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EFFECTS OF SOCIAL INTERACTION ON MORPHINE CONDITIONED PLACE PREFERENCE IN ADOLESCENT MALE RATS

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DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

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ABSTRACT OF DISSERTATION

EFFECTS OF SOCIAL INTERACTION ON MORPHINE CONDITIONED PLACE PREFERENCE IN ADOLESCENT MALE RATS

The fact that adolescents commonly initiate drug use in social settings is well established. Both clinical and preclinical research has investigated how social interaction is altered by a variety of drugs of abuse. What is less understood is how the rewarding value of drugs of abuse is affected by the presence of social peers. This dissertation aimed to investigate the interaction of morphine and social play on conditioned place preference (CPP) in adolescent male Sprague Dawley rats, using both behavioral and immunohistochemistry (IHC) methods. Rats were exposed to morphine (0, 1, or 3 mg/kg; s.c.), social interaction, or a combination of both and tested in a modified CPP procedure. Behavioral results indicate that, while doses of morphine used produced only weak CPP across experiments, they were sufficient to reduce the rewarding effect of social interaction. IHC results suggest that this finding may be due to reduced activation in NAc shell. Taken together, the results of this dissertation may help to provide an explanation as to why persons with opioid use disorder spend less time interacting with social peers, compared to non-dependent persons.

KEYWORDS: Morphine, Conditioned Place Preference, Social Interaction, Adolescence, Rats

Virginia G. Weiss
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CHAPTER 1

Introduction

In 2014, over 47,000 people in the United States died due to an opioid overdose, including 3907 persons under the age of 25 (Rudd, 2016). This is a 200% increase from the year 2000, leading opioids to account for 61% of all overdose fatalities. The 2015 National Survey on Drug Use and Health (SAMHSA, 2016) estimated that 97.5 million people age 12 years and older in the United States have used prescription pain relievers in the past year. Alarmingly, within this population, 53.7% said that they obtained the pain relievers from a friend or relative, while only 34% received a prescription from a doctor. In fact, of those who misuse a prescription, there is a 3-in-5 chance that the drug is an opioid. The most common opioid used in the United States is hydrocodone, which an astonishing 58.3 million people used at least once in the last year (accounting for 21.8% of the population). Furthermore, an estimated 828,000 people age 12 years and older used heroin in the past year (SAMHSA, 2016). Perhaps most worrying of all, however, is that among adolescents age 12-17 years old, it is estimated that 276,000 misuse prescription opioids (SAMHSA, 2016). The impact of the misuse of opioids in the United States goes far beyond those addicted and their families. The cost to society of opioid addiction has been estimated to be over $78.5 billion (Florence et al., 2016). This sum includes not only the cost of health care, but also treatment, criminal justice, and loss of productivity.

These drastic statistics about the misuse of prescription opioids, and the fatalities associated with them, has led the Center on Disease Control to proclaim an “opioid

epidemic," as well as the introduction of "prescription opioid and heroin epidemic awareness week" in September by Former President Obama (The White House, 2016). Legislation to decrease the number of prescriptions written to those without a medical need has led to the development of Prescription Drug Monitoring Programs (PDMPs). These PDMPs are regulated by each state with the purpose of collecting, monitoring, and analyzing electronic prescription and dispensing data that has been submitted by pharmacies and practitioners.

Although PDMPs are helpful in decreasing the probability that someone being prescribed a pain reliever for the first time will become addicted, they do not solve the problem that an estimated 20 million people aged 12 years and older have a opioid use disorder (SAMHSA, 2016). Furthermore, while there are several FDA-approved pharmacotherapies for opioid use disorder (e.g., methadone and buprenorphine), only about 14% of people who meet criteria for substance abuse therapy receive treatment (SAMHSA, 2016). Even within the population that receives treatment, approximately 40-60% of people will relapse (NIDA, 2014).

Many factors affect the likelihood that a therapeutic treatment will be effective, as measured by abstinence. Among these factors, evidence indicates that maintaining abstinence is best achieved when the patient avoids the drug-associated environment experienced before rehabilitation (Kelly et al., 2014). This stems from the fact that drug cues play a role in craving and relapse (O'Brien et al., 1992; Kosten et al., 2006). These cues can be both contextual (neighborhood) and discrete (drug paraphernalia). In addition to these inanimate cues, considerable evidence indicates that affiliation with drug-using social peers also plays an important role in relapse (Brown et al., 1989;

Brewer et al., 1998; Beattie, 2001; Sun, 2007). Compared to adults, adolescents are significantly more likely to relapse in the presence of peers (Ramo and Brown, 2008). Furthermore, the relationship with social peers appears to play a large role in relapse, as recovering addicts are more likely to report relapse when they are in the presence of a friend that they knew prior to treatment, compared to a friend they made following treatment (Brown et al., 1989). Given that relapse rates among recovering persons is so high, it is important to investigate factors that could potentially decrease relapse rate, whether they are pharmacotherapies or behavioral therapies.

Historical Context

In order to understand how our society ended up in the troubling position that we are in regarding the opioid epidemic, it is important to understand the general history of opioids within society, both in the medical field as well as in recreational use and abuse. A detailed history has been described previously (Levinthal, 2010). Although it is well accepted that the opium poppy has been used for thousands of years for its medicinal properties, it was not until the early 1500s that opium began to be thought of as a therapeutic drug by physicians in Europe. During that time, an opium drink became popular, and was recommended as a treatment for practically every known disease. In the late 1700s, Britain started using opium as a major trade item with China (whose residents had taken up the practice of smoking opium). This led to the widespread use of opium within the Chinese population, and opium dependence soon became a major social problem. In 1839, the Opium War began between China and Western countries, ending in China signing over Hong Kong and legalizing opium within its borders. Within

Britain, opium was commonly consumed within a drink, or in medicines such as advertised to treat toothaches, coughs, diarrhea, colic, or merely to keep infants and children quiet. Smoking opium, on the other hand, was viewed as barbaric (Levinthal, 2010).

Meanwhile, in Germany, the primary active ingredient in opium was isolated in 1803. It was given the name morphine after the Greek god of dreams, Morpheus. Morphine makes up only 10% of the total weight of opium, but was found to be 10 times stronger. Morphine quickly became the main ingredient in many opioid-containing medicines. In 1856, Dr. James Wood developed the hypodermic syringe, allowing opioids to be directly injected into the blood stream. This timing was particularly important given the upcoming Civil War in the United States, and allowed wounded soldiers more instant relief than they would have received through oral administration of opioids, which have to undergo metabolism within the gastrointestinal system prior to having any analgesic effects. Of course, this more direct route of administration also led to more soldiers becoming dependent on opioids and remaining so in the years that followed their return home. With increasing concerns about opioid dependence, the Bayer Company (known today primarily for aspirin) introduced heroin to the market in 1898. The company marketed this new drug as free from the dependence-causing properties, even though heroin is more potent than morphine. The abuse potential of heroin was not realized until 1910. This realization occurred because morphine addicts discovered that the euphoric effects of heroin could be enhanced by intravenous injection (Hosztafi, 2001).

In the U.S., opioid consumption closely paralleled that in Britain. Opium drinks and medicine were commonly used among middle and upper class citizens, with a discrimination of opium smoking due to anti-Chinese prejudice seen within the West when men were brought in to help build railroads in the 1850s. However, by 1900, opioid dependence among citizens was so great that it was causing a significant disruption in American society. Opioids were no longer seen as acceptable for everyday use, and a movement to implement government regulation began to grow.

The Harrison Act of 1914 was the first step in changing how opioids and their abusers were treated in the United States. The Harrison Act did not originally make opioids illegal, but rather imposed a tax on physicians who wished to prescribe them to their patients. It was not until 1920 that the Supreme Court prohibited physicians from prescribing opioids for nonmedical use. While prohibition decreased prescriptions, those already addicted to opioids were now forced to obtain opioids illegally, which led to a rise in black market heroin sales. Furthermore, without a legal way to obtain heroin, the price increased up to 50-fold.

Although this legislation significantly changed the social stigma surrounding heroin use, and use was relatively low, heroin came back in popularity in the 1960s and 1970s. This newfound popularity was, in part, due to the "hippie" culture that was flourishing. This time was filled with unconventional fashions and anti-establishment attitudes. In unprecedented numbers, middle and upper class people experimented with illegal drugs to get "high," thus making heroin addiction become a national concern (Zackon, 1993). It was during this time that the Vietnam War had U.S. soldiers in an area of the world where heroin was cheap and 90-98% pure, compared to the expensive

heroin that was only 2-10% pure found in the United States. It was estimated that approximately 11% of soldiers regularly used heroin while in Vietnam. Fortunately, only 1-2% of Vietnam veterans were regular heroin users one year following their return from overseas (Robins, 1974). One possibility for this drastic difference in usage is that the context cues and motivational factors associated with heroin use in Vietnam were not present in the soldiers' daily life back in the United States.

In the 1980s, crack cocaine took precedence over opioid abuse; but heroin abuse still continued, especially with the introduction of black tar, a relatively pure and inexpensive form of Mexican heroin. Other synthetics were also created in illegal laboratories, including China White, which is derived from fentanyl. Due to a legal loophole in drug laws, these "designer drugs" were technically legal because they were not chemically identical to heroin. In 1986, however, the Controlled Substance Analogue Act closed that loophole. As time went on, heroin became more pure and less expensive. With regard to opioid pain relievers, prescription rates have been on the rise in the past 25 years. In 1991, 76 million prescriptions were filled for opioid pain relievers. By 2013, the number of prescriptions filled was 207 million, a 172% increase from 1991 (IMS, 2014).

Today, the U.S. is the biggest consumer of prescription opioids globally, accounting for close to 80% of the world total of oxycodone (Board, 2008). Due to availability, it is estimated that 828,000 people in the United States used heroin within the past year (SAMHSA, 2016).

Opioid System

Unlike some other neurotransmitter systems (e.g. dopamine and serotonin), which follow specific pathways in which they follow, the opioid system is apparent throughout the entire brain. Detailed coverage of the opioid system has been described elsewhere (Meyer, 2005) and is merely summarized briefly here.

There are several types of opiate receptors, but the three most important are mu, delta, and kappa. Each of these receptor subtypes has their own, distinct distribution in the brain. The mu-receptor is the one most commonly linked with the euphoric effects of opioid drugs (e.g. morphine). These receptors are widely distributed in both the brain and spinal cord and are concentrated in areas associated with analgesia (e.g. medial thalamus, periaqueductal gray, and median raphe) and positive reinforcement (e.g. nucleus accumbens). Mu-receptors are generally perisynaptic, meaning that they can be localized both postsynaptically on dendrites and cell bodies, and they can also be localized presynaptically on axon terminals (Williams, 2001). The delta receptors have similar distributions to the mu receptors, but are more restricted (low expression in the hypothalamus, thalamus, mesencephalon, and brain stem; Le Merrer et al., 2009). These receptors are more commonly found in the forebrain (e.g. neocortex, striatum, substantia nigra, and nucleus accumbens). The location of delta receptors suggest that, like mu, they are involved in reinforcement, as well as cognitive function. The kappa receptors are found in areas different from mu and delta receptors. Kappa receptors are found in the striatum and amygdala, but also the hypothalamus and pituitary. It is thought that kappa receptors participate in pain perception, digestion, and dysphoria.

Each of these receptor subtypes has at least one endogenous ligand that acts upon it. Endomorphins (e.g. endomorphin-1) activate the mu receptor, whereas endorphins (e.g. β-endorphin) activate both mu and delta receptors. Enkephalins (e.g. met-enkephalin) activate delta receptors and dynorphins (e.g. dynorphin A) activate kappa receptors. Each of these peptides was originally part of larger propeptides, which are broken into smaller active peptides. Specifically, endorphins are cleaved from the propeptide proopiomelanocortin (POMC), which is expressed in the pituitary and the hypothalamus. Enkephalins, on the other hand, come from proenkephalin, which is widely distributed throughout the central nervous system. Prodynorphin is the propeptide for dynorphins, and is also widely distributed throughout the brain. At the current time, the propeptide for endomorphins is still unknown (Meyer, 2005).

All three opioid receptor subtypes are coupled to inhibitory G proteins that inhibit adenylyl cyclase, which usually synthesizes the second messenger cyclic adenosine monophosphate (cAMP). Opioids also work by opening K^+ channels and closing Ca^{2+} channels. The overall effects that opioids have on cell function is reducing membrane excitability, slowing cell firing, and inhibiting neurotransmitter release.

Due to its role in the rewarding effects of opioid drugs, the mu-opioid receptor (MOR) has been most widely studied for its role in abuse and dependence. The MOR consists of an extracellular N-terminal domain, seven transmembrane helical domains (connected by three extracellular and three intracellular loops), and an intracellular C-terminal tail (Chen et al., 1998). Binding of ligands to the MOR occurs at a large open mouth at the extracellular side of the binding pocket (Xu et al., 2013). This may explain why this receptor can bind with a wide array of molecules, including the large

endogenous polypeptides (e.g. endorphins). When a mu ligand enters the binding cavity, the internal conformation of the receptor changes, which disrupts the basal hydrophobic and hydrophilic interactions of the transmembrane helical domains (Lopez and Salome, 2009). After binding, the ligands trigger signaling cascades. Activation of the MOR stimulates catalytic GDP-GTP exchange. Doing so requires dissociation of the G-protein complex, which leads independently to activation or inhibition of multiple down-stream effects. For instance, when G_{α} dissociates to $G_{\beta\gamma}$, stimulation of adenylyl cyclase occurs (Chakrabarti et al., 2005) as well as activation of phospholipase C (Williams et al., 2001). G_{α}, in contrast, inhibits adenylyl cyclase activity (Diel et al., 2008).

Chronic opioid use can lead to alterations in gene expression, particularly in the mesocorticolimbic system. One of the most characterized transcription factors that produce alterations in cell function is ΔFosB. All members of the Fos family proteins are rapidly induced in specific brain regions following administration of many drugs of abuse, including opioids (Hope et al., 1994; Nye et al., 1995; Moratalla et al., 1996; Pich et al., 1997), and these cellular signals are particularly prominent in the nucleus accumbens (Koob et al., 1998). Following repeated opioid exposure, modified isoforms of ΔFosB accumulate within the nucleus accumbens (and other brain regions where acute administration also leads to increases in ΔFosB). This is in contrast to other Fos family members, which do not accumulate over time (Nestler et al., 2001). It is believed that this accumulation occurs because of the extraordinarily long half-lives of the ΔFosB isoforms (Chen et al., 1995). The accumulation of ΔFosB in drug addiction has been well studied, particularly with the use of transgenic mice. For instance, mice that show overexpression of ΔFosB show an enhanced sensitivity to the rewarding effects of

morphine in a conditioned place preference (CPP) paradigm (Kelz et al., 1999). Induction of this transcription factor is not specific to opioids, however, since mice expressing ΔFosB also show increased locomotor activation in response to cocaine, work harder to self-administer cocaine, and show enhanced anxiolytic effects of alcohol (Dobrazanski et al., 1991; Kelz et al., 1999). These findings suggest that ΔFosB increases the sensitivity to various drugs of abuse and also promotes drug-seeking behavior.

While ΔFosB is a long-lasting adaptation that occurs in the adult brain, it is not permanent. ΔFosB is no longer detected in the brain after 1-2 months of drug withdrawal (Chen et al., 1997). Therefore, ΔFosB is not solely responsible for the more permanent behavioral changes following chronic opioid use. One possibility for the long-lasting behavioral changes is that other transcription factors may mediate long-lived changes in neuronal morphology (Nestler et al., 2001). For example, there is an increase in the density of dendritic spines in hippocampal pyramidal neurons and medium spiny neurons in the nucleus accumbens following drug administration (Robinson and Kolb, 1999; Luscher et al., 2000; Scannevin and Huganir, 2000; Gipson et al., 2013).

Pharmacokinetics

As the prototypic opioid, morphine is primarily metabolized in the liver, and has two known metabolites (De Gregori et al., 2012). The major morphine metabolite is morphine-3-glucuronide (M3G), which is analgesically inactive, but has been reported to antagonize morphine (Smith, 2000). The minor morphine metabolite is morphine-6-glucuronide (M6G), which has a greater analgesic potency than morphine. Within the

liver, uridine-5'-diphosphate (UDP) glucoronosyltransferase is the primary enzyme that metabolizes morphine into M3G and M6G. However, different amounts of each metabolite are produced, with up to 90% of morphine being metabolized into M3G, while only 10% is metabolized into M6G. Morphine can also be metabolized within the central nervous system (CNS) and produce nanomolar concentrations of M3G and M6G. Interestingly, the M6G formed directly in the CNS appears to be able to penetrate the blood brain barrier (BBB) at a greater rate than when M6G is produced in the liver (Yamada et al., 2003).

Once the morphine or its metabolites cross the BBB, they act upon the MOR. There are multiple genetic variants that can moderate the effectiveness of morphine. One such variant occurs in the μ-receptor gene (OPRM1), which provides instructions for making MORs. The OPRM1 gene, however, is highly polymorphic. For example, a polymorphism at the nucleotide position 118 can have a large impact on therapeutic efficacy. In particular, people with OPRM1 118 G homozygote or heterozygote genotypes (i.e. 118 GG or 118 AG) require higher doses of morphine for pain relief compared to people with the OPRM1 118 A homozygote genotype (i.e. OPRM1 118 AA; Wu et al., 2009).

Similarly, catechol-o-methyl transferase (COMT) polymorphisms also affect morphine's efficacy. COMT is a relatively nonspecific enzyme that is responsible for the metabolism of dopamine, norepinephrine, and epinephrine. Morphine is believed to increase dopamine release through inhibition of GABAergic neurons in the VTA, which synapse on dopaminergic neurons in the NAc (Tepper et al., 1995; Jalabert et al., 2011). Like OPRM1, polymorphisms of COMT can have a large impact on opioid metabolism.

For instance, the amino acid at position 158 has two forms. The Val158 form is fully active, but the MET158 variant is much less active. Therefore, persons with the Val/Val homozygous genotype require more morphine compared to people with the Val/Met heterozygous or Met/Met homozygous genotype (Allegri et al., 2010). These genetic variations are relevant not only due to decreased pain relief by opioids, but also because if someone requires more morphine to mange pain, they are at higher risk of respiratory depression.

Dopaminergic System

As mentioned previously, the dopamine system follows specific pathways within the brain. As above, this section is a brief summary, as detailed coverage of the dopamine system has been described elsewhere (Carlson, 2007; Meyer, 2005).

The monoamine system is made up of cell bodies, with axons that branch off to a number of other brain regions. The cell bodies for dopamine are primarily located in the midbrain, specifically in the substantia nigra and ventral tegmental area (VTA). Cell bodies located in the substantia nigra that project to the neostriatum (caudate nucleus and putamen) make up what is known as the nigrostriatal pathway. This pathway is an important part of the basal ganglia, which is heavily involved in movement. There are two pathways with cell bodies located in the VTA. First, there is the mesolimbic pathway, which includes projections from the VTA to brain regions included in the limbic system such as the nucleus accumbens, amygdala, and hippocampus. The limbic system, particularly the nucleus accumbens, plays an important role in reinforcement/reward of both natural (e.g. food, social interaction) and drug stimuli. The

second pathway involves the cell bodies located in the VTA that project to the prefrontal cortex, i.e., the mesocortical pathway. This pathway is important for short-term memory, planning, and problem solving.

