated Brain Area in Clinical Neuroscience, Psychiatry, and Neurology. *Trends in Neurosciences*, *40*(4), 200–207.

Naqvi, N. H., Gaznick, N., Tranel, D., & Bechara, A. (2014). The insula: a critical neural substrate for craving and drug seeking under conflict and risk. *Annals of the New York Academy of Sciences*, *1316*(1), 53–70.

Naqvi, N., Rudrauf, D., Damasio, H., & Bechara, A. (2007). Damage to the Insula Disrupts Addition to Cigarette Smoking. *Science*, *315*(26), 531–535.

Neisewander, J. L., Peartree, N. A., & Pentkowski, N. S. (2012). Emotional valence and context of social influences on drug abuse-related behavior in animal models of social stress and prosocial interaction. *Psychopharmacology*, *224*(1), 33–56.

Neuroscience, H., Sliz, D., & Hayley, S. (2012). *Major depressive disorder and alterations in insular cortical activity : a review of current functional magnetic imaging research*. *6*(December), 1–14.

Olive, M. F. (2017). *Understanding Drugs: Heroin ,*. New York: Chelsea House Publishers.

Ostling, P. S., Davidson, K. S., Anyama, B. O., Helander, E. M., Wyche, M. Q., & Kaye, A. D. (2018a). America's Opioid Epidemic: a Comprehensive Review and Look into the Rising Crisis. *Current Pain and Headache Reports*, *22*(5), 32.

Panlilio, L. V., & Goldberg, S. R. (2007). Self-administration of drugs in animals and humans as a model and an investigative tool. *Addiction*, *102*(12), 1863–1870.

Peck, J., Galaj, E., Eshak, S., Newman, K, & Ranaldi, R. (2015). Environmental enrichment induces early heroin abstinence in an animal conflict model. *Pharmacology Biochemistry and Behavior*, *138*, 20–25.

Pooriamehr, A., Sabahi, P., & Miladi-Gorji, H. (2017). Effects of environmental enrichment during abstinence in morphine dependent parents on anxiety, depressive-like behaviors and voluntary morphine consumption in rat offspring. *Neuroscience Letters*, *656*, 37–42.

Raz, S., & Berger, B. D. (2010). Social isolation increases morphine intake: Behavioral and psychopharmacological aspects. *Behavioural Pharmacology*, *21*(1), 39–46.

Rech, R. H., Mokler, D. J., & Briggs, S. L. (2012). Effects of combined opioids on pain and mood in mammals. *Pain Research and Treatment*, *2012*.

Ribeiro Do Couto, B., Aguilar, M. A., Manzanedo, C., Rodríguez-Arias, M., Armario, A., & Miñarro, J. (2006). Social stress is as effective as physical stress in reinstating morphine-induced place preference in mice. *Psychopharmacology*, *185*(4), 459–470.

Robinson, T. E., & Berridge, K. C. (2000). Animal models in craving research. The psychology and neurobiology of addiction: an incentive – sensitization view. *Addiction*, *95*(2), 91–117.

Rotge, J.-Y., Cocker, P. J., Daniel, M.-L., Belin-Rauscent, A., Everitt, B. J., & Belin, D. (2017). *Bidirectional regulation over the development and expression of loss of control over cocaine intake by the anterior insula*.

Rudd, R. A. (2016). Increases in drug and opioid-involved overdose deaths—United States, 2010–2015. *MMWR.Morbidity and Mortality Weekly Report*, *65*, 2010–2015.

Russell, B. S., Trudeau, J. J., & Leland, A. J. (2015). Social Influence on Adolescent Polysubstance Use: The Escalation to Opioid Use. *Substance Use and Misuse*, *50*(10), 1325–1331.

Substance Abuse and Mental Health Services Administration, Center for Behavioral Health Statistics and Quality. *Treatment Episode Data Set (TEDS): 2004-2014. National Admissions to Substance Abuse Treatment Services. BHSIS Series S-84, HHS Publication No. (SMA) 16-4986*. Rockville, MD: Substance Abuse and Mental Health Services Administration; 2016.

Sato, N., Tan, L., Tate, K., & Okada, M. (2015). Rats demonstrate helping behavior toward a soaked conspecific. *Animal Cognition*, 1039–1047.

Schenk, S., Hunt, T., Colle, L., & Amit, Z. (1982). Isolation versus grouped housing in rats: Differential effects of low doses of heroin in the place preference paradigm. *Life Sciences*, *32*, 1129–1134.