Dopamine itself is synthesized within neurons from the precursor tyrosine, which is an amino acid that is obtained via the diet. Tyrosine is converted into L-DOPA by an enzyme called tyrosine hydroxylase, which adds a hydroxyl group to the catechol ring. L-DOPA, in turn, is converted to dopamine by an enzyme called DOPA decarboxylase, which removes the carboxylic acid from the molecule.

There are several different subtypes of dopamine receptors. All dopamine receptors are metabotropic, meaning they involve G-protein coupled second-messenger systems. The two most common receptor subtypes are D_1 and D_2 receptors. Dopamine D_1 receptors appear to be found only postsynaptically, whereas D_2 receptors are found both postsynaptically and presynaptically in the brain. Unlike opioids, which are almost always inhibitory, dopamine can be either excitatory or inhibitory. For instance, stimulation of D_1 receptors increases production of the second messenger cyclic AMP, whereas stimulation of D_2 receptors decreases it. Because of these inhibitory effects, D_2 receptors found presynaptically can act as autoreceptors, which decrease neuronal firing as part of a feedback loop for the system. Along with D_1 and D_2 receptors, there are D_1-like family receptors and D_2-like family receptors. D_1-like receptors include D_5 receptors, and D_2-like receptors include D_3 and D_4 receptors. Each of these receptor subtypes is not as prevalent as D_1 or D_2 receptors, but, depending on the family, they act in similar ways.

Modulation of synaptic transmission can occur either presynaptically or postsynaptically (for a detailed review see Iversen et al., 2009). Possible reasons for presynaptic modulation includes: a change in the firing frequency of the neuron; a change in the reuptake or transport of a neurotransmitter; a change in the synthesis, storage, or release of a neurotransmitter; and blocking of ion channels or changing in open time of the channel. Possible reasons for postsynaptic modulation include: down regulation of receptors; a change in the affinity of a ligand for a receptor; and changes in second messenger systems. One way that second messenger systems can modulate synaptic transmission is via protein phosphorylation. For instance, when phosphorylated, DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of 32 kDa) acts as a protein phosphatase inhibitor.

DARPP-32 is found in D_1 dopaminoceptive medium spiny neurons, and is highly concentrated in the putamen, nucleus accumbens, olfactory tubercle, and amygdala (for a review see Svenningsson et al., 2004). Interestingly, MORs are also expressed on medium spiny neurons and are colocalized with D_1 receptors in striatonigral neurons (Georges et al., 1999). Stimulation of D_1 receptors enhances cAMP formation and leads to stimulation of protein kinase A (PKA), which leads to phosphorylation of DARPP-32. When DARPP-32 is phosphorylated at Thr75, it is potent inhibitor of PKA (Bibb et al., 1999). Alternatively, when DARPP-32 is phosphorylated at Thr34, it is an inhibitor of protein phosphatase-1 (Hemmings et al., 1984). Once phosphorylated, DARPP-32 is inactivated by protein phosphatase -2A and -2B and Thr34 and Thr75, respectively.

Although it appears that social interaction does not have a long-lasting impact on DARPP-32 (Zakharova et al., 2009), there is evidence that drugs of abuse alter DARPP-

32 activity. For instance, acute administration of low-doses of the MOR agonist DAMGO inhibit phosphorylation of DARPP-32 via activation of D_1 receptors (Lindskog et al., 1999). However, repeated administration of morphine does not lead to any long-term modifications of DARPP-32 phosphorylation (Scheggi et al., 2004). Conversely, repeated administration of cocaine leads to an increase in phosphorylation of DARPP-32 at Thr75 and a decrease in phosphorylation at Thr34 in both the nucleus accumbens and caudate putamen (Scheggi et al., 2004). Evidence also suggests that while DARPP-32 activation is necessary for morphine's psychomotor stimulation, it is not required for morphine reward (Borgkvist et al., 2007). To our knowledge, there is currently no literature on changes in DARPP-32 activity with regards to social play.

As mentioned previously, the dopamine system is involved in reward/reinforcement. Studies using 6-hydroxydopamine (6-OHDA) to lesion various parts of the dopamine pathways lead to severe behavioral dysfunction, including sensory neglect, motivational deficits, and motor impairments (Retailleau et al. 2013; Bonito-Oliva et al., 2014; Ma et al., 2014). Conversely, intracerebral electrical self-stimulation of the mesolimbic dopamine pathway is highly reinforcing (Wise, 199; Ewan and Martin, 2012; Hernandez et al., 2012). In addition to the endogenous opioid system, there is also a dopaminergic component that contributes to opioid reinforcement. Opiods that are injected either systemically or directly into the VTA leads to an increase of dopaminergic cell firing, which increases the release of dopamine in the nucleus accumbens (Leone et al., 1991; Fields and Margolis, 2015).

Adolescence as a Period of Risk for Drug Abuse

Adolescence is a time associated with maturation of both neuronal and peripheral body systems. During this age period, peer interactions are typically extensive due to school and extracurricular activities. These peer interactions also are apparent with crimes and antisocial behaviors committed by adolescents, which typically occur in a group setting (McCord, 2005). This type of social facilitation of antisocial behavior in adolescents contrasts with adults, who often act alone. It is unknown, however, why there are age differences related to crimes committed in peer groups vs. alone. One possibility is that, because adolescents are in the presence of peers more often than adults, they simply show increased odds of committing a crime within a group context.

The phenomenon of social facilitation of disruptive behaviors is not solely associated with crimes such as robbery, destruction of property, or vandalism. In particular, the initiation of drug use during adolescence also generally happens in a group setting. Early research has found that for most drugs, initiation of use begins around age 18 (being a little older for prescription opioids) and discontinuation often occurs around age 21 (Kandel and Logan 1984). The easiest way to predict whether or not an adolescent will initiate drug use is whether or not their friends use drugs (Bahr et al., 2005). While most adolescents experiment with illicit substances and relatively few continue use to the point where a substance use disorder becomes manifest, drug addiction is still prevalent within this population. For example, in 2015, an estimated 450,000 adolescents initiated pain reliever use, and that 122,000 persons aged 12-17 met criteria for a substance use disorder of pain relievers (SAMHSA, 2016).

Although it is well accepted that adolescents initiate drug use within a social setting, most laboratory research has focused on decision-making and drug abuse-related behaviors measured in subjects tested in the absence of social peers. However, the field of social psychology has contributed significantly to our understanding of risk taking. This work has shown that adolescents are more vulnerable to peer pressure and advice compared to adults.

In one important study, Gardner and Steinberg (2005) evaluated social influence on risk-taking using a computer task called "Chicken," in which the participants controlled when a car driving along a course should stop. In the task, subjects gained points based on the distance their car traveled. However, if a brick wall appeared in front of their car, causing a crash, the subject lost all points for that trial. In this task, adolescents were significantly more risky than adults, such that they would allow the car to travel further before stopping it. Furthermore, adolescents were more influenced by social peers to travel further along the course than adults, who showed no significant increases in risk taking in the presence of peers. Examination of gender differences revealed that males gave greater weight to benefits of a risky decision and less weight to the negative consequences compared to females, suggesting that males are more likely to engage in these risky behaviors. When asked about the same risk taking situations in a group setting, males further increased the perceived weight of the benefits as compared to the negative consequences. Importantly, all participants in the study knew the other members of the group to which they were assigned prior to participating. This may be an important factor for enhancing the effect of social influence, as friends tend to pressure others more than acquaintances (McPhee, 1996).

Interestingly, peers typically advise friends to make riskier decisions than they would choose for themselves (Beisswanger, 2003). This appears to be true only for social situations and does not generalize other situations (e.g. financial decisions). For example, items included on surveys that participants commonly said they would encourage a friend to do included: (1) introducing themselves to an attractive member of the opposite sex, (2) giving someone attractive their phone number, (3) going home with a member of the opposite sex after a party, and (4) moving in with a significant other. However, when completing a gambling task, participants rated their willingness to accept a bet similarly to how likely they would be to encourage a peer to accept the same bet (Stone, 2002).

Research also shows that members of various social groups will either reinforce or punish the use of drugs based on the norms that have been decided upon by that group (Kandel, 1986). Within most social groups, the status of the leader is a critical determinant of substance use norms within the group (Jones, 2007). However, while group affiliation can determine drug use norms for individual members, there is also evidence that individuals who possess traits that increase risk for drug use will seek each other out in order to form peer clusters (Donohew, 1999), thus suggesting that predisposing individual differences in biological risk may precede peer group identification. In this regard, preclinical research may be useful for assessing social x biological interactions involved in drug use.

Neuroimaging and Decision-making

In clinical research, it is possible to directly examine the neurobiological processes associated with drug abuse or social interaction using modern neuroimaging

technology. Magnetic resonance imaging (MRI) allows for 3-demensional imaging of brain structure. A review of MRI literature reveals that administration of almost all drugs of abuse lead to decreases in cortical volume, and increases in subcortical structures (Suckling and Nestor, 2017). For instance, previous research with opioid dependent subjects has shown decreases in gray matter volume, including the amygdala, when compared to healthy controls (Upadhyay et al., 2010). In another study, one-month of oral morphine administration in opioid naïve participants also led to structural changes in gray matter structures, including decreases in the amygdala and hippocampus, and increases in the hypothalamus and anterior cingulate. These morphine-induced changes were sustained following a 5-month cessation period (Younger et al., 2011). These morphological changes, particularly in the amygdala and hippocampus, provide evidence that opioids lead to fast alterations in reward-learning circuits, and that they are sustained throughout chronic use.

Unlike MRI, which only allows for the investigation of structural differences or changes, functional magnetic resonance imaging (fMRI) allows for investigation of metabolic function. In order to qualitatively measure metabolic function, fMRI makes use of changes in blood oxygen levels to indicate changes in activity, which can be compared across multiple experimental groups. fMRI can be conducted during a resting state or while a participant performs a behavioral task. Research involving administration of a drug during a fMRI scan has been termed pharmacological functional magnetic resonance imaging (phMRI). With regard to opioid phMRI, in opioid naïve participants, morphine increases in neural activity (i.e. blood oxygen levels) in reward-relevant regions, such as the nucleus accumbens, amygdala, orbitofrontal cortex, and

hippocampus, compared to controls (Becerra et al., 2006). Furthermore, acute precipitated opioid withdrawal in males led to increases in neural activity of reward-relevant regions, such as the caudate, orbitofrontal gyrus, and putamen (Chu et al., 2015). As these regions have previously been associated with the processing of rewarding cues (Staudinger et al., 2011; Langleben et al., 2014), one would predict that naloxone-precipitated withdrawal would decrease activation in these regions. However, other research has shown that morphine decreases neural activity in the same areas (Khalili-Mahani et al., 2012), and it is common for withdrawal to produce opposite effects of the drug itself.

fMRI has also been used to investigate social reward processing in humans. With regard to social reward, it is well established that anticipation of positive social interaction activates the dopaminergic mesocorticolimbic system, including the nucleus accumbens (Knutson et al., 2001, 2005; Gasic et al., 2009; Dichter et al., 2012). However, due to the restrictions of fMRI, procedures have relied primarily on static face images to serve as the social rewards. Given that non-verbal signals (e.g. body posture and gestures) are also important components of social reinforcement (Skinner, 1953), it can be argued that more ecologically valid stimuli should be introduced into the fMRI paradigms.

A recent study has incorporated more dynamic stimuli (i.e. short video clips) into a social reward paradigm in order to address this concern. Kohls and colleagues (2013) used an incentive delay task to examine motivation to receive social rewards. The incentive delay task is a simple speeded button press task, which tests a person's ability to quickly and accurately respond to a target symbol. Within the task, a cue signals the

trial type (i.e. approval, disapproval, or control), which is followed by a symbol that is present for varying amounts of time, and finally a target symbol that signals the need for a response. During approval trials, "hits" resulted in a short video clip of a person showing happy facial expressions and giving a "thumbs up" sign, while "misses" resulted in a video clip of a person showing a neutral expression. During avoidance trials, "hits" resulted in a video clip of a person showing a neutral facial expression, while "misses" resulted in a video clip of a person frowning and giving a "thumbs down" sign. During control trials, "hits" and "misses" resulted in a video clip of a person with their eyes closed and neutral expressions, pretending to listen to music while snapping their fingers. Results revealed that participants responded more quickly during approval and avoidance trials, compared to control trials. However, responses during avoidance trials were also significantly faster than approval trials. Most important, there was stronger activity in the nucleus accumbens during the social approval and social avoidance trials compared to control trials, implicating an overlapping neural system between social and drug reward.

Preclinical Models of Drug Abuse

There is no equivalent of human drug addiction in rodents because laboratory animals show no volition to desist in drug taking. For instance, part of the DSM-V criteria for substance use disorder includes "continuing use, even when it causes problems in relationships" and "giving up important social, occupational, or recreational activities because of substance use" (American Psychiatric Association, 2013). Rodents do not have the same social structures that humans do, and therefore we cannot model these situations. Nonetheless, there are a variety of behaviors and procedures that can be

used to model various aspects of drug abuse using laboratory animals. Of course, it is not possible to measure subjective feelings or the impact that drugs have on criminal behavior in laboratory animals. It is, however, possible to model reward value and how it changes across the addiction cycle.

One of the simplest procedures used to examine the behavioral effects of opioids in rats is locomotor activity. With this procedure, animals are first habituated to a large chamber, which contains a number of laser beams that can detect motion and location. Once animals have habituated to the chamber, they are administered an opioid and are placed in the chamber for a specified amount of time (typically between 30 to 60 minutes). Using specialized software, the researcher can determine if the drug increases or decreases movement. This procedure is primarily used when determining whether a pharmacotherapy has the ability to block hyperactivity associated with a drug of abuse. With high doses of morphine, there is an initial period of hypoactivity, followed by a rebound of hyperactivity (Bajic et al., 2015). With repeated administrations, tolerance develops to the hypoactivity phase and sensitization occurs to the hyperactive phase (Li et al., 2010; Wei and Li 2014). These locomotor effects are blocked by naloxone and μ-selective antagonists (Brady and Holtzman 1981; Smith et al., 2009). Therefore, when testing a novel compound thought to treat opioid abuse, researchers can first determine if their new drug blocks the hyperactivity caused by morphine.

Baseline levels of hyperactivity in the absence of drug are also known to be associated with impulsivity and increased drug use (Garcia and Kirkpatrick, 2013; Yamamoto et al., 2013; Ferland et al., 2014). Therefore, a researcher can also use locomotor movement as a screen prior to a behavioral task or drug self-administration

(described below). While locomotor activity is not a direct measure of abuse liability, it is easy to conduct and is known to be dependent on dopamine activity in the mesolimbic reward pathway (Shim et al., 2014; Oh et al., 2015).

One of the most widely used tests to study the rewarding properties of natural and opioid rewards in laboratory animals is conditioned place preference (CPP; Bardo and Bevins, 2000). CPP has been measured in various species, including rats, mice, chickens, quail, prairie voles, and humans (Liu et al., 2011; Bolin et al., 2012; Jones et al., 2012; Cole et al., 2013; Astur et al., 2014; Weiss et al., 2015), and many studies have shown that morphine produces robust CPP in rats (Bali et al., 2015; Moaddab et al., 2015; Koek, 2016). CPP requires a special chamber, made up of three compartments. Each compartment has different colored walls (e.g. white, gray, and black) and floors (e.g. wire mesh, solid plastic, and metal rods) to distinguish them from the other compartments. Between the two end compartments and the middle compartment are guillotine doors than can be opened or closed.

A standard CPP procedure takes 10 sessions (days). The first session is a pretest, where all guillotine doors are open, and animals are allowed to explore all three compartments for a total of 15 minutes. Time spent in each compartment is measured by photo beams (a similar system to that used in the locomotor procedure). The next 8 sessions (2 through 9) are conditioning sessions, where the end compartments are each paired with a different reward. On alternating days, animals are confined to one of the two compartments for a set period of time (typically between 10 and 30 minutes, depending on the drug). Therefore, on session 2 the animal will be administered the drug, on session 3 the animal will be administered vehicle, and so on.

Although the standard CPP procedure only includes one session every day during conditioning, there are other procedures where the animal is exposed to both drug and vehicle trials every day (i.e. having two sessions every day, separated by a specified amount of time). The last session (day 10) is the test session. As with the pretest, during the test session, all guillotine doors are open and animals are allowed to explore all three compartments for a total of 15 minutes. The drug is considered to be rewarding if the animal spends more of its time in the compartment paired with the drug compared to the compartment paired with saline. This preference is typically measured by converting the time spent in the two compartments into a preference ratio. A ratio of 0.5 would designate no preference for either compartment, and ratios closer to 1 or 0 would show a preference or aversion for the drug, respectively. Different doses of a drug can also be directly compared to each other. For morphine specifically, CPP is established reliably in adult rats between doses of 1 and 10 mg/kg (Bardo et al., 1995), which is at or below the effective analgesic threshold.

Following acquisition of CPP, researchers can also investigate extinction and reinstatement. During extinction, animals are placed into the CPP chamber, without prior administration of any drug, and allowed to explore all three compartments for multiple sessions. Generally, it is believed that the strength of the rewarding drug stimulus is directly correlated with the number of sessions required before the animal shows no preference for either compartment. During a reinstatement session, animals are pre-exposed to the rewarding drug stimulus outside of the apparatus, and then allowed to explore all three compartments. Reinstatement is said to occur if the animal spends significantly more time in the compartment that was previously paired with the drug

during the test. This procedure has been used to investigate the reinstatement of both morphine and social CPP (Trezza et al., 2009; Cordery et al., 2014; Gawel et al., 2014).

There is significant evidence that the nucleus accumbens is important for the induction of morphine CPP. Inactivation of the nucleus accumbens, and antagonism of glutamate receptors mGLuR5, TRPV1, or the mitogen-activated protein kinase p38, can lead to reduction of morphine CPP acquisition (Esmaeili et al., 2012; Zhang et al., 2012; Heng et al., 2014; Roohi et al., 2014). Intracerebroventricular administration of oxytocin, on the other hand, enhances morphine CPP expression (Moaddab et al., 2015). Furthermore, morphine CPP led to increased dendritic complexity (e.g. increased dendritic count, length, and intersections) in the nucleus accumbens core, compared to morphine administration in a homecage (Kobrin et al., 2016). Following the extinction of morphine CPP, lesioning the nucleus accumbens via radio-frequency lesioning electrodes inhibits reinstatement (Wang et al., 2008).

Contrary to the nucleus accumbens, evidence suggests that the dorsal striatum (which consists of the caudate nucleus and the putamen) is not heavily involved in CPP learning. For example, rats that receive injections of amphetamine directly into the dorsolateral caudate nucleus do not develop CPP (Carr and White, 1983). In the same study, rats that received injections of amphetamine directly into the nucleus accumbens showed significant preference for the amphetamine-paired environment. In another study, researchers investigated the effects of dorsolateral and dorsomedial quinolinic lesions on stimulus-response learning. Following recovery from surgery, rats were trained in both an operant discrimination task and CPP. While rats with lesions of the dorsolateral striatum were impaired on the operant discrimination task (with a food

reinforcer), lesions of either the dorsolateral or dorsomedial striatum had no significant impact on the rats' ability to develop food CPP (Featherstone and McDonald, 2004).

There are several advantages and disadvantages of CPP. One advantage of CPP is that it is a choice paradigm, wherein an animal is given the opportunity to make a decision about what compartment to spend time in during the post-test session. Unlike locomotor activity, this allows for an interpretation of reward value of various conditions, which can be directly compared to each other. CPP is also a short paradigm, allowing researchers to obtain results quickly. This quicker result is of particular importance when studying adolescent animals, as a rat is only in the adolescence stage for approximately 25 days (generally equating to post-natal days 28-55; Sengupta, 2013). CPP can also be conducted with animals of any age. Paradigms that require surgery (e.g. self-administration; detailed below) are limited to animals that are fully grown and healthy enough to survive the procedure, generally eliminating adolescents and aged animals from being subjects.

Disadvantages of CPP include difficulty to create a dose-response curve, interpretation of results, and compartment biases by the animal (Bardo and Bevins, 2000). When using a CPP paradigm, it is not possible to use a within subjects design to produce a dose-response curve. This is due to the conditioning of the compartments that has already occurred. Therefore, in order to fully investigate the rewarding value of a drug of interest, multiple groups of animals must be used. The interpretation of results for CPP can also be difficult, particularly because animals can have compartment preferences prior to conditioning (i.e., during the pretest). One way to resolve this issue

is by pairing the drug of interest with the non-preferred compartment, known as a "biased design."

In contrast to CPP, a more direct assessment of opioid reinforcement is the self-administration paradigm. Considered to be the "gold standard" for studying the abuse potential, self-administration allows researchers to observe voluntary drug intake. Since drugs of abuse are typically not consumed voluntarily by the oral route in rodents, drug self-administration requires animals to undergo surgery to implant a catheter into a vein (typically a jugular vein) that can then be connected to an automated syringe pump. The apparatus used during self-administration is an operant conditioning chamber. Standard operant chambers used for self-administration typically require two levers, two cue lights (usually placed above the levers), and a house light. Animals are required to make responses on one of the levers in order to receive an infusion of drug; responding on the other lever produces no programmed consequence. The number of responses required and/or the time during which an infusion can be earned is dependent on the schedule of reinforcement. Using this general procedure, it has been clearly demonstrated that morphine and related μ agonists serve as reliable reinforcers in rats, engendering steady, but relatively low, rates of responding compared to cocaine (Balster and Lukas, 1985; Mierzejewski et al., 2003).

Two common schedules of reinforcement for self-administration include a fixed-ratio schedule and a fixed-interval schedule. During a fixed-ratio schedule, the delivery of the drug (via infusion) occurs after a certain number of responses have been emitted. For example, under a fixed-ratio 10 schedule (designated FR 10), drug delivery occurs following the 10^{th} response on the lever. During a fixed-interval schedule, delivery of the

drug occurs after a certain amount of time has elapsed. For example, under a fixed-interval 10-second schedule (designated FI 10 second), drug delivery occurs following the first response after 10 seconds has elapsed. Under both of these schedules, when rates of responding are plotted as a function of the drug dose, the relationship is represented by a biphasic function (Pickens and Thompson, 1968). Biphasic functions (also known as an "inverted U-shaped functions") are, unfortunately, difficult to interpret. While it is generally believed that dose-dependent decreases in response rates on the descending limb of the function are related to positive physiological effects and receptor occupancy (Haney and Spealman, 2008), it is also possible that aversive effects are responsible.

One behavioral task that allows for an easier interpretation of self-administration data is the progressive ratio (PR) schedule of reinforcement (Richardson and Roberts, 1996). During a PR schedule, the number of responses required to receive the reinforcer increases following every trial. For example, the first reinforcer may occur after 10 responses, the second after an additional 20 responses, the third after an additional 30 responses, and so on. The primary dependent variable under a PR schedule is known as the break point, which is defined as the final response ratio requirement completed either after a predetermined period of time without receiving the reinforcer or at the end of an experimental session. Therefore, a PR schedule provides a measure of how many responses a subject is willing to make to receive a reinforcer before they stop responding, which is thought to be associated with the strength of a reinforcer. The PR schedule is also used to determine levels of drug seeking behavior within animals. The PR schedule

has been used with opioid drugs such as heroin and morphine (Roberts and Bennett, 1993; Grasing et al., 2003; Li et al., 2003).

The length of the opioid self-administration session can also impact behavior. Giving rats access to opioids for a limited amount of time every day (e.g. 1-4 hour sessions) typically results in stable levels of self-administration (Vendroscolo et al., 2014). This short access paradigm (ShA) is generally used to model recreational drug use. Extending access to the drug (e.g. 6-12 hour sessions) will result in a progressive increase in daily intake (Picetti et al., 2012; Wade et al., 2015). This long access paradigm (LgA) yields an escalation of drug use, including heroin (Lenoir and Ahmed, 2007; Picetti et al., 2012), which is thought to more closely mimic dysregulated drug addiction behavior in humans. It is worth noting, however, that not all rats self-administer drugs at high rates during ShA, and not all rats escalate their use over time during LgA. Therefore, it is likely that neurobiological differences may underlie drug abuse vulnerability.

Following drug self-administration, the extinction-reinstatement procedure allows researchers to model relapse, including opiate relapse (Shaham et al., 2003; Bossert et al., 2016). During extinction, animals are placed into the operant chamber and allowed to respond on the levers. A response on the lever during extinction, however, does not result in drug administration. Instead, responses either have no programmed response, or activate cues previously associated with drug self-administration (e.g. cue lights, tones, etc.). Animals are run in the extinction paradigm until average daily responses are below a set criteria (usually either a percentage of responding compared to self-administration, or a set number of responses). To induce reinstatement, the animal is re-exposed to a

stimulus that was not extinguished. Stimuli used for reinstatement most often include either drug priming or a drug-associated cue. For drug-induced reinstatement, animals are administered a low dose of the drug previously being self-administered prior to being placed into the operant chamber. During cue-induced reinstatement, a drug-associated cue that was not present during extinction is added back to the extinction paradigm. Depending on the strength of either the reinforcer (in the case of drug-induced reinstatement) or the cue, animals will respond significantly more during a reinstatement session compared to an extinction session. Data from our laboratory has shown that a social peer can act as a cue for cue-induced reinstatement of cocaine self-administration (Weiss et al., under review); however, it is unclear if a similar effect would be observed with opioids.

Development Across the Adolescent Period in Rats

While adolescence in rats is roughly defined as taking place during postnatal day (PND) 28-42 (Spear, 2000), there is still development of neural systems occurring during this time. For instance, while the blood brain barrier is established between PND 1-3 (Engelhardt, 2003), myelination is not detectable until PND 20 (Wiggins, 1986). PND 20 is also the timepoint where cortices of rats reach 90% of their adult weight (Semple et al., 2013).

The most notable changes across the adolescent time period in rats is the development of synapses and neurotransmitter systems. As with prenatal development, postnatal development involves overproduction and pruning of multiple systems. For example, it is estimated that rats will lose up to half of the synapses in cortical neurons by

the time they reach adulthood (Rakic et al., 1994). Volume of the prefrontal cortex also decreases across adolescence (van Eden et al., 1990).

The dopamine system also undergoes significant changes during this time period. Dopamine transporters are at 70% of adult levels by weaning (Coulter et al., 1996) and increase in number until the animals mature (Akbari et al., 1992). While dopamine input increases across adolescence (Kalsbeek et al., 1988), basal dopamine synthesis consistently increases from PND 1 until PND 30, then decreases until PND 40 in the prefrontal cortex (Boyce et al., 1996; Anderson et al., 1997). Interestingly, dopamine neurons in adolescence show decreases in release under basal conditions compared to adults (Stamford, 1989), but show increases in dopamine release when neurons are stimulated (Galvan, 2010). This suggests that rewarding events may lead to greater dopamine release in adolescents, compared to adults.

Changes across development may help to explain differences in drug sensitivity between adolescents and adults. In general, adolescents tend to be less sensitive to drugs of abuse than adults (Laviola et al., 1995; Bolanos et al., 1998; Snyder et al., 1998). Evidence suggests that this blunted response is not due to drug metabolism (Wang et al., 2005). As rats are growing substantially across adolescence, and undergoing both neural as well as hormonal changes, is possible that changes in body composition and organ function associated with growth spurts and rising gonadal steroid titers may alter drug distribution (Spear, 2000).

Adolescents also show changes in social interaction across development. For instance, up until PND 20 rats show adult-like defensive behaviors in the presence of a peer (Pellis et al., 1997). Alternatively, rats between PND 30-40 engage in play fighting

behavior (Fassino et al., 1981; Primus et al., 1989). By the time rats reach adulthood at PND 60, they revert back to defensive behaviors around peers. While evidence suggests that the emergence of novelty-suppression of social interaction in adult rats is dependent on gonadal hormones (Primus et al., 1990), the temporary switch from adult-typical defense strategies to play does not appear to be hormone-dependent, as the change in social behavior is not influenced by castration (Smith et al., 1998).

Preclinical Social Play Behavior

In adolescent rats, the term "social play" refers to a set of behaviors that are known together as "rough and tumble play." This behavior includes actions such as pouncing, pinning, and boxing (See Figure 1, taken from Trezza et al., 2010). Pouncing is a way of initiating play, with one rat attempting to nose or rub the nape of the neck of another rat (Panksepp and Beatty, 1980). Pinning is a behavior that can occur following pouncing, where the rat that was pounced on rotates so that its back is to the ground, leaving the soliciting rat standing over it. Another alternative behavior in response to pouncing could be running away, which will typically lead to chasing by the soliciting rat. Rather than another pounce, this chasing could result in boxing when running ends. During boxing, rats stand upon their back legs and engage each other with their front legs. These behaviors can be easily quantified, as well as other social behaviors such as sniffing and grooming.

Since social play behavior involves behaviors that are similar to other social interactions (e.g. aggressive and sexual behavior), it is important for the rats to provide signals to peers in order to insure that their intention is playful. In adolescent rats, high-

frequency vocalizations are associated with rewarding activities (Knutson et al., 2002; Palagi et al., 2016), and can also possibly act as a signal of playful intent (Wohr and Schwarting, 2013). Another behavior associated with playful intent is a jumpy gait, which is not seen during aggressive or sexual behavior. This specific type of social play behavior is seen most often beginning in the juvenile phase and continues until mid-adolescence (McCutcheon and Marinelli, 2009; Willey et al., 2009). Although these play behaviors are specific to the species, there are strain differences, which need to be considered when making experimental manipulations (Siviy et al., 2003; Reinhart et al., 2006; Siviy et al., 2011). For instance, Sprague-Dawley rats have higher baseline levels of social play compared to Wistar rats (Manduca et al., 2014).

While adolescent play behavior is abundantly observed in the animal kingdom, scientists have yet to pinpoint an obvious direct function. With specific regard to rats, evidence indicates that social play behavior facilitates the development of social, cognitive, emotional, and motor skills that allow the animal to be flexible in an ever-changing environment (Spinka et al., 2001; Trezza et al., 2010; Vanderschuren and Trezza, 2014). Evidence for this theory primarily comes from social isolation studies, in which adolescent rats are separated from social peers for a specific amount of time, ranging from a few hours to a full day. Results show that these animals show deficits in social situations. While they are capable of displaying behaviors associated with both social play and aggression, they are less likely to alter their behavior following initiation. In the case of aggressive behavior, socially isolated rats show more aggression, incur more injuries, and take longer to assume a submissive posture (van den Berg et al., 1999; Von Frijtag et al., 2002). These socially isolated rats also show deficits in cognitive

tasks, such as reversal learning, probabilistic discounting, and the 5-choice serial reaction time task (Einon and Morgan, 1977; Einon et al., 1978; Baarendse et al., 2013). Social isolation is also associated with increased drug self-administration (Alvers et al., 2012; Puhl et al., 2012), including oral morphine (Alexander et al., 1981). Conversely, environmental enrichment (where rats have access to other conspecifics and objects) positively affects social behavior (Renner and Rosenzweig, 1986; Belz et al., 2003; Tanas et al., 2015), as well as decreasing the long-term effects of prenatal stress (Morley-Fletcher et al., 2003), and can even decrease rates of drug self-administration (Alvers et al., 2012; Puhl et al., 2012). Most important for this dissertation, social enrichment has been shown to decrease opioid self-administration (Hofford et al., 2017).

Drugs of Abuse and Social Play Behavior

The effects that drugs have on various facets of adolescent social play have been widely investigated. For example, it is well established that administration of low doses of morphine generally increase rough and tumble play (Panksepp et al., 1985; Niesink and Van Ree, 1989; Normansell and Panksepp, 1990; Vanderschuren et al., 1995; Trezza and Vanderschuren, 2008), while administration of amphetamine generally decreases play (Thor and Holloway, 1983; Beatty et al., 1984; Siviy et al., 1996).

The endogenous opioid system is perhaps the most widely studied neuromodulator system of social play. The opioid receptor agonist morphine increases play behavior, and it does so not by changing the structure of the behavior, but rather by increasing the length of social interactions (Vanderschuren et al., 1995). Conversely, administration of opioid receptor antagonists (e.g. naloxone and naltrexone) decreases

social play behavior (Beatty and Costello, 1982; Siegel et al., 1985; Jalowiec et al., 1989).

These changes in play behavior altered by opioids are generally attributed to action upon MORs (Niesink and Van Ree, 1989; Vanderschuren et al., 1995; Trezza et al., 2011). Evidence for this comes from studies showing that systemic treatment with MOR agonists increase social play behavior and treatment with MOR antagonists reduce it. In contrast, treatment with delta-opioid receptor agonists has no effect on social play, and treatment with kappa-opioid receptor agonists decreases social play behavior.

While there is extensive literature on the effect that various drugs of abuse have on play behavior, there has been significantly less research on whether or not these changes in social play behavior are associated with changes in the rewarding value of drugs of abuse. Preclinical research indicates that social interaction during the adolescent period is a rewarding event. Calcagnetti and Schechter (1992) were the first to demonstrate that social play could be used as a reward with CPP. Testing for social CPP was conducted using a standard CPP chamber, as described previously. Rats were exposed to each end of the compartment on alternating days, with a social partner placed in one compartment and no partner placed in the opposite compartment. On the test session, individual rats were allowed access to all three compartments and the duration in each compartment was recorded. Upon testing in the absence of any partner, the social chamber was preferred over the isolation chamber.

Douglas and colleagues (2004) further investigated the rewarding properties of social interactions in both adolescent and adult rats, using both males and females that were housed in either individual or pair-housed conditions. Results from that study

revealed that the rewarding effect of social interaction was dependent not only on gender, but also on the housing condition of the subjects (Douglas et al., 2004). Adolescents that were housed in isolated conditions found social interaction rewarding, especially in males. In contrast, animals that were housed in social conditions did not find social interaction rewarding, although adolescents eventually began to show a preference for the chamber previously paired with a partner following multiple test sessions. Interestingly, if a socially housed animal was paired with an animal that was housed in isolation, the socially housed animal found the interaction aversive; this effect was stronger for the adolescent than the adult rats. Thus, this study demonstrated that age, sex, and housing all have a significant impact on the rewarding value of social interaction. Furthermore, this study established that individually housed, adolescent, male rats find social interaction more rewarding than any other group.

Interestingly, it appears that there is a positive correlation between the total amount of pins and pounces during conditioning sessions and the magnitude of CPP, such that the more animals play, the larger the CPP (Vanderschuren et al., 2016). In contrast to that finding, it has also been shown that social interaction without physical engagement can also lead to CPP, although an increased number of pairings is required (Kummer et al., 2011; Peartree et al., 2012). Thus, these results suggest that, while social play is not required for the development of CPP, being able to actively engage with another animal noticeably increases the rate of acquisition.

Following these findings, other researchers investigated the impact that drugs of abuse have on social CPP. While little work has been conducted with opioids, it has been demonstrated that social interaction can either serve as an alternative rewarding stimulus

compared to stimulant drugs (Fritz et al., 2011; Watanabe, 2013, Yates et al., 2013; Weiss et al., 2015), or can enhance the rewarding value of a stimulant (Thiel et al., 2008, 2009; Watanabe, 2011; Grotewold et al., 2014; Bastle et al., 2016), depending on the specific paradigm used. For instance, when cocaine and social interaction are conditioned against each other in different environments, rats show a decreased preference for both environments compared to rats that are conditioned to either cocaine or social interaction alone (Fritz et al., 2011). However, that study was conducted using adult male rats that were individually housed. As previously mentioned, adolescents find social interaction rewarding regardless of housing condition, but adults only find social interaction rewarding if they are individually housed (Douglas et al., 2004). Additionally, evidence indicates that adolescents are more sensitive to the locomotor effects of stimulants than adults (Laviola et al., 1995).

In order to address these concerns, our laboratory recently conducted several experiments to further investigate the effects that age, sex, and housing conditions have on concurrent choice for social interaction and amphetamine. Using a standard CPP paradigm, rats were exposed to a sex- and age-matched conspecific in one compartment, and amphetamine (1 mg/kg, s.c.) in the other compartment. Results revealed that adolescent male rats preferred the compartment previously paired with a social peer relative to the compartment paired with amphetamine (Yates et al., 2013). This effect was not observed in adolescent females, who showed no preference for either compartment (Weiss et al., 2015). In addition, adult rats of either gender showed preference for the amphetamine compartment compared to the social-paired compartment (Yates et al, 2013; Weiss et al., 2015). Taken together, these results suggest that

adolescent males housed in isolation are most sensitive to social CPP. Additionally, since females are known to be more sensitive to stimulant reward (Glick and Hinds, 1984; Russo et al., 2003; Reichel et al., 2012; Orsini et al., 2016), an enhanced sex-dependent preference for the amphetamine-paired compartment may have reduced choice for the social-paired compartment among females.

In another study, mice were paired together in both compartments, but only one of the mice received methamphetamine (2 mg/kg) in each compartment (i.e. Mouse A received methamphetamine in Compartment 1, and Mouse B received methamphetamine in Compartment 2). Results indicated that mice receiving a different drug treatment spend significantly more time in the non-drug paired compartment compared to mice that had no social interaction (i.e. controls; Watanabe, 2011). These results suggest that, for stimulants, it can be aversive for rodents to be paired with a social peer that has been administered saline. However, it should be noted that only one dose of methamphetamine (2 mg/kg) was used in this study.

There is also literature demonstrating that social interaction and stimulant drugs can have synergistic effects in rats, as the time spent in an environment where conditions are paired together is greater compared to either condition on its own (Thiel et al., 2008, 2009; Watanabe, 2011, 2013; Grotewold et al., 2014). In these studies, low doses of cocaine and nicotine, as well as short periods of play were used that did not produce CPP on their own. However, when these conditions were combined in one environment, there was a synergistic effect that led to robust CPP (Thiel et al., 2008, 2009). A similar effect has been seen with methamphetamine in mice. When mice were paired with a mouse that had received the same dose of methamphetamine, they spent more time in the

methamphetamine-paired compartment compared to mice that were conditioned in the absence of a social peer (Watanabe, 2011). Together, these results indicate that there is not a direct correlation between the total amount of social play and the rewarding value of a drug of abuse. Rather, the findings from these studies suggest that dose and subjective drug effects may play a more important role in CPP than the absolute reward value of the two stimuli themselves.