Scherbaum, N., & Specka, M. (2008). Factors influencing the course of opiate addiction. *International Journal of Methods in Psychiatric Research*, *17*(S1), S39–S44.

Schwartz, L. P., Silberberg, A., Casey, A. H., Kearns, D. N., & Slotnick, B. (2017). Does a rat release a soaked conspecific due to empathy? *Animal Cognition*, *20*(2), 299–308.

Seip, K. M., Reed, B., Ho, A., & Kreek, M. J. (2012). Measuring the incentive value of escalating doses of heroin in heroin-dependent Fischer rats during acute spontaneous withdrawal.

Seip-Cammack, K.M., Reed, B., Zhang, Y., Ho, A., Kreek, M.J. (2013). Tolerance and sensitization to chronic escalating-dose heroin following extended withdrawal in Fischer rats: A possible role of mu-opioid receptors. *Psychopharmacology.* 225(1), 127-140

Shaham, Y., Shalev, U., Lu, L., De Wit, H., & Stewart, J. (2003). The reinstatement model of drug relapse: History, methodology and major findings. *Psychopharmacology*, *168*(1–2), 3–20.

Sherman, S. G., Smith, L., Laney, G., & Strathdee, S. A. (2002). Social influences on the transition to injection drug use among young heroin sniffers: A qualitative analysis. *International Journal of Drug Policy*, *13*(2), 113–120.

Silberberg, A., Allouch, C., Sandfort, S., Kearns, D., Karpel, H., & Slotnick, B. (2014). Desire for social contact, not empathy, may explain "rescue" behavior in rats. *Animal Cognition*, *17*(3), 609–618.

Solinas, M., Chauvet, C., Thiriet, N., El Rawas, R., & Jaber, M. (2008). Reversal of cocaine addiction by environmental enrichment. *Proceedings of the National Academy of Sciences*, *105*(44), 17145–17150.

Solinas, M., Thiriet, N., Chauvet, C., & Jaber, M. (2010). Prevention and treatment of drug addiction by environmental enrichment. *Progress in Neurobiology*, *92*(4), 572–592.

Stanton, M. D., & Shadish, W. R. (1997). Outcome, attrition, and family-couples treatment for drug abuse: A meta-analysis and review of the controlled, comparative studies. *Psychological Bulletin*, *122*(2), 170–191.

Stein, C. (2016). Opioid Receptors. *Annual Review of Medicine*, *67*(1), 433–451.

Steketee, J. D., & Kalivas, P. W. (2011). Drug Wanting: Behavioral Sensitization and Relapse to Drug-Seeking Behavior. *Pharmacological Reviews*, *63*(2), 348–365.

Tomek, S. E., Stegmann, G. M., & Olive, M. F. (2019). Effects of heroin on rat prosocial behavior. *Addiction Biology*, *24*(4), 676–684.

Torabi, M., Pooriamehr, A., Bigdeli, I., & Miladi-Gorji, H. (2017). Maternal swimming exercise during pregnancy attenuates anxiety/depressive-like behaviors and voluntary morphine consumption in the pubertal male and female rat offspring born from morphine dependent mothers. *Neuroscience Letters*, *659*(August), 110–114.

Tzschentke, T. M. (2007). Measuring reward with the conditioned place preference (CPP) paradigm: Update of the last decade. *Addiction Biology*, *12*(3–4), 227–462.

Varghese, S., Montalvo-Ortiz, J., Csernansky, J., Eiger, R., Herrold, A., Koola, M., & Dong, H. (2015). Early life stress as a risk factor for substance use disorders: Clinical and neurobiological substrates. *Indian Journal of Psychological Medicine*, *37*(1), 36.

Vendruscolo, L. F., Schlosburg, J. E., Misra, K. K., Chen, S. A., Greenwell, T. N., & Koob, G. F. (2011). Escalation patterns of varying periods of heroin access. *Pharmacol Biochem Behav*; 98: 570-4

Walsh, S. L., Comer, S. D., Lofwall, M. R., Vince, B., Levy-Cooperman, N., Kelsh, D., … Kim, S. (2017). Effect of buprenorphine weekly depot (CAM2038) and hydromorphone blockade in individuals with opioid use disorder: A randomized clinical trial. *JAMA Psychiatry*, *74*(9), 894–902.

Wasserman, D. A., Stewart, A. L., & Delucchi, K. L. (2001). Social support and abstinence from opiates and cocaine during opioid maintenance treatment. *Drug and Alcohol Dependence*, *65*(1), 65–75.