In contrast to stimulants, opioids can produce opposite results, at least with adult mice. When mice were conditioned using morphine (1 mg/kg) and placed in a compartment with a social peer that had also received morphine, they spent significantly more time in the non-drug compartment on the post-conditioning test, compared to controls that were conditioned in the absence of a social peer (Watanabe, 2013). This effect was dose-dependent, however, because the effect was not observed when the dose of morphine was lower (0.1 mg/kg) or higher (3 mg/kg). Further, when mice were conditioned using morphine (3 mg/kg) and placed into a compartment with a mouse that had received saline, they spent significantly more time in the drug-paired compartment compared to controls that were conditioned in the absence of a social peer (Watanabe, 2013). However, when the dose of morphine was lower (0.1 or 1.0 mg/kg), this difference was not seen. Because social facilitation was seen at 3 mg/kg and social suppression was seen at 1 mg/kg, there may be different mechanisms for social facilitation and social suppression by morphine. In any case, in contrast to mice, there is little known about social facilitation and/or suppression of morphine CPP in either adolescent or adult rats.

Together, this research indicates that drugs from different classes are affected differently by social interaction. Based on the current literature, it appears that, when both subjects are exposed to social interaction in the presence a stimulant, a synergistic effect occurs to increase the rewarding value of the condition over either state alone. In contrast, the opposite effect is seen with morphine, where social interaction in the absence of the drug is more rewarding than when both the drug and social interaction are present simultaneously. Importantly, however, most of the literature mentioned above used adult rodents, which are affected by drugs and social interaction differently than adolescents.

Social Housing and Genetic Factors in Drug Abuse Vulnerability

A host of studies have examined the influence of social housing on subsequent drug use. As mentioned earlier, animals reared in an enriched environment self-administer drugs at lower rates than rats that are reared in an isolated environment (Alvers et al., 2012; Puhl et al., 2012). Housing conditions can also influence morphine sensitization and withdrawal. Hofford et al. (2012) discovered that twice daily injections of morphine led to locomotor sensitization in both adolescent and adult rats that were housed with other rats that also received morphine treatment. Locomotor sensitization was absent if the morphine treated rats were housed with other rats that only received saline. Further research by Bates et al. (2014) showed that morphine-treated adolescent mice have decreased withdrawal symptoms (e.g. jumping) when housed with saline-treated mice, compared to when housed with other morphine-treated mice.

The effects that social housing conditions have on behavioral expressions of a morphine dependence also carry over to CPP. In adolescent mice, those housed with mice receiving only saline acquired morphine CPP more slowly than mice housed with mice receiving morphine (Cole et al., 2013; Bates et al., 2014). Furthermore, extinction of morphine CPP occurred more quickly in morphine-treated adolescent mice housed with saline-treated mice, compared to adolescent mice housed with morphine-treated mice (Bates et al., 2014).

Other research has incorporated various strains of mice that are relatively asocial (e.g. BALB/cj) and more prosocial (e.g. C57BL/6j) to examine how baseline social behavior can interact with housing to affect social and drug reward. For example, Kennedy and colleagues (2012) investigated how housing (which can affect the motivation to be with social peers) influences the effects of social context on morphine CPP. Two strains of mice, chosen for their differences is sociability, were housed either in isolation or mixed-sex social groups (two males, two females). The social environment was then manipulated during each conditioning session. The mice experienced morphine/saline conditioning either in a social group or as isolated individuals. Results revealed that the prosocial C57BL/6j mice showed robust morphine CPP regardless of social condition, with the exception of animals that were housed in isolation and then conditioned in a social environment. Within this latter group of mice, there was a significant preference for the compartment paired with social interaction and saline compared to the compartment paired with social interaction and morphine. These results are similar to those found by Watanabe (2013), suggesting that with opioids, social interaction can act as a strong alternative reward. Conversely, the asocial BALB/cj

mice showed robust morphine CPP, regardless of social manipulations, suggesting that there is a threshold level of social motivation required for social interaction to affect morphine reward.

Peer Influences in Drug Self-Administration

Other studies have examined how the presence of a social peer during the drug self-administration session influences drug intake. Since intravenous drug self-administration requires a catheter attached to an infusion system, placing two catheterized rats into the same operant chamber is not practical from a logistical point of view. In addition, even with orally self-administered drugs, putting two rats into an operant chamber simultaneously would lead to responses made by both individuals, with no accountability for which rat was making each response. To avoid these logistical problems, a novel apparatus has been devised that consists of two standard operant chambers, connected by removing one side on each and replacing it with wire mesh (Smith, 2012). This allows the animals to have limited tactile, olfactory, auditory, and visual contact with one another, but prevents one rat from interfering with the responses made by the other. Thus, these social chambers allow for two animals to run at the same time and allow the experimenter to collect data from the influence of social interaction during task performance.

Using these social chambers, Smith (2012) assessed peer influences on cocaine self-administration. Rats lived in the chambers throughout the experiment, with one rat in each chamber. Half of the rats were paired with a partner that also had access to cocaine, and the other half had a non-drug paired partner. Results showed that self-

administration was facilitated in rats if both partners had access to cocaine, but was inhibited if only one had access. These findings indicate that it is not simply the presence of a peer that is important for initial acquisition of drug self-administration, but that the peer must also have access to the drug acquisition to be facilitated.

In another experiment using a social chamber apparatus, Gipson et al. (2011) first trained rats to self-administer amphetamine in the absence of any social partner. After stable responding was established, a novel same-sex partner was introduced into the adjacent chamber during the self-administration session; the social partner did not have access to drug. Results showed that amphetamine self-administration of the trained rat was increased in the presence of the partner, but that this social facilitation did not occur past the first self-administration session. Importantly, social facilitation of responding for sucrose pellets did not occur, indicating that the effect was specific to amphetamine. While it is not yet clear if social facilitation also occurs with opioid self-administration, results to date parallel the human literature showing an increase in drug use in the presence of using peers and further suggesting that animal models may be useful for studying social influences on drug use. They also provide evidence that social influence can have either a positive or negative impact on drug use.

Recently, an operant conditioning paradigm for social play reinforcement in rats has been developed where rats are trained to lever press for brief episodes of social play following various periods of isolation (Achterberg et al., 2016). During this task, a PR schedule of reinforcement was implemented to assess the motivational properties of social play. The results of the study showed that break points were positively correlated with isolation time, such that the longer an animal was in isolation, the higher their break

point was. Furthermore, longer periods of isolation also lead to increases in social play behavior. This latter finding is consistent with other literature showing that social isolation increases social play (Niesink and Van Ree, 1989; Panksepp and Beatty, 1980; Vanderschuren et al., 1995).

Drugs of Abuse, Social Play, and the Nucleus Accumbens

The nucleus accumbens (NAc) is well known to be a key neuroanatomical substrate involved in drug reward. The NAc is an integral part of the basal ganglia, which generates voluntary motor output on the basis of cognitive information (Mogenson et al., 1980; Cardinal et al., 2002; Voorn et al., 2004). Rats will self-administer opioids directly into the NAc (Olds, 1982). Acute morphine administration also increases dopamine release in the NAc (Di Chiara and Imperato, 1988; Johnson and North, 1992). It is believed that this extracellular dopamine increase occurs via inhibition of GABAergic neurons in the VTA and rostromedial tegmental nucleus that synapse on dopaminergic neurons in the NAc (Tepper et al., 1995; Jalabert et al., 2011). Interestingly, autoradiographic research has shown that neither acute nor chronic administration of morphine leads to changes in MOR density in the NAc (core and shell) of rats (Turchan et al., 1999).

The NAc also plays a key role in opioid withdrawal. Microinjections of naloxone (a MOR antagonist) into the NAc leads to conditioned place aversion (Koob et al., 1992). Dopamine release is decreased in the NAc during morphine withdrawal (Rossetti et al., 1992; Diana et al., 1995; Bonci and Williams, 1997), and can be attenuated by administration of a D_2-like receptor agonist directly into the NAc (Harris and Aston-

Jones, 1994). The NAc's GABAergic neurons also play a role in opioid withdrawal (Chieng and Williams, 1998). Agonists of $GABA_A$ receptors (e.g. muscimol) and $GABA_B$ receptors (e.g. baclofen) are able to attenuate withdrawal symptoms in rodents of both sexes (Diaz et al., 2006; Cabral et al.. 2009).

The NAc has also been identified as an important region for social play behavior. In nonhuman primates, for example, striatum size is correlated with the rate of social play behavior (Graham, 2011). In adolescent rats, social play behavior reduces in vivo opioid receptor binding, which suggests that social play leads to the release of opioid peptides (Vanderschuren et al., 1995). Furthermore, administration of a MOR agonist (e.g. morphine or DAMGO) directly into the NAc increases play behavior, whereas MOR antagonists (e.g. CTAP) decrease social play behavior (Trezza et al., 2011). Another study found that an infusion of amphetamine and apomorphine (a dopamine receptor agonist) directly into the NAc also increases social play, and that alpha-flupenthixol (a non-selective dopamine receptor antagonist) inhibits the effects of morphine on social play behavior (Manduca et al., 2016). Inactivation of the NAc core with baclofen/muscimol increased social play duration (Carlezon and Thomas, 2009), further implicating a critical role for this structure in social play.

Social play also increases cFos expression in multiple brain regions, including the nucleus accumbens (Gordon et al., 2002; van Kerkhof et al., 2014). cFos is a member of the FOS family of genes that, as mentioned earlier, function as regulators of cell proliferation, differentiation, and transformation. cFos is considered an immediate early gene, meaning that it is activated relatively quickly by cellular stimuli. Because cFos is often expressed when neurons fire action potentials (Dragunow and Faull, 1989;

VanElzakker et al., 2008), upregulation of cFos mRNA indicates recent activity (Day et al., 2008). Most research on social play induced cFos activation, however, has been done following acute exposure to social play after a set period of isolation. To our knowledge, there are no studies that have examined the changes in cFos activation following multiple episodes of social play.

Drugs of Abuse, Social Play, and the Dorsal Striatum

The dorsal striatum is made up of the caudate nucleus and putamen. Like the NAc, the dStr is also part of the basal ganglia. Also like the NAc, the dStr plays role in social play behavior. Previous research has shown that social play during adolescence leads to an increase in cFos+ cells in the dStr following 24 hrs of isolation (van Kerkhof et al., 2013). There are, however, differences in the roles of the dStr and NAc with regard to social behavior. Where the NAc appears to be involved in externally guided behavior, the dStr is more involved in internally guided behavior. That is, the NAc is more likely to be activated when an individual is making a change in behavior based on the behavior of those around them, whereas the dStr is activated when the individual changes behavior based on internal cues and the environment (van den Bos, 2015).

With regard to opioids, electrolytic lesions to the dStr significantly decrease responding for low-doses of morphine (0.125 and 0.5 mg/kg, i.v.) under a progressive ratio schedule of reinforcement, although not to the same extent as lesions of the NAc (Suto et al., 2011). In another study, C57BL/J mice that received an injection of morphine (20 mg/kg, s.c.) showed significant increases in both dopamine and its main metabolite, DOPAC, in the dStr (Fadda et al., 2005). The dStr also appears to be

involved in symptoms of opioid withdrawal. Rats dependent on morphine that were then administered naloxone (10 μg) directly into the dStr elicited symptoms of withdrawal, including paw tremor, chewing, teeth chattering, and ejaculation (Tremblay and Charton, 1981).

Statement of Hypothesis

The purpose of this dissertation was to investigate the interaction between morphine and social play on CPP in adolescent male rats. This population was chosen due to previous literature showing that adolescent males find social interaction/play more rewarding that adults and females (Douglass et al., 2004; Yates et al., 2013; Weiss et al. 2015). In order to investigate this relationship, CPP procedures were used to directly compare how social play can affect morphine CPP. Immunofluorescence was also used to examine similarities and differences in neural activation in NAc (core and shell) and dStr following multiple exposures to morphine and/or social play.

We hypothesized that during behavioral experiments, there would be a synergistic interaction between morphine and social play. For the immunofluorescence experiments, it was hypothesized that cFos activation would differ between morphine and social play groups. Specifically, it was hypothesized that social play would increase cFos+ cells more than morphine (primarily due to the low doses of morphine used). Furthermore, when morphine and social play are combined, it was hypothesized that there would be a synergistic effect on neural activation.

We believe the present research question is relevant for a number of reasons. First, adolescents are at the highest risk of initiating drug use (Kandel and Logan, 1984). This is of particular importance due to strong correlation between age of initiation and the

likelihood of becoming addicted to drugs of abuse (Bahr, 2005). Second, initiation typically occurs in a social situation (Bahr, 2005; Kandel and Logan, 1984). Therefore, the interaction between a drug of abuse and social experience may have an impact on whether a person continues to use illicit substances. Third, the data from this dissertation may be useful from a clinical perspective when targeting adolescent-age males through programs designed to decrease drug use within this population.

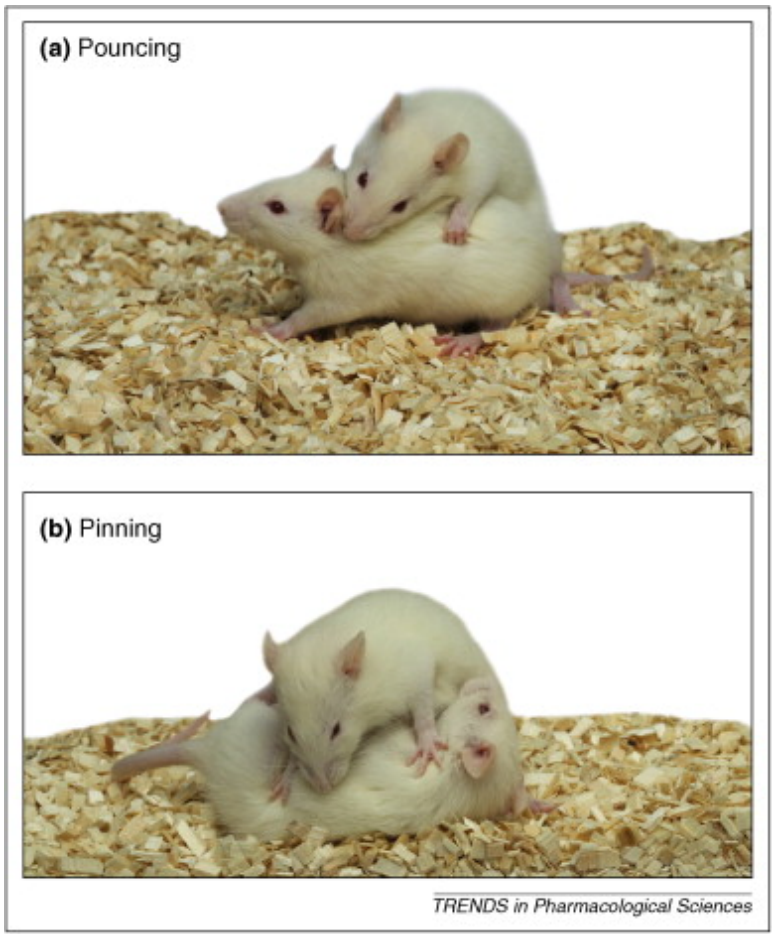

Figure 1. The most characteristic postures of social play in adolescent rats. A) Pouncing. B) Pinning. Taken from Trezza et al., 2010.

CHAPTER 2

Preliminary Experiments

Preliminary Experiment 1: Low Dose Morphine CPP

Rationale

Doses of morphine that are used to investigate behavioral changes in social play tend to be low (≤ 1 mg/kg). As such, there is very little research on whether or not these doses can reliably produce CPP. Furthermore, most of the literature focuses on drug reward in adult rodents. As adolescent rats can show differences in sensitivity to opioids (Kennedy et al., 2011; Koek et al., 2012, 2014), it is important to investigate whether or not doses that are applicable to social play behavior are also applicable to drug reward. Thus, this experiment assessed morphine CPP at doses from 0.1 to 1 mg/kg. It was hypothesized that 1 mg/kg morphine would produce CPP.

Animals

Male Sprague Dawley rats (n=47; Harlan Industries, Indianapolis, IN, USA) were used. Rats arrived at postnatal day (PND) 21, and were individually housed for the entire experiment. Rats were housed in a temperature- and humidity-controlled colony room that was maintained on a light-dark cycle in which lights were on from 7:00 a.m. to 7:00 p.m. Rats were allowed to acclimate to the colony for 7 days before the start of the experiment. Rats had ad libitum access to food and water in their home cage for the entire experiment. All procedures were in accordance with the "Guide for the Care and

Use of Laboratory Animals" and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Apparatus

A 3-compartment chamber (68 x 21 x 21 cm; ENV-013; MED Associates, St. Albans, VT) located inside a sound-attenuating chamber (ENC-020 M; MED Associates) was used to measure CPP. The three compartments were separated by sliding guillotine doors. The middle compartment (12 x 21 x 21 cm) had gray walls with a smooth gray PVC floor. The end compartments (28 x 21 x21 cm) provided distinct contexts, with one compartment having black walls with a stainless steel rod floor (6 gauge rods, spaced 1.55 cm apart) and the other end compartment having white walls with a stainless steel mesh floor (16 gauge mesh, spaced 1.27 cm apart). Recessed trays for bedding were located 2 cm below each compartment. A computer controlled the experimental trial using MED-IV software. A series of infrared photobeams (6 beams in the black and white compartments and 3 beams in the gray compartment) were used to detect the rats' presence in a particular compartment and record the amount of time spent in that compartment, as well as to record locomotor activity during conditioning trials.

Procedure

Rats were tested for CPP using a 10-day procedure. On day 1 (pre-conditioning test), the guillotine doors were opened, and adolescent (PND 28) rats were placed in the center gray compartment and were allowed to explore all three compartments for 15 min. The duration spent in each compartment was recorded. Following the pre-conditioning

test, rats went through 8 conditioning trials (days 2-9) in which rats were confined by the guillotine door to either the black or white compartment for 30 min. On every other day, each rat was given an injection of morphine (0.1, 0.3, or 1.0 s.c.) or saline (s.c.) and was placed immediately into either the white or black compartment. On alternating days, each rat received saline (s.c.) and was placed immediately into the opposite chamber. Doses and route of administration of morphine were chosen based on previous literature investigating the effects of morphine on adolescent play behavior (Vanderschuren et al., 1995; Manduca et al., 2014). On the post-conditioning test (day 10), each rat was placed in the center gray compartment with the guillotine doors open and was allowed to explore all three compartments for 15 min. The chamber in which rats received morphine was counterbalanced across dose. Time spend in each compartment was recorded.

Drug

Morphine sulfate (gift from NIDA) was prepared in sterile 0.9% NaCl (saline) and was injected subcutaneously in a volume of 1ml/kg. The dose was calculated based on the salt weight.

Statistical Analyses

A preference ratio was calculated by dividing the amount of time spent in the compartment paired with morphine by the time spent in both the white and black compartments. A preference ratio of 0.5 indicated no preference for either compartment, whereas ratios above 0.5 designated a place preference and ratios below 0.5 designated a place aversion. Preference ratios were analyzed by a one-way ANOVA, with dose as a

factor, followed by Student's *t* tests to determine if each preference ratio was significantly different from saline controls. In addition, in order to determine if the sample means for each treatment group was significantly different from a preference ratio of 0.5, exploratory one-sample t-tests were performed as described previously (Yates et al., 2013). All tests were considered significant at $p < .05$.

Results and Discussion

Results from the 4 different treatment groups are summarized in Figure 2. ANOVA revealed no significant effect of dose ($F(3,42) = 1.53$, $p = 0.221$). Planned comparison independent samples and one-sample t-tests revealed that adolescent rats developed morphine CPP at the 0.3 mg/kg s.c. dose ($t(22) = 2.152$, $p = 0.044$ and $t(11) = 3.579$, $p = 0.004$, respectively), but not at either 0.1 or 1.0 mg/kg. Given the unusual inverted U-shaped curve and the lack of literature surrounding CPP at these low doses, it is possible that the dose range selected is at the threshold required to produce CPP, thus requiring the relatively large number of rats used in this study (n=11-12 per group). In particular, we cannot rule out the possibility that the significant effect observed at the 0.3 mg/kg dose reflects a Type-I Error, where the null hypothesis has been incorrectly rejected (i.e. a "false positive"). Conversely, it is possible that the non-significant effect observed at 1.0 mg/kg reflects a Type-II Error, where the null hypothesis has been incorrectly retained (i.e. a "false negative"). However, if the inverted-U is a robust and reproducible finding, these results would suggest two different mechanisms may be engaged within this dose range. For example, perhaps the neural substrates responsible for increases in social play behavior were engaged primarily by the 0.3 mg/kg dose,

whereas the higher dose (1.0 mg/kg) also began to engage the neural substrates involved analgesia and catalepsia. Because this dissertation is more interested in doses that have abuse liability, the primary experiments used doses that are socially relevant, as well as doses that have already been shown to result in reliable and robust CPP using a fewer number of rats.

Preliminary Experiment 2: Morphine and Social Interaction Induced cFos Expression

Rationale

Although it is established that both morphine and social play lead to increases in cFos expression (Gordon et al., 2002; Van Kerkhof et al., 2014), there is no literature on the effect that combining these two rewarding conditions has on neural activation. Therefore, this experiment aimed to investigate changes in cFos expression following exposure to morphine and/or social interaction, using a dose of morphine (0.3 mg/kg) that has been shown to reliably increase social play behavior (Vanderschuren et al. 1995; Manduca et al., 2014) and that produced CPP in Preliminary Experiment 1. It was hypothesized that there would be a significant increase in cFos expression following social play, and that the addition of morphine would lead to an enhancement in cFos expression.

cFos was chosen over other early gene markers (e.g. ERG-1) for a number of reasons. First, Fos proteins are more extensively used to examine changes following drug administration (Beckmann and Wilce, 1997), while ERG is more common in literature that examines sensory stimuli (Herdegen and Leah, 1998). Secondly, while cFos and ERG-1 both show induction of MRNA and protein at similar time points, cFos

is more transient, with levels returning to baseline more quickly (Torres et al., 1998; Zangehpour and Chaudhuri, 2002). This means that it is less likely that differences in cFos expression between groups are due to an unrelated stimulus, compared to ERG-1.

Animals

Male Sprague Dawley rats (n=46; Harlan Industries, Indianapolis, IN, USA) were used. Twenty-two rats were from Preliminary Experiment 1 and 24 rats arrived at postnatal day (PND) 36. Rats were kept in their home cages, with free access to food and water, for 1 week prior to the beginning of Preliminary Experiment 2. All rats were PND 43 at the beginning of the experiment. All procedures were in accordance with the "Guide for the Care and Use of Laboratory Animals" and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Apparatus

The apparatus was a commercial open field chamber used to measure locomotor activity (ENV-515S-A; MED Associates, St. Albans, VT). Each chamber was made of clear acrylic and measured 42 x 42 x 30 cm.

Behavior

Rats were allowed to acclimate to the test chambers for 30 min the day prior to testing. On testing day, animals (PND 45) were assigned to one of four groups: (1) saline (s.c.; n=5; SAL), (2) morphine (0.3 mg/kg s.c.; n=5; MOR), (3) saline and social interaction (s.c.; n=6; SOC), or (4) morphine and social interaction (0.3 mg/kg s.c.; n=6;

MOR + SOC). Rats were injected with either saline or morphine and immediately placed into a test chamber either alone or with a weight- and age-matched peer for 30 min.

Drug

Morphine sulfate (gift from NIDA) was prepared in sterile 0.9% NaCl (saline) and was injected subcutaneously in a volume of 1ml/kg. The dose was calculated based on the salt weight.

Perfusion and brain sampling

Following the 30 min of being confined in the test chambers, rats were placed back into their home cages for 90 min. Rats were then deeply anesthetized with ketamine/xylazine cocktail and perfused transcardially with cold saline solution (0.9 percent NaCl) followed by cold 4 percent paraformaldehyde. After perfusion, brains were extracted and placed in 4 percent paraformaldehyde solution overnight, followed by 30 percent sucrose solution for 48-72 hours or until immersion. Using a frozen medium (Hitsoprep, Fisher Scientific, USA), brains were then immersed under liquid nitrogen for 20 seconds. Consecutive coronal sections at 40 μm were obtained using a cryostat (Ag Protect Leica CM 1860, Leica Biosystems, USA) and stored in a freezing solution (30 percent ethylene glycol, 25 percent glycerol and 30 percent sucrose in Phosphate Buffer Saline (PBS) at -20 °C.

Immunofluorescence

Free-floating sections were rinsed three times for 15 min each with Triton X-100:1x tris-PBS (TPBS; Tris–HCl 10 mM, sodium phosphate buffer 10 mM, 0.9 percent NaCl, pH 7.4) and incubated with the following primary antibody: rabbit polyclonal anti-cFos antibody (RPCA-c-Fos-AP, Encor Biotechnology, FL, USA), diluted 1:200. Incubations were at 4°C for 24 hours in TPBS 0.1 M Triton X-100 containing 3 percent of donkey serum. After rinsing, tissue was incubated for 2 hours at room temperature protected from light with the following secondary antibody with conjugated fluorochrome: Alexa Fluor 488 donkey anti-rabbit (A-21206, Life Technologies, CA, USA), diluted 1:250. Once the fluorescence reaction occurred, sections were mounted using Mowiol 4-88 reagent (475904-100GM, EMD Millipore, USA).

Neuronal Counting

Three fluorescent-labeled sections for the NAc (core and shell) were examined using a confocal microscope (Nikon Eclipse-1C, Nikon Instruments, Americas, USA) by an observer who was not aware of the treatment condition for each section. Confocal images were taken in single XY planes, 1-µm thick, at a resolution of 1024 × 1024 and 100-Hz speed. Laser intensity, gain and offset were maintained constant in each analysis. Image analyses were made using the Nikon NIS Elements software (AR 4.20.02 64 bit) software. In each brain area, three selected slices for c-Fos-IR were estimated in a region of interest of 20 000 μm^2. For c-Fos-IR, we considered a cell positive if it showed full nuclear and intense labeling. Immunofluorescence results are represented as the average

cell counts collected from the three brain slices selected. Representative images were selected based on similarity to the mean of each group.

Statistical Analyses

The number of cFos+ cells were analyzed by a repeated measure ANOVA, with drug and social condition as between subject factors, and region as a within subject factor. Significant effects were further investigated with student's t tests.

Results and Discussion

Due to unusable tissue, 4 animals were excluded from analyses. A 2 x 2 (drug x social condition) repeated measure ANOVA, with region as the within subject factor, revealed a significant triple interaction ($F(35) = 7.466$, $p = 0.01$). As shown in Figure 3, student's t tests revealed significant increases in cFos expression in the NAc core compared to saline in both the Social ($t(18) = 2.60$, $p = 0.018$) and MOR + SOC groups ($t(18) = 2.98$, $p = 0.008$), as well as a trend towards significance between the Morphine and MOR + SOC groups ($t(18) = 1.94$, $p = 0.068$). In the NAc shell, the MOR + SOC group had significantly more cFos expression compared to both the Saline ($t(18) = 2.382$, $p = 0.029$) and Morphine groups ($t(18) = 2.395$, $p = 0.027$). These data and representative images can be seen in Figures 3-6.

These data suggest a possible synergistic effect between morphine and social interaction, at least in NAc shell. Surprisingly, however, morphine alone did not increase expression of cFos in either the NAc core or shell. The most likely explanation for lack of statistically significant increases in cFos expression may be that the dose of morphine

used (0.3 mg/kg) was too low. In juvenile rats, previous research has shown that reliable increases in cFos expression due to morphine does not begin to occur until a dose of 10 mg/kg or higher is administered (Gordon et al., 2002).

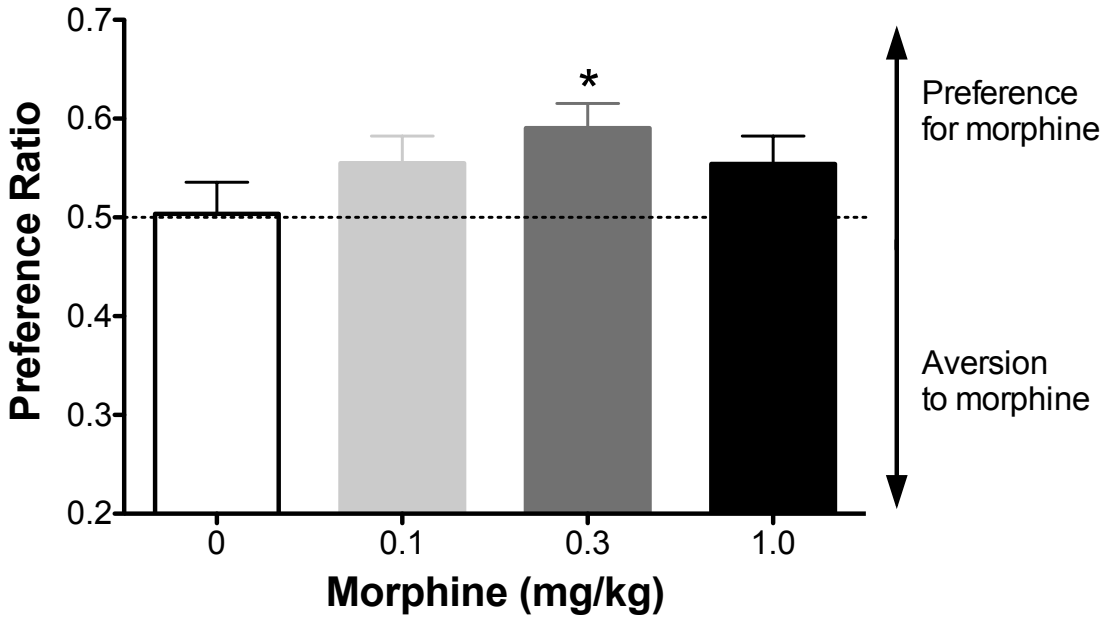

Figure 2. Preference ratio for adolescent males following morphine in Preliminary Experiment 1. Bar represents mean (±SEM) preference ratio, with the dashed line indicating equal preference for both compartments. Asterisk (*) represents a difference relative to saline controls, $p < 0.05$. n = 12 per treatment group.

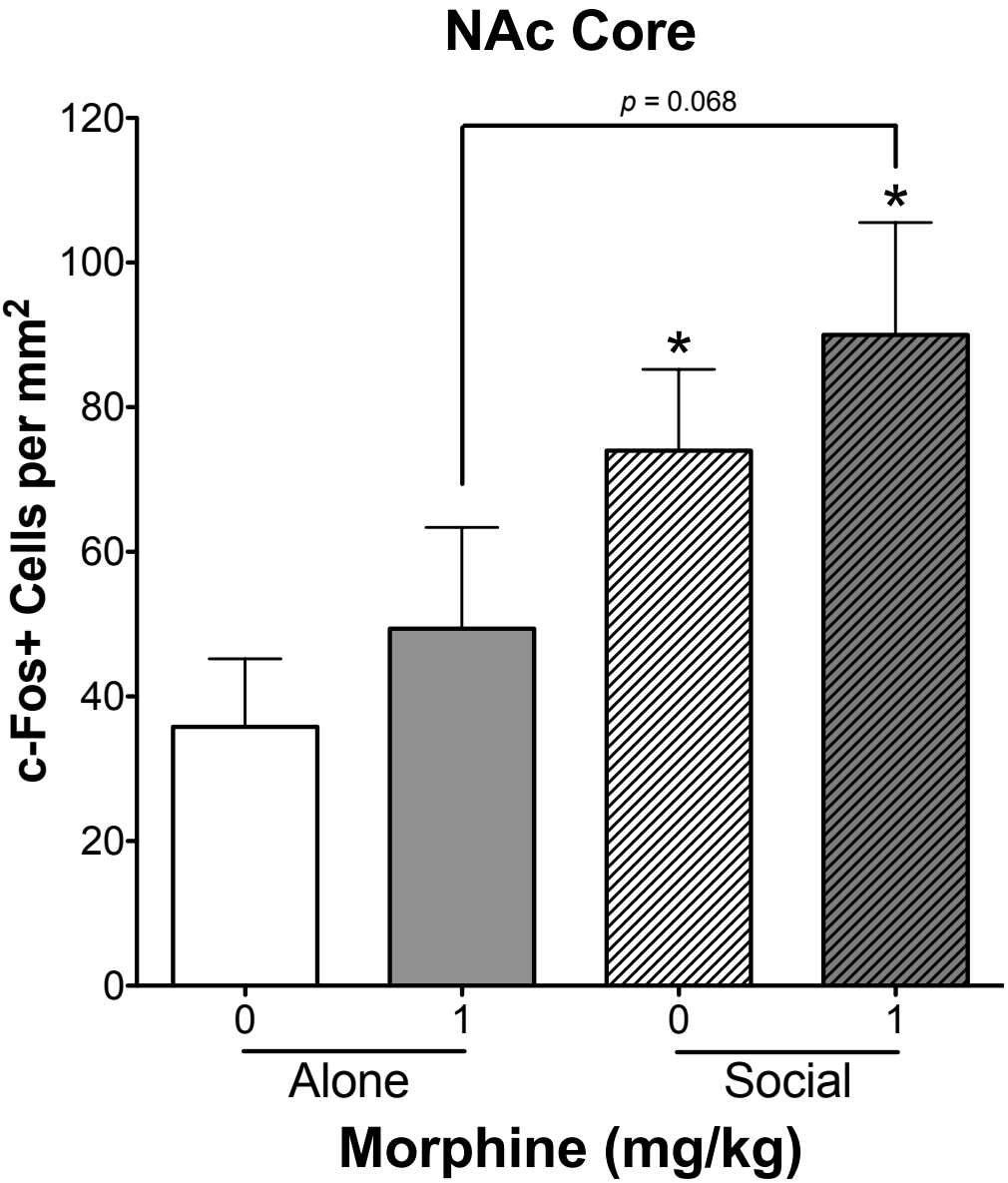

Figure 3. Immunofluorescence results from NAc core showing the mean (±SEM) number of cFos+ cells per mm^2 at 120 min after drug administration and/or social interaction in Preliminary Experiment 2. * $p < 0.05$ compared to saline control. n = 9 per treatment group.

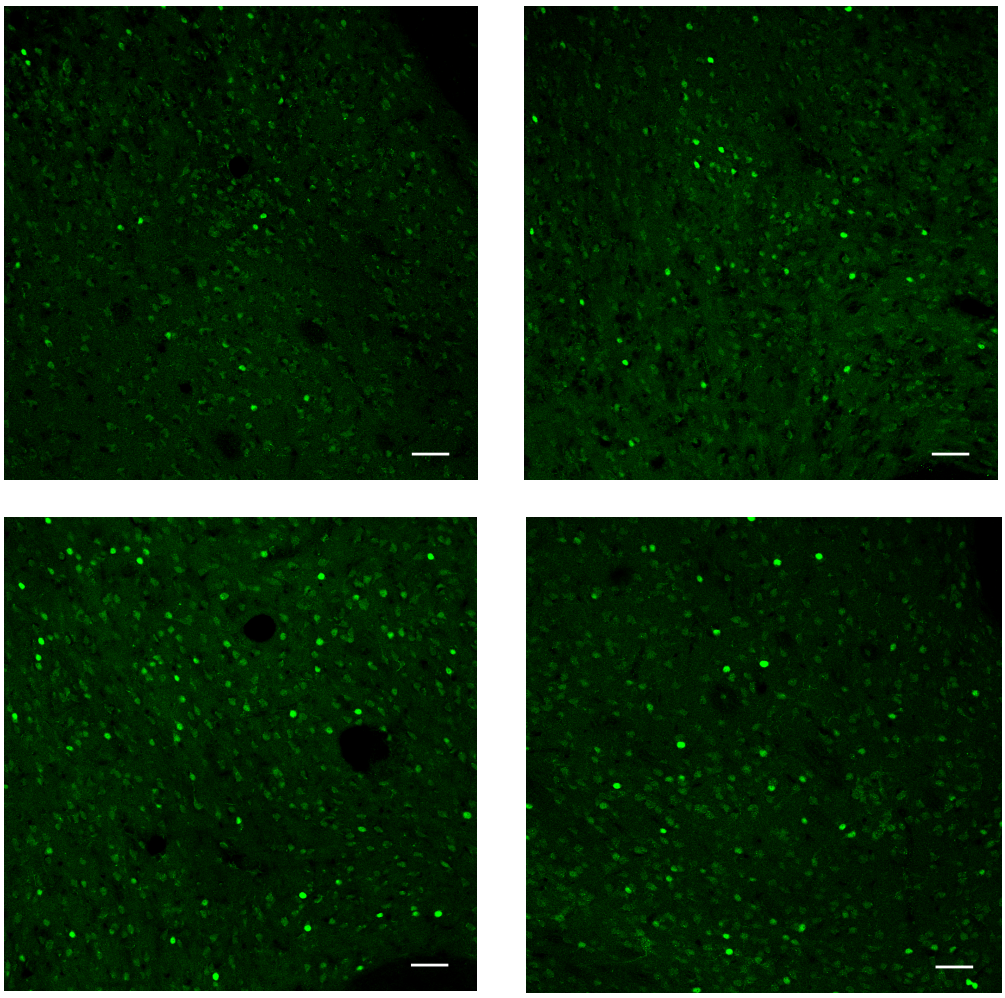

Figure 4. Confocal images showing representative fluorescent cells in the NAc core expressing cFos in Preliminary Experiment 2. Going clockwise from the top left: saline, 0.3 mg/kg morphine, social, and 0.3 mg/kg morphine with social. Scale bar represents 50 μm. Images were acquired with a 20x lens.