Watterson, L. R., Kufahl, P. R., Nemirovsky, N. E., Sewalia, K., Grabenauer, M., Thomas, B. F., … Olive, M. F. (2014). Potent rewarding and reinforcing effects of the synthetic cathinone 3,4-methylenedioxypyrovalerone (MDPV). *Addiction Biology*, *19*(2), 165–174.

Weiss, F. (2005). Neurobiology of craving, conditioned reward and relapse. *Current Opinion in Pharmacology*, *5*(1), 9–19.

What are the immediate (short-term) effects of heroin use? | National Institute on Drug Abuse (NIDA). (2017). Retrieved April 7, 2017, from https://www.drugabuse.gov/publications/research-reports/heroin/what-are-immediate-short-term-effects-heroin-use

Williams, C. T., & Latkin, C. A. (2007). Neighborhood Socioeconomic Status, Personal Network Attributes, and Use of Heroin and Cocaine. *American Journal of Preventive Medicine*, *32*(6 SUPPL.), 203–210.

Xigeng, Z., Yonghui, L., Xiaojing, L., Lin, X., Dongmei, W., Jie, L., … Nan, S. (2004). Social crowding sensitizes high-responding rats to psychomotor-stimulant effects of morphine. *Pharmacology Biochemistry and Behavior*, *79*(2), 213–218.

Xu, Z., Hou, B., Gao, Y., He, F., & Zhang, C. (2007). Effects of enriched environment on morphine-induced reward in mice. *Experimental Neurology*, *204*(2), 714–719.

Zorrilla, E. P., Logrip, M.L., Koob, G. F. (2014). *Corticotropin Releasing Factor: A Key Role in the Neurobiology of Addiction*. *35*(2), 234–244.

APPENDIX A

PHARMACOLOGY AND TOXICOLOGY OF SYNTHETIC CATHINONES

Burrows, B.T., **Tomek, S.E**., Nagy, E, Popeski, C., Olive, M.F. (2017). Pharmacology and Toxicology of Synthetic Cathinones (“Bath Salts”) *Horizons in Neuroscience* 33, 103-129

Abstract: Synthetic cathinones, frequently referred to as "bath salts," are amphetamine-like psychostimulants that emerged onto drug markets in the late 2000's as "legal" alternatives to illicit stimulants such as cocaine and amphetamines. While their pharmacological mechanisms of action are similar to those of the drugs they are intended to mimic, their adverse psychiatric effects can be more severe and result in agitated delirium, psychosis, and violent behaviors. These problems are further complicated by a constantly shifting landscape of newer cathinone analogues designed to circumvent legislative control efforts. In this chapter, we will review the known pharmacological mechanisms of action of first- and second generation Synthetic cathinones in the central nervous system in comparison to those of cocaine and amphetamines. We will also review their use patterns, adverse psychological and physiological effects, and recent studies that have assessed the potential toxic effects of Synthetic cathinones as well as their ability to induce neuroinflammation and cognitive dysfunction.

APPENDIX B

THE WINDING ROAD TO RELAPSE: FORGING A NEW UNDERSTANDING OF CUE-INDUCED REINSTATEMENT MODELS AND THEIR ASSOCIATED NEURAL MECHANISMS.

Namba, M.D., **Tomek, S.E.,** Olive, M.F., Beckmann J.S., Gipson, C.D. (2018). The Winding Road to Relapse: Forging a New Understanding of Cue-Induced Reinstatement Models and Their Associated Neural Mechanisms. *Frontiers in behavioral neuroscience* 12, 17

Abstract: In drug addiction, cues previously associated with drug use can produce craving and frequently trigger the resumption of drug taking in individuals vulnerable to relapse. Environmental stimuli associated with drugs or natural reinforcers can become reliably conditioned to increase behavior that was previously reinforced. In preclinical models of addiction, these cues enhance both drug self-administration and reinstatement of drug seeking. In this review, we will dissociate the roles of conditioned stimuli as reinforcers from their modulatory or discriminative functions in producing drug-seeking behavior. As well, we will examine possible differences in neurobiological encoding underlying these functional differences. Specifically, we will discuss how models of drug addiction and relapse should more systematically evaluate these different types of stimuli to better understand the neurobiology underlying craving and relapse. In this way, behavioral and pharmacotherapeutic interventions may be better tailored to promote drug use cessation outcomes and long-term abstinence.