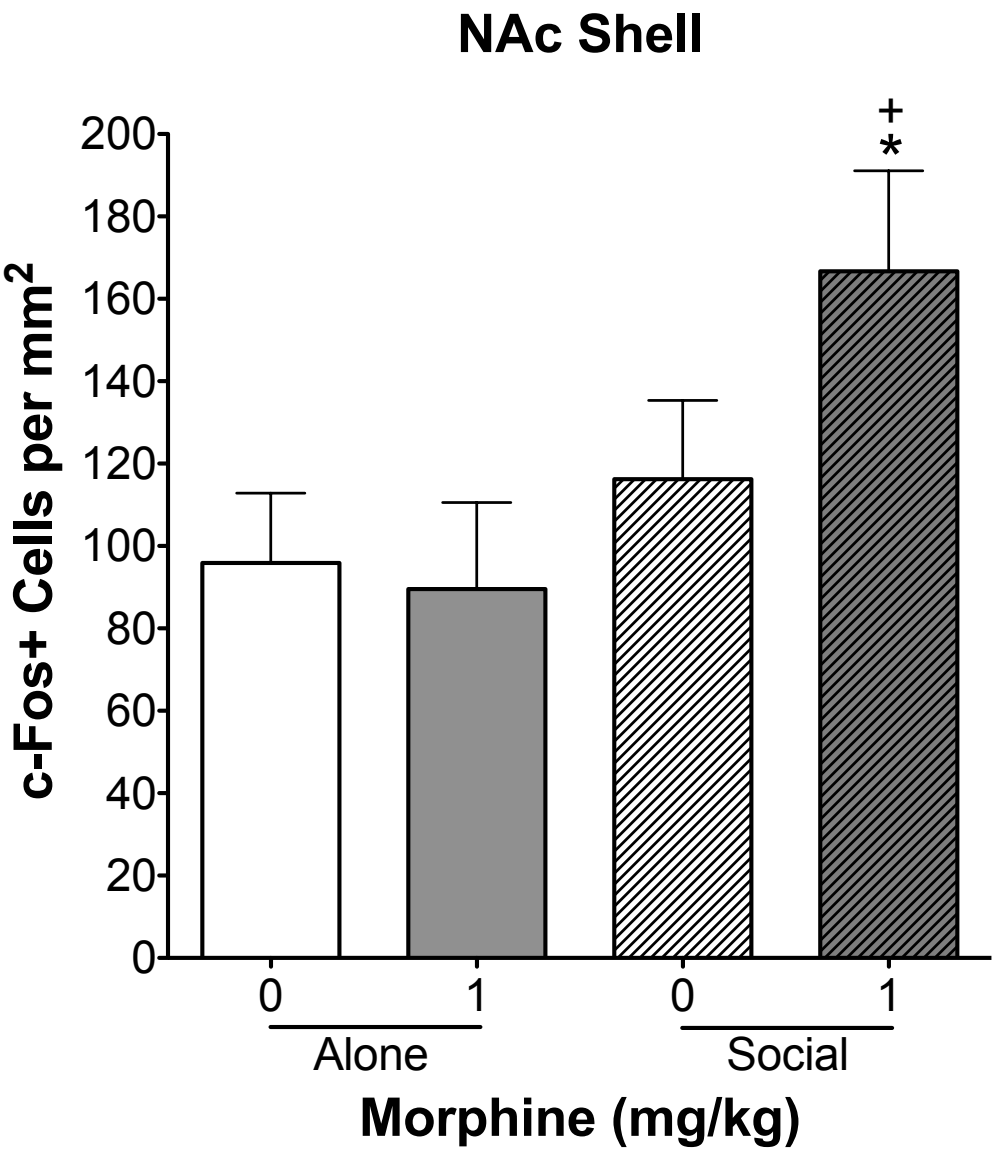

Figure 5. Immunofluorescence results from NAc shell showing the mean (±SEM) number of cFos+ cells per mm^2 at 120 min after drug administration and/or social interaction in Preliminary Experiment 2. * $p < 0.05$ compared to saline control. + $p < 0.05$ compared to morphine group. n = 9 per treatment group.

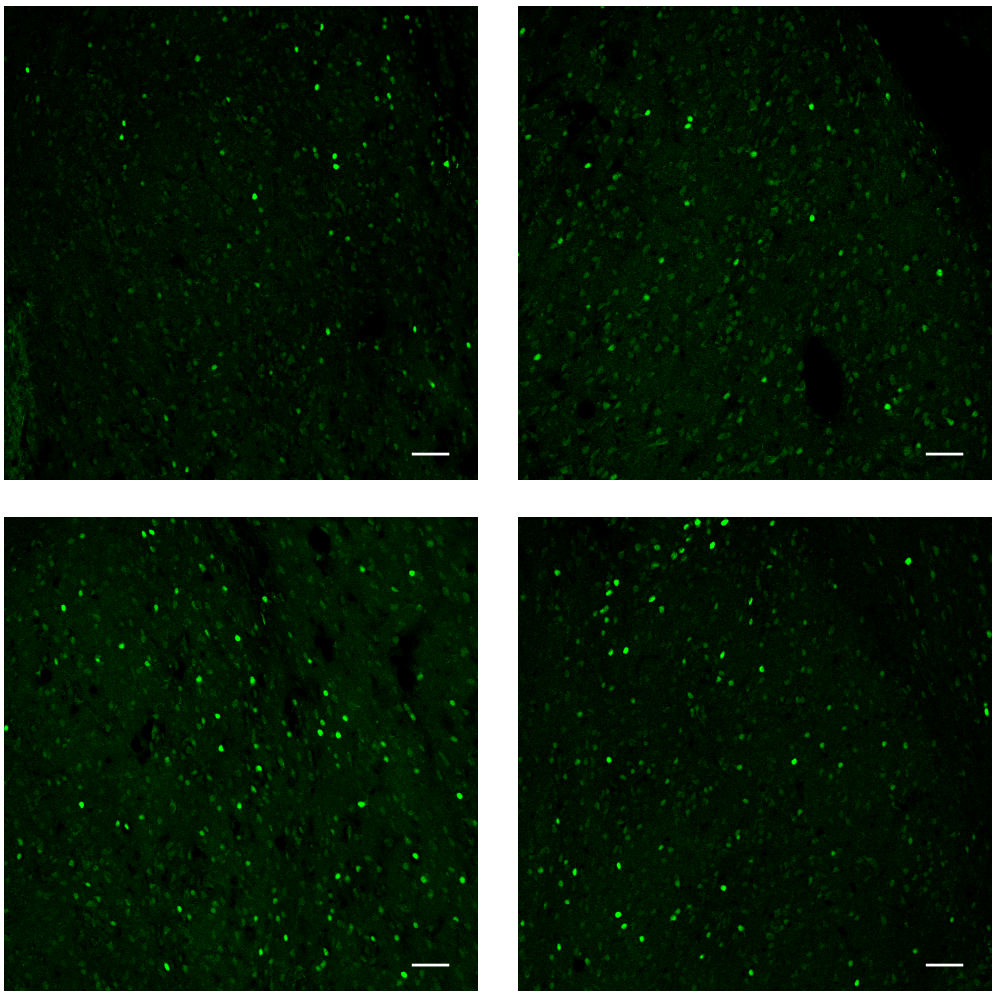

Figure 6. Confocal images showing representative fluorescent cells in the NAc shell expressing cFos in Preliminary Experiment 2. Going clockwise from the top left: saline, 0.3 mg/kg morphine, social, and 0.3 mg/kg morphine with social. Scale bar represents 50 μm. Images were acquired with a 20x lens. n = 5 per treatment group.

CHAPTER 3

Primary Experiments

Experiment 1: Effect of Social Interaction on Morphine Reward using CPP in Adolescent Male Rats with Standard Flooring

Rationale

Although there is abundant research on morphine and social CPP separately, there is little research that has investigated the effects of both conditions when paired together. Importantly, the primary emphasis has been placed on examining how drugs alter social behavior, rather than on how social play can alter drug reward (Achterberg et al., 2014; Trezza et al., 2014; Blanco-Gandia et al., 2015). In those studies, examining the effects of drugs on social play typically has used low doses of drugs that are at or below the doses required to induce robust reward. As shown in Preliminary Experiment 1, for example, the dose range of morphine used to induce social play (≤ 1 mg/kg) is only marginally effective in producing CPP (see Figure 2). As male adolescents are clinically the most likely group to initiate drug use, it is important to investigate the effects that social interactions may have on drug reward. Thus, this experiment assessed the influence of male social peers on morphine reward using doses relevant to social play, as well as doses previously shown to produce CPP. It was hypothesized that both 3 mg/kg morphine and social play alone would produce CPP, and that the compound stimulus of morphine and social play would produce greater CPP than either condition alone.

Animals

Adolescent male rats (PND 21 upon arrival; n=72) were housed individually in a temperature- and humidity-controlled colony room that was maintained on a light-dark cycle in which lights were on from 7:00 a.m. to 7:00 p.m. Rats were allowed to acclimate to the colony for 7 days before the start of the experiment. Rats had ad libitum access to food and water in their home cage for the entire experiment.

Apparatus

The apparatus was identical to the CPP chambers used in Preliminary Experiment 1.

Procedure

Rats were tested using a modified CPP procedure. On day 1 (pre-conditioning test), the guillotine doors were opened, and adolescent (PND 28) rats were placed in the center gray compartment and allowed to explore all three compartments for 15 min. The duration spent in each compartment was recorded. Following the pre-conditioning test, rats went through 8 conditioning trials (days 2-9) in which rats were confined by the guillotine door to either the black or white compartment for 20 min. The trial duration was chosen because it reliably establishes morphine CPP (see Bardo et al., 1995 for a meta-analysis). Although a 10 min trial duration is commonly used to establish social interaction CPP (Calcagnetti and Schechter, 1992; Douglas et al., 2004; Peartree et al., 2012; Van den Berg et al., 1999), other work has shown that trial duration does not affect social interaction CPP (Thiel et al., 2008).

Rats were randomly assigned to one of 6 different experimental groups (See Table 1). Half of the rats (n=36) were given an injection of morphine (0, 1, or 3 mg/kg; s.c.) and placed immediately into either the white or black compartment, every other day. On alternating days, each rat received saline (s.c.) and was placed immediately into the opposite compartment. The other half of the rats (n=36) were given an injection of morphine (0, 1, or 3 mg/kg; s.c.) and were placed immediately into either the white or black compartment that contained a weight-and age-matched male partner, every other day. On alternating days, each rat received saline (s.c.) and was placed immediately into the opposite compartment with no social partner. Rats received the same dose of morphine throughout the entire experiment, and the chamber in which rats received morphine (and/or social interaction) was counterbalanced. Rats that received a compartment paired with social interaction received the same partner during each conditioning session. Doses and route of administration of morphine were chosen based on previous literature (Bardo et al., 1995; Campbell et al., 2000). During the post-conditioning test (day 10), each rat was placed in the center gray compartment with the guillotine doors open and allowed to explore all three compartments for 15 min. Time spent in each compartment was recorded. Rats then underwent an additional round of CPP conditioning (days 11-18) and a second post-conditioning test (day 19).

In order to assess the effect of morphine on social play, during the first and fourth sessions where social interaction occurred, animals were recorded using a camera located above the compartment. A blind observer coded social play behavior for occurrences of pinning, pouncing, and boxing as described previously (Vanderschuren et al., 1995). See Figure 7 for a timeline of the experiment.

Drug

Morphine sulfate (gift from NIDA) was prepared in sterile 0.9% NaCl (saline) and was injected subcutaneously in a volume of 1ml/kg. The dose was calculated based on the salt weight.

Statistical Analyses

A preference ratio was calculated by dividing the amount of time spent in the compartment paired with morphine, or morphine and social interaction, by the time spent in both the white and black compartments. A preference ratio of 0.5 indicated no preference for either compartment, with ratios above 0.5 designating a preference and ratios below 0.5 designating an aversion.

Preference ratios were analyzed with ANOVA, with dose and social interaction as between-subject factors. Significant interactions were probed with student's *t* tests. Independent samples t-tests were performed to determine if each preference ratio was significantly different from saline controls. As an exploratory measure, one-sample *t*-tests were performed to determine if each preference ratio was significantly different from 0.5. Social play behaviors were analyzed separately with one-way ANOVAs, with dose as a between-subject factor. Significant effects were probed with student's t tests. All tests were considered significant at $p < .05$.

Results

Results for the first post-conditioning test are summarized in Figure 8. A 3 x 2 univariate ANOVA (drug x social condition) revealed a significant main effect of drug

($F(2,66) = 4.563$, $p = 0.014$), but no significant interaction. Independent samples t-tests revealed that 1 mg/kg morphine produced CPP ($t(22) = 2.696$, $p = 0.013$), as well as when morphine (1 and 3 mg/kg) was combined with social interaction ($t(22) = 2.973$, $p = 0.007$); $t(22) = 2.864$, $p = 0.009$). One sample t-tests revealed that both 1 and 3 mg/kg morphine produced significant CPP ($t(11) = 4.006$, $p = 0.002$; $t(11) = 2.314$, $p = 0.041$), as well as when morphine (1 and 3 mg/kg) was combined with social interaction ($t(11) = 3.7$, $p = 0.003$; $t(11) = 4.428$, $p = 0.001$). Thus, animals that received morphine, either with or without social interaction, developed CPP after 8 conditioning trials.

Results for the second post-conditioning test are summarized in Figure 9. A 3 x 2 univariate ANOVA (drug x social condition) revealed a significant main effect of both drug ($F(2,65) = 3.705$, $p = 0.03$) and social condition ($F(1,65) = 4.394$, $p = 0.04$), but no significant interaction. Planned independent samples and one sample t-tests revealed that rats that received both social interaction and either 1 or 3 mg/kg morphine showed significant CPP ($t(22) = 2.762$, $p = 0.011$ and $t(11) = 4.752$, $p = 0.001$; $t(22) = 4.243$, $p < 0.001$ and $t(11) = 7.870$, $p < 0.001$), but rats that received morphine alone no longer showed CPP. Thus, although the interaction effect was not statistically significant, planned comparison tests provided some evidence that the combination of morphine and social interaction produced greater CPP than either stimulus alone after 16 conditioning trials.

A one-way ANOVA revealed a significant effect of morphine dose on pouncing during the first play pairing ($F(2,23) = 9.921$, $p = 0.001$) and pinning during the fourth play pairing ($F(2,31) = 5.886$, $p = 0.007$). Student's t test revealed that, during the 1st play pairing, there was a significant decrease in pouncing following administration of 3

mg/kg morphine when compared to saline controls ($t(16) = 4.946$, $p < 0.001$). During the 4th play pairing, student's t test revealed significant decrease in pinning following administration of both 1 mg/kg ($t(20) = 2.641$, $p = 0.016$) and 3 mg/kg ($t(22) = 2.97$, $p = 0.007$) morphine. Results for all play behaviors are summarized in Figures 10 and 11.

Discussion

Two doses of morphine (1 and 3 mg/kg, s.c.) were used to investigate the effects of social interaction on morphine CPP in adolescent male rats, using a modified CPP procedure. Rather than a single round of CPP (i.e. 4 drug pairings), rats underwent 2 rounds of CPP, with each round followed by a test. In contrast to Preliminary Experiment 1, the conditioning session time was decreased from 30 min to 20 min. This change was made based on a meta-analysis of morphine CPP, which determine that the ideal conditioning session time for morphine was either 20 min and shorter, or 45 min and longer (Bardo et al. 1995).

Results from this experiment revealed that on the first post-conditioning test, 1 mg/kg morphine produced significant CPP, regardless whether rats were given social interaction. In addition, 3 mg/kg morphine in combination with social interaction produced CPP. In contrast, social interaction alone did not produce significant CPP. Following the second round of CPP, however, only rats that had received morphine *and* social interaction displayed significant preference for the paired compartment. These latter results following the second round of CPP conditioning trials provide support for our original hypothesis that social interaction will enhance morphine reward. Further, these latter findings are congruent with the cFos results from Preliminary Experiment 2

showing that NAc shell activity was enhanced in rats given morphine and social interaction combined, but not in rats given either morphine or social interaction alone.

Interestingly, there was a dose-dependent decrease in pinning during both the first and fourth play pairings. This is contradictory to previous literature, which consistently shows that 1 mg/kg morphine increases play behavior in adolescent male rats (for review see Blanco-Gandia et al., 2015). However, while coding video, it was observed that the limbs of the adolescent rats were slipping through the wire grid and metal rod flooring. Therefore, we decided that it was likely that the play behavior may have been suppressed due to the unstable floor arrangement.

Of interest, the decrease in play behavior observed with morphine doses that produced CPP mimics previous findings with stimulants. At low doses, stimulants decrease social play behavior, but when combined with social interaction, the combination is more rewarding than either stimulus alone (Thiel et al., 2008). Thus, with both stimulants and opioids, the ability of social interaction to enhance drug reward is not due to a drug-induced elevation in social play.

Because we were interested in replicating literature that shows increases in play behavior at the 1 mg/kg dose of morphine, we decided to alter the flooring in the CPP compartments to provide a solid surface in the next experiment.

Experiment 2: Effect of Social Interaction on Morphine Reward using CPP in Adolescent Male Rats with Non-Standard Flooring

Rationale

Following the previous experiment, it was observed while coding videotaped play behaviors that the adolescent rats' limbs were slipping through the stainless steel mesh

and grid flooring during social interaction sessions. As a result, lower levels of play behavior were observed than were to be expected based on previous literature (Manduca et al., 2014; Trezza et al., 2014). Therefore, in this experiment, rubber floor mats were introduced to provide more stable flooring for elicitation of play behavior. Like Experiment 1, it was hypothesized that both 3 mg/kg morphine and social play alone would produce CPP, and that the compound stimulus of morphine and social play would produce greater CPP than either condition alone.

Animals

Adolescent male rats (PND 21 upon arrival; n=36) were housed individually in a temperature- and humidity-controlled colony room that was maintained on a light-dark cycle in which lights were on from 7:00 a.m. to 7:00 p.m. Rats were allowed to acclimate to the colony for 7 days before the start of the experiment. Rats had ad libitum access to food and water in their home cage for the entire experiment.

Apparatus

The apparatus was identical to the CPP chambers used in Experiment 1, with the exception of the flooring. Rather than stainless steel rod and mesh flooring in the black and white compartments, plastic or rubber mats were inserted over top of the original flooring. Clear plastic mats were added to the white compartments, while black rubber mats were added to the black compartments. The plastic and rubber mats differed in texture, with the black mats having grooves while the clear mats were relatively smooth.

Procedure

The procedure used in Experiment 2 was identical to the procedure used in Experiment 1, with the addition of recording social interaction during the 8th pairing.

Drug

Morphine sulfate (gift from NIDA) was prepared in sterile 0.9% NaCl (saline) and was injected subcutaneously in a volume of 1ml/kg. The dose was calculated based on salt weight.

Statistical Analyses

A preference ratio was calculated by dividing the amount of time spent in the compartment paired with morphine, or morphine and social interaction, by the time spent in both the white and black compartments. A preference ratio of 0.5 indicated no preference for either compartment, with ratios above 0.5 designating a preference and ratios below 0.5 designating an aversion.

Preference ratios were analyzed using SPSS 24 statistical software. An ANOVA, with dose and social interaction as between-subject factors, was performed. Significant interactions were probed with student's t-tests. Independent samples t-tests were performed to determine if each preference ratio was significantly different from saline controls. As an exploratory measure, one-sample t-tests were performed to determine if each preference ratio was significantly different from 0.5. Social play behaviors were analyzed separately with one-way ANOVAs with dose as a between-subject factor. Significant effects were probed with student's t tests. Multiple regression analyses were

conducted with SAS statistical software to examine the relationship between CPP preference ratios and social play behaviors. All tests were considered significant at $p < .05$.

Results

A univariate ANOVA revealed a significant main effect of morphine for the first post-conditioning test ($F(2,30) = 3.534$, $p = 0.042$), but no main effect of social condition, and no significant interaction (see Figure 12). Independent samples t-tests revealed no significant differences from saline controls. However, one-sample t-tests revealed that the 1 mg/kg dose of morphine in rats tested without social interaction produced significant CPP ($t(5) = 2.923$, $p = 0.33$).

Results for the second post-conditioning test are summarized in Figure 13. Results from the second post-conditioning test revealed no main effect of either drug or social condition, but the drug x social condition interaction reached near significance ($F(2,30) = 3.281$, $p = 0.051$). Independent samples t-test revealed no significant differences from saline controls. However, one-sample t-tests revealed that both social interaction alone and social interaction combined with 1 mg/kg morphine produced significant CPP ($t(5) = 2.959$, $p = 0.032$; $t(5) = 2.905$, $p = 0.034$), while 1 mg/kg morphine alone did not. Morphine at the 3 mg/kg dose without social interaction produced nearly significant CPP ($t(5) = 2.556$, $p = 0.051$). Thus, in contrast to the previous experiment, these results following 16 conditioning trials indicated that while either morphine or social interaction alone each produced CPP, these effects were negated when morphine and social interaction were combined.

As shown in Figure 14, univariate ANOVAs revealed significant effects of morphine on social play behaviors during the 1st pairing for pouncing ($F(2,9) = 12.964$, $p = 0.002$) and was trending for pinning ($F(2.9) = 3.961$, $p = 0.058$), but not boxing. Student's t tests revealed that both 1 and 3 mg/kg morphine *decreased* pouncing, compared to saline ($t(6) = 2.087$, $p = 0.024$; $t(6) = 5.457$, $p = 0.002$).

As shown in Figure 15, during the 4th play pairing, univariate ANOVAs revealed significant effects of morphine on both pinning ($F(2,13) = 10.958$, $p = 0.002$) and boxing ($F(2,13) = 15.641$, $p < 0.001$), but not pouncing. Student's t tests revealed that 3 mg/kg morphine *decreased* pouncing compared to both saline ($t(10) = 7.140$, $p < 0.001$) and 1 mg/kg morphine ($t(8) = 2.741$, $p = 0.025$). For boxing, 1 mg/kg morphine *increased* the behavior compared to both saline ($t(8) = 3.896$, $p = 0.005$) and 3 mg/kg morphine ($t(8) = 4.16$, $p = 0.003$). Finally, as shown in Figure 16, results from the 8th play pairing once again revealed significant effects of morphine on both pinning ($F(2,15) = 20.962$, $p < 0.001$) and boxing ($F(2,15) = 4.216$, $p = 0.035$), but not pouncing. Student's t tests revealed that morphine *decreased* pinning at both the 1 mg/kg ($t(10) = 4.209$, $p = 0.002$) and 3 mg/kg ($t(10) = 13.99$, $p < 0.001$) doses. Morphine at the 1 mg/kg dose *decreased* boxing compared to saline ($t(10) = 3.008$, $p = 0.013$).

Together, these play behavior results showed that morphine has differential effects on the type of social behavior observed. With pouncing, morphine dose dependently decreased pouncing during the first pairing, but these effects tolerated following multiple pairings. In contrast, with pinning, morphine had no significant effect during the first play pairing, although dose dependent decreases develop with further pairings. Finally, with boxing, behavior tended to increase as animals get older, with the

exception of animals that received 1 mg/kg morphine, which showed no consistent pattern of boxing behavior across time.

In an attempt to determine if there was any relation between play behavior and CPP, multiple regression analyses were performed comparing the 8th play pairing and CPP preference ratios from the second post-conditioning test. These data are summarized in Table 3 and Figures 17-19. Play behaviors were analyzed as either an action performed by a rat, or an action that occurred to a rat. Results revealed there was a significant difference in intercept when a rat pounced on his social peer for both 1 mg/kg ($t(12) = 3.23$, $p = 0.007$) and 3 mg/kg ($t(12) = 2.18$, $p = 0.049$) morphine compared to saline. There was also a significant difference in the slope between saline and 3 mg/kg morphine ($t(12) = 3.12$, $p = 0.008$). Rats that had been pounced on also showed a significant difference in intercept between saline and 1 mg/kg morphine dose ($t(12) = 2.29$, $p = 0.041$), and reached near significance for the slope ($t(12) = 2.15$, $p = 0.0527$). Rats that received 3 mg/kg morphine showed a significant difference in slope compared to saline controls when a rat was pinned by their social peer ($t(12) = 2.30$, $p = 0.039$). Boxing (which is a behavior that requires engagement by both rats) was trending towards significance at 1 mg/kg morphine for both the intercept ($t(12) = 2.02$, $p = 0.065$) and slope ($t(12) = 2.06$, $p = 0.061$). Therefore, it appears as though morphine is not only altering play behavior, but that morphine is also possibly influencing the subjective experience of social play on a dose-dependent basis.

Discussion

In order to provide more solid flooring in all of the CPP compartments, clear plastic mats were added to the white compartments, and black rubber mats were added to the black compartments. In all other regards, the procedure used in this experiment were identical to those used in Experiment 2.

Similar to the results from Experiment 1 using the standard floor, there was no significant effect of social interaction alone after either 8 or 16 conditioning trials. As for the effect of morphine, results revealed that no conditions produced CPP, which was unexpected based on the results from Experiment 1 and previous literature (Bardo et al., 1984, 1995).

Interestingly, similar to the results of Experiment 1, morphine produced dose-dependent decreases in social play behaviors during the 1st, 4th, and 8th pairings. The only exception was during the 4th play session, where 1 mg/kg morphine lead to an increase in boxing compared to both saline and 3 mg/kg morphine. Thus, our results are contradictory to previous literature (Blanco-Gandia et al., 2015; Trezza et al., 2014), and indicate that flooring, timing, and apparatus size may all play crucial roles in rodent play behavior. Most research on social interaction uses a larger apparatus with a solid floor (Achterberg et al., 2014; Vanderschuren et al., 2016). Given that the adolescent rats used for this dissertation had difficulty stabilizing themselves on the non-solid floors of standard CPP compartments and has less room to engage in rough and tumble play, it is understandable that results of Experiment 1 was not consistent with previous literature. With regard to timing, the session lengths for all experiments were based off literature that primarily used adult subjects. However, adolescents respond differently to drugs of

abuse than adults (Campbell et al., 2000; Wiley et al., 2008; Kennedy et al., 2011; Koek et al., 2012, 2014; Dannenhoffer and Spear, 2016), and it is therefore possible that the times chosen were not appropriate for the age of the animals studied.

Correlations between CPP preference ratios from the second post-conditioning test session and play behavior during the 8th play session led to another interesting finding. While both social interaction alone and social combined with 1 mg/kg morphine produced significant CPP, the correlations between CPP and play were in opposite directions. In general, social interaction paired with saline showed a negative correlation, whereas social interaction paired with 1 mg/kg morphine showed a positive correlation. This negative correlation between CPP and social play is contradictory to previous literature (Vanderschuren, Achterberg et al. 2016); however our data were collected based on individual rats, whereas the study by Vanderschuren et al. (2016) collected data as the dyad of both rats. Unfortunately, the number of subjects used in this dissertation prohibits us from appropriately doing similar statistical analyses for a direct comparison of results to those provided in Vanderschuren et al. (2016). Also, two different types of flooring were used, with the previous study using standard flooring, and this experiment having used non-standard flooring.

In any case, the current behavioral findings showed that none of the conditions used in this dissertation produces CPP following either 8 or 16 conditioning trials. Data correlating CPP preference ratios with social play behaviors revealed that while the saline group showed a negative correlation, the 1 mg/kg morphine group showed a positive correlation. Because of this finding, we were interested whether there were differences in the underlying neural mechanisms involved.

Experiment 3: Measuring Differences in cFos Expression Due to Morphine and Social Interaction using Immunofluorescence in Adolescent Male Rats

Rationale

As mentioned earlier, much of the literature on the interaction between drugs of abuse and social play has used drugs as a method of investigating the mechanisms underlying social play behavior, rather than how social play can affect drug reward. Furthermore, although it is established that both morphine and social play lead to increases in cFos expression, there is no literature on the effect that combining these two rewarding stimuli has with regard to neural activation. Finally, most literature regarding social play focuses on acute effects, while CPP measures behavior following repeated exposure. Therefore, it is of interest to investigate neuronal changes that occur after multiple pairings of morphine and/or social interaction.

The dose of morphine (1 mg/kg) used in this experiment was chosen based upon both CPP and multiple regression results presented in Experiment 2. The group of rats conditioned with 1 mg/kg morphine in combination with social interaction in Experiment 2 showed a positive correlation between social play and CPP. This posed an interesting research question as to whether there were neuronal differences between these two groups who showed similar CPP behavior, but opposite play behavior. We hypothesized that social interaction alone would increase cFos expression compared to saline controls, but that the compound stimulus of morphine and social interaction would not show the same enhancement in cFos that was observed in Preliminary Experiment 2, and would possibly show a decrease compared to saline controls.

Animals

Adolescent male rats (PND 21 upon arrival; n=24) were housed individually in a temperature- and humidity-controlled colony room that is maintained on a light-dark cycle in which lights were on from 7:00 a.m. to 7:00 p.m. Rats were allowed to acclimate to the colony for 7 days before the start of the experiment. Rats had ad libitum access to food and water in their home cage for the entire experiment.

Apparatus

The apparatus was identical to the CPP chambers used in Experiment 2 using the plastic and rubber mat floor inserts.

Behavior

Rats underwent a procedure similar to the CPP procedure in Experiments 1 and 2, but with a few key differences. Although the same experimental groups as Experiment 2 were used (See Table 2 for details about experimental groups), only one dose of morphine was used (1 mg/kg; s.c.). Rats underwent a pre-test on day 1, conditioning trials on days 2-9, a post-test on day 10, and then an additional 7 or 8 conditioning trials (days 17 and 18). Rats were killed following the final morphine conditioning trial (the 8th pairing of morphine and/or social interaction) to determine cFos activation following repeated treatment.

As with Experiment 2, during the 1st, 4th, and 8th sessions when social interaction occurred, animals were recorded using a camera located above the compartment. A blind

observer coded social play behavior for occurrences of pinning, pouncing, and boxing. See Figure 20 for a timeline.

Drug

Morphine sulfate (gift from NIDA) was prepared in sterile 0.9% NaCl (saline) and was injected subcutaneously in a volume of 1ml/kg. The dose was calculated based on salt weight.

Perfusion and brain sampling

Following the final drug conditioning trial (day 17 or 18), rats were placed back into their home cages for 90 min. Rats then underwent immunohistochemistry procedures similar to those described in Preliminary Experiment 2. Slices were collected from the NAc (core and shell) and dStr.

Immunofluorescence

Free-floating sections were rinsed three times for 15 min each with Triton X-100:1x tris-PBS (TPBS; Tris–HCl 10 mM, sodium phosphate buffer 10 mM, 0.9 percent NaCl, pH 7.4) and incubated with rabbit polyclonal anti-c-Fos antibody (sc-7202, Santa Cruz Biotechnology, CA, USA), diluted 1:200. Incubations were at 4°C for 48 hours in TPBS 0.1 M Triton X-100 containing 3 percent of donkey serum. After rinsing, tissue was incubated for 2 hrs at room temperature protected from light with secondary antibody with conjugated fluorochromes Alexa Fluor 488 donkey anti-rabbit (Jackson Immunoresearch Laboratories, PA, USA), diluted 1:250. Once the fluorescence reaction

occurred, sections were mounted using Mowiol 4-88 reagent (475904-100GM, EMD Millipore, USA).

Neuronal Counting

Three fluorescent-labeled sections for the NAc (core and shell) and dStr were examined using a confocal microscope (Nikon Eclipse-1C, Nikon Instruments, Americas, USA) by an observer who was not aware of the treatment condition for each section. Confocal images were taken in single XY planes, 1-μm thick, at a resolution of 1024 × 1024 and 100-Hz speed. Laser intensity, gain and offset were maintained constant in each analysis. Image analyses were made using the Nikon NIS Elements software (AR 4.20.02 64 bit). In each brain area, three selected slices for c-Fos-IR were estimated in a region of interest of 20,000 μm^2. For c-Fos-IR, we considered a cell positive if it shows full nuclear and intense labeling. Immunofluorescence results are represented as the average cell counts collected from the three brain slices selected. Representative images were selected based on similarity to the mean of each group.

Statistical Analyses

A preference ratio was calculated by dividing the amount of time spent in the compartment paired with morphine, or morphine and social interaction, by the time spent in both the white and black compartments. A preference ratio of 0.5 indicated no preference for either compartment, with ratios above 0.5 designating a preference and ratios below 0.5 designating an aversion.

Using SPSS 24 statistical software, preference ratios were analyzed with ANOVA, with dose and social interaction as between-subject factors. Significant interactions were probed with student's t-tests. Independent samples t-tests were performed to determine if each preference ratio was significantly different from saline controls. As an exploratory measure, one-sample t-tests were performed to determine if each preference ratio was significantly different from 0.5. Play behaviors were analyzed separately with student's t tests. Multiple regression analyses were conducted with SAS statistical software to examine the relationship between CPP preference ratios and social play behavior. cFos expression was analyzed using a repeated measures ANOVA, with region as a within subject factor. Significant interactions were further probed with student's t tests. All tests were considered significant at $p < .05$.

Results

As shown in Figure 21, a univariate ANOVA of preference ratios revealed a near significant interaction for drug x social condition ($F(1,20) = 4.29$, $p = 0.051$), but no main effects of either drug or social condition. Both independent samples and one-sample t-tests revealed that only the social group showed significant CPP ($t(22) = 3.362$, $p = 0.003$ and $t(5) = 4.361$, $p = 0.007$, respectively).

All play behaviors are summarized in Figures 22-24. For play behavior, the only difference between the groups was the amount of boxing during the eighth play pairing ($t(8) = 19.31$, $p < 0.001$), with rats that had received 1 mg/kg morphine showing an *increase* in the amount of boxing compared to saline controls.

As shown in Table 4 and Figures 25-27, multiple regression of CPP and play behavior (collapsed with data from Experiment 2) from round 1 of CPP revealed that there were significant differences in pinning, when performed both by a rat and to a rat, between saline and 1 mg/kg morphine in the intercept ($t(18) = 3.03$, $p = 0.007$; $t(18) = 3.33$, $p = 0.004$) as well as the slope for "done to" ($t(18) = 2.30$, $p = 0.034$), with the slope for "did" near significance ($t(18) = 2.06$, $p = 0.054$). There was also a significant difference in the intercept between saline and 1 mg/kg morphine for boxing ($t(18) = 2.87$, $p = 0.01$).

Results and representative images of cFos expression are summarized in Figures 28-31. A 2 x 2 x 2 repeated measures ANOVA (region x drug x social condition) analysis of cFos expression revealed a significant triple interaction ($F(2,19) = 6.162$, $p = 0.008$). In NAc shell, there was also a significant drug x social interaction ($F(1,20) = 4.753$, $p = 0.041$). In NAc core, there was a trend towards a main effect of social condition ($F(1,20) = 3.767$, $p = 0.067$). In dStr, there was a significant main effect of drug ($F(1,20) = 18.270$, $p < 0.001$). Student's t tests revealed that in NAc shell, only the social group showed an increase in cFos expression compared to saline controls ($t(10) = 2.457$, $p = 0.034$). In NAc core, there were no significant differences between any of the experimental groups. In dStr, student's t tests revealed that 1 mg/kg morphine increased cFos expression compared to saline controls either when the rat was alone ($t(10) = 2.95$, $p = 0.015$) or paired with a social peer ($t(10) = 3.711$, $p = 0.004$). Animals paired with a social peer that received morphine also showed an increase in cFos compared to rats paired with a social peer that received saline ($t(10) = 3.099$, $p = 0.011$) in dStr.

Discussion

Male adolescent rats underwent CPP training using a similar procedure to Experiment 2, with two major differences. Similar to Experiment 2, rats underwent the first round of CPP, along with the post-conditioning test, as well as the additional conditioning sessions during the second round of CPP. However, in contrast to Experiment 2, only a single dose of morphine was used (1 mg/kg), and instead of undergoing a second post-conditioning test, rats were anesthetized and perfused after their last morphine and/or social pairing session. The NAc (shell and core) and dStr were examined for cFos expression.

Results from the post-conditioning test revealed that social interaction alone produced CPP, whereas morphine alone or morphine paired with social interaction did not. These behavioral results generally replicate the CPP results obtained in Experiment 2, although fewer conditioning trials (8 vs 16) and lower morphine dose (1 vs 3) were needed in this experiment to reach the same conclusion that social CPP is negated when combined with morphine. Surprisingly, unlike previous experiments, there were no significant differences in pinning or pouncing during the 1st, 4th, or 8th social pairing. However, only the lower dose of morphine (1 mg/kg) was used in this experiment, whereas the effects observed in the previous experiment were obtained primarily with a higher dose (3 mg/kg). In the current experiment, 1 mg/kg morphine increased boxing compared to saline during the 8th pairing.

Data from Experiment 3 collapsed with data from Experiment 2 was used to correlate preference ratios with play behavior from the 4th social pairing. Once again, there was a negative correlation in the group that received saline and social interaction,

but a positive correlation in the group that received 1 mg/kg morphine and social interaction, but only for pinning.

Immunofluorescence results from Experiment 3 revealed that the social alone group showed a significant increase in cFos expression in NAc shell, but not the NAc core or dStr. There was also a main effect of social interaction in the NAc core, such that there was more cFos expression in social groups, relative to isolated groups. In contrast, morphine, either with or without social interaction, lead to greater cFos expression in the dStr. These results differ from the results of Preliminary Experiment 2, which showed significant increases in cFos expression in NAc core following social interaction given either alone or combined with morphine (0.3 mg/kg), which is similar to previous findings (van Kerkhof et al., 2014). Increases in cFos expression were also observed in NAc shell following social combined with morphine (0.3 mg/kg), but not social alone.

Morphine, in the absence of social interaction, did not significantly alter cFos expression in any regions in Preliminary Experiment 2, which likely relates to the low dose of morphine administered (Liu et al., 1994; Hamlin et al., 2009). There are, however, a number of reasons why we expect differences. Not only was the dose of morphine lower in Preliminary Experiment 2, but Preliminary Experiment 2 also examined cFos expression following a single pairing, whereas Experiment 3 assessed cFos expression following repeated pairings. Therefore, we would expect to observe lower levels of activation, in general, because of habituation and tolerance. By generally comparing cFos levels in the saline control groups, it is clear that the chronic exposure in Experiment 3 lead to less cFos expression than what is seen following acute exposure in Preliminary Experiment 2.

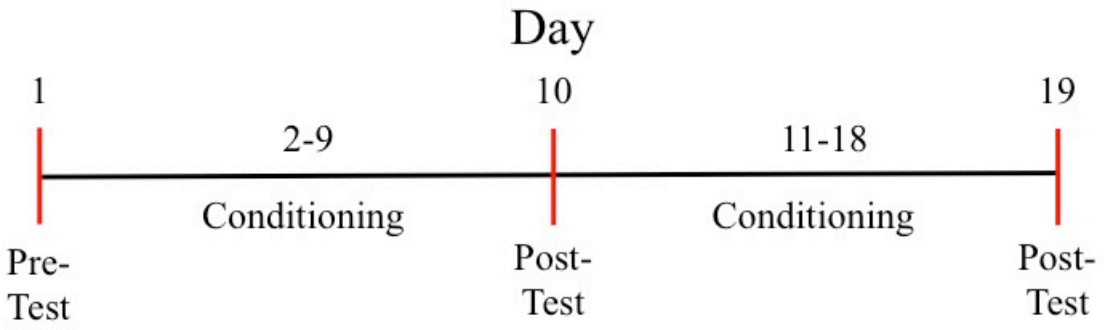

Figure 7. Timeline for Experiments 1 and 2.

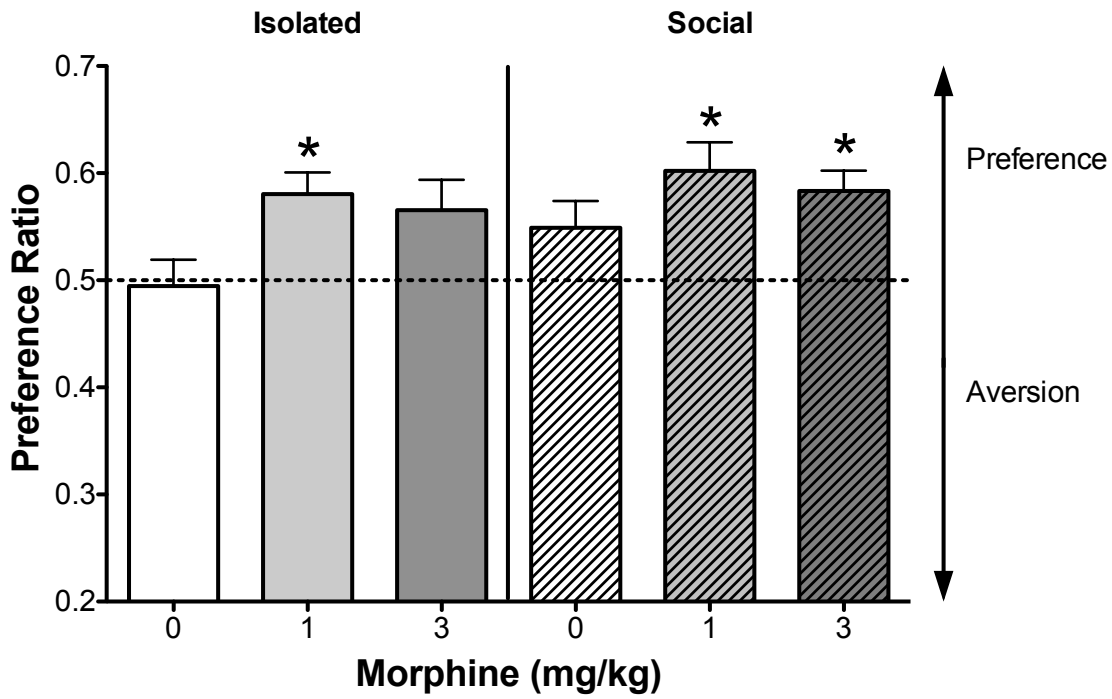

Figure 8. Preference ratio for isolated and socially paired adolescent males following morphine and/or social interaction for the first post-conditioning test in Experiment 1. Bar represents mean (±SEM) preference ratio, with the dashed line indicating equal preference for both compartments. Asterisk (*) represents a difference relative to saline controls, $p < 0.05$. n = 12 per treatment group.

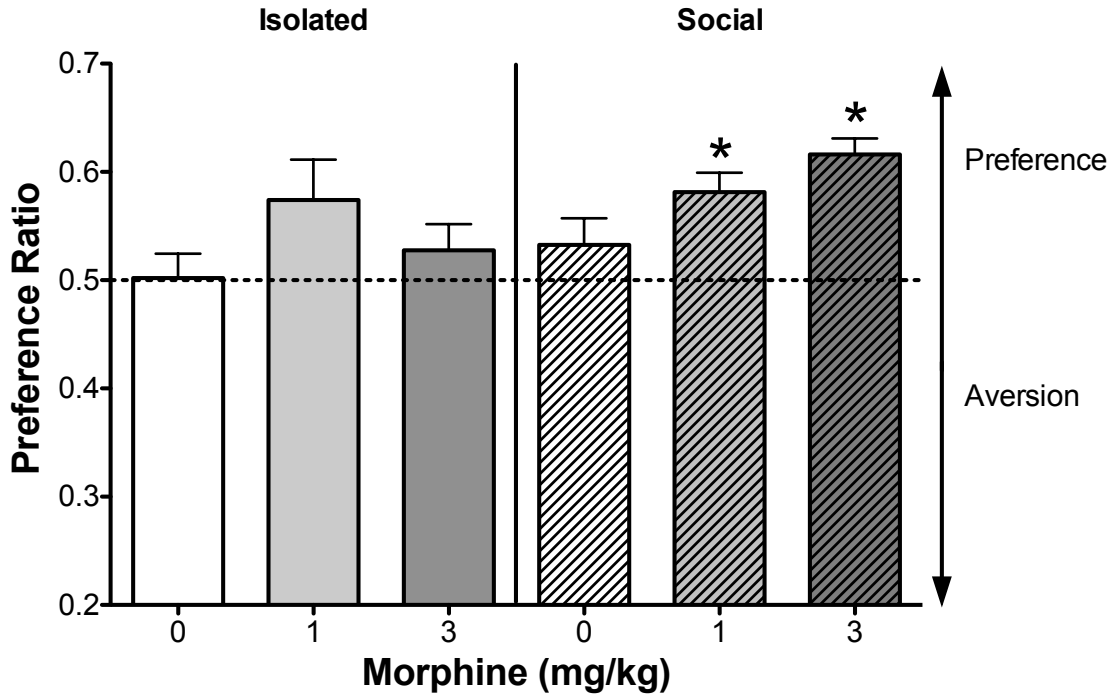

Figure 9. Preference ratio for isolated and socially paired adolescent males following morphine and/or social interaction for the second post-conditioning test in Experiment 1. Bar represents mean (±SEM) preference ratio, with the dashed line indicating equal preference for both compartments. Asterisk (*) represents a difference relative to saline controls, $p < 0.05$. n = 12 per treatment group.

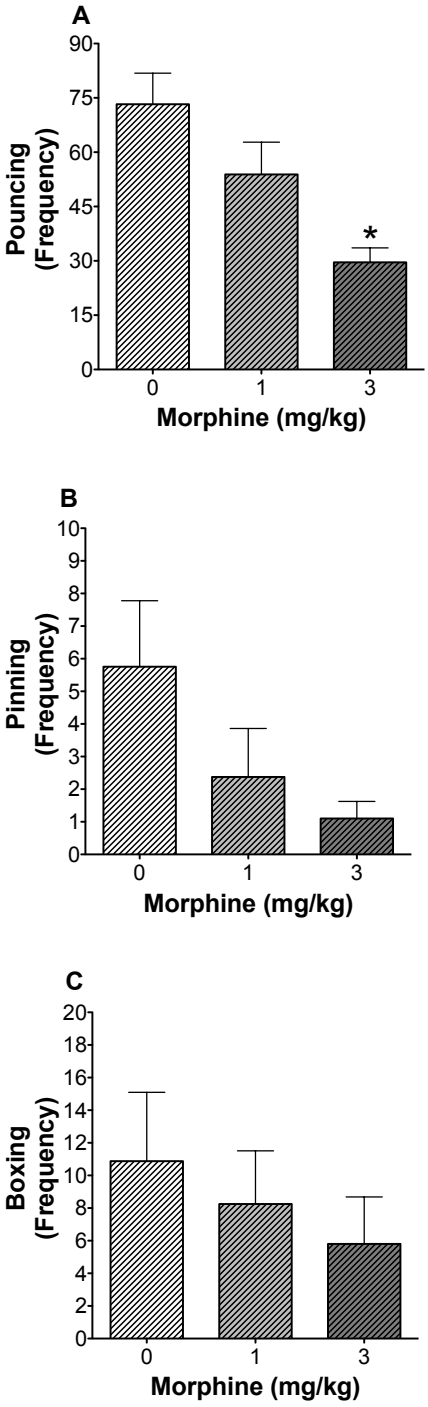

Figure 10. Mean (±SEM) frequency of (A) pouncing, (B) pinning, and (C) boxing during the first social pairing in Experiment 1. * $p < 0.05$ vs saline. n = 10-12 per treatment group.

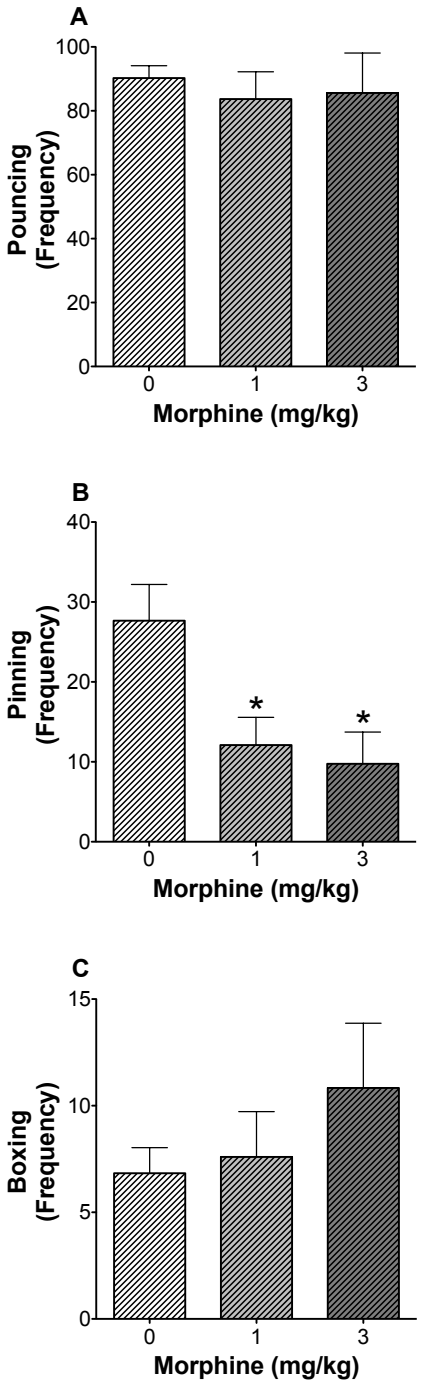

Figure 11. Mean (±SEM) frequency of (A) pouncing, (B) pinning, and (C) boxing during the fourth social pairing in Experiment 1. * $p < 0.05$ vs saline. n = 10-12 per treatment group.

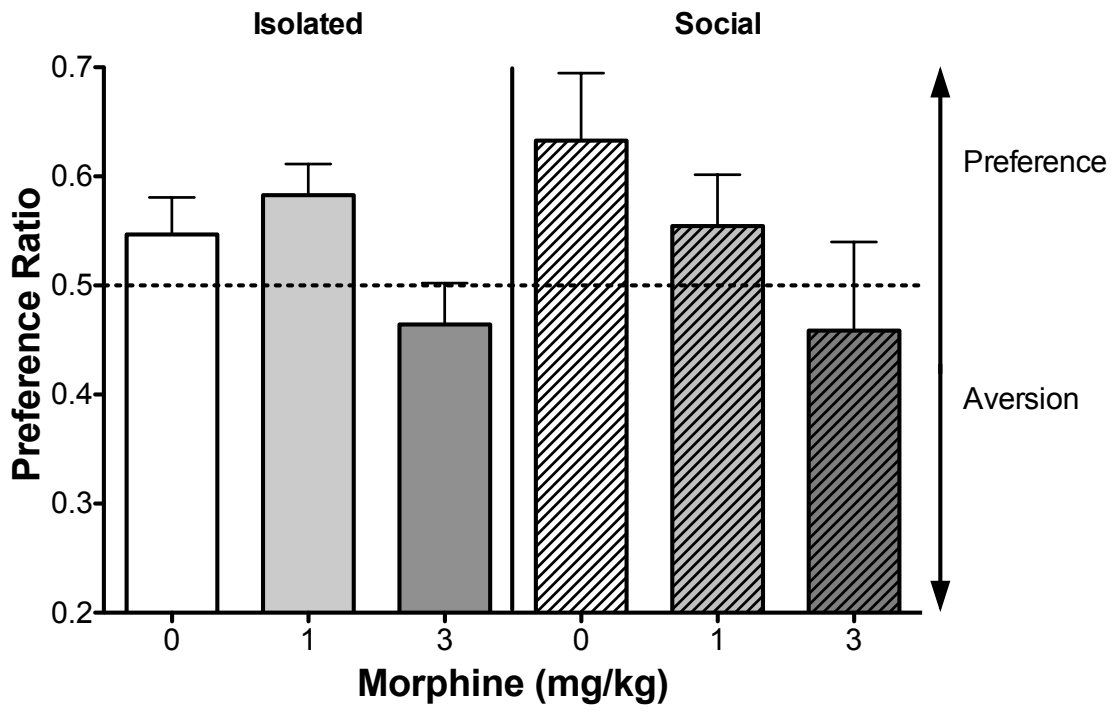

Figure 12. Preference ratio for isolated and socially paired adolescent males following morphine and/or social interaction for the first post-conditioning test in Experiment 2. Bar represents mean (±SEM) preference ratio, with the dashed line indicating equal preference for both compartments. n = 6 per treatment group.

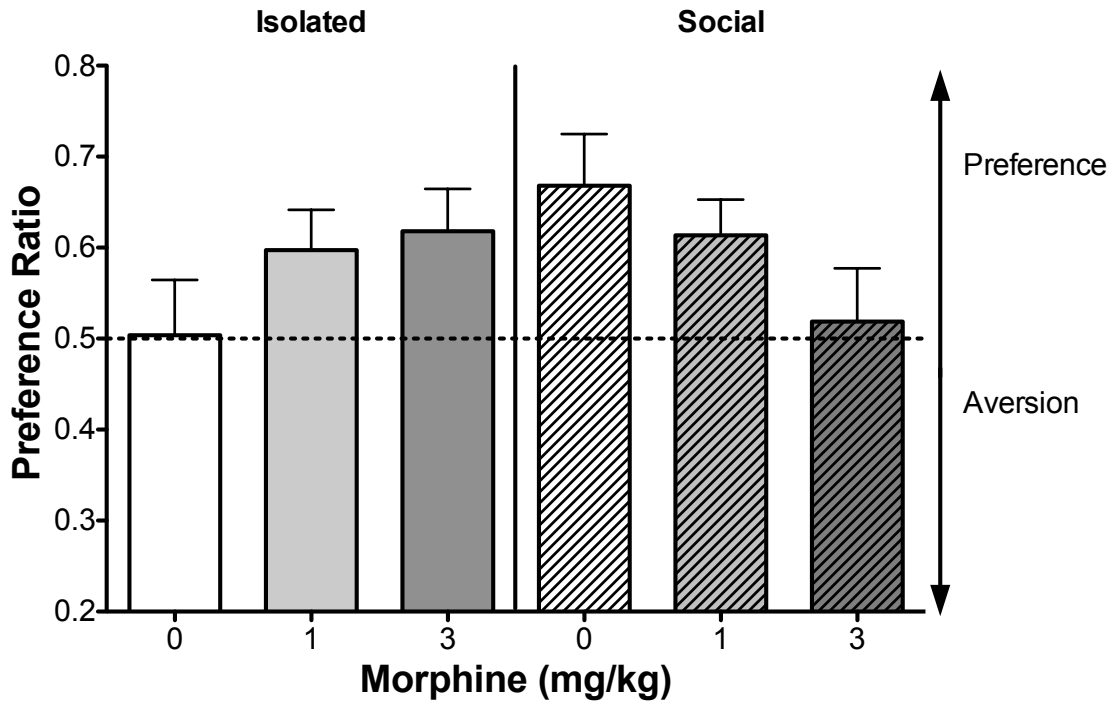

Figure 13. Preference ratio for isolated and socially paired adolescent males following morphine and/or social interaction for the second post-conditioning test in Experiment 2. Bar represents mean (±SEM) preference ratio, with the dashed line indicating equal preference for both compartments. n = 6 per treatment group.

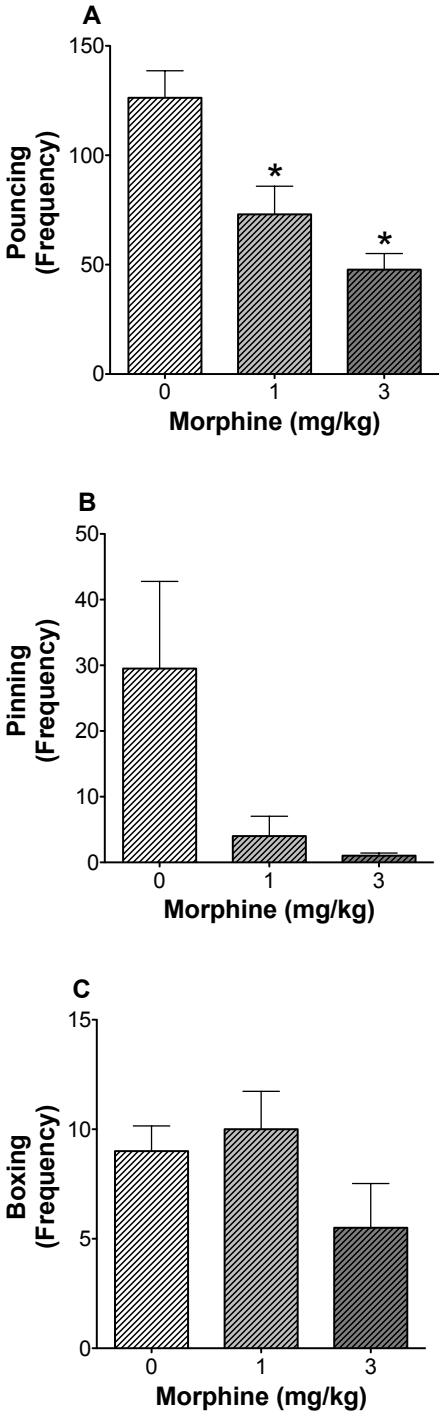

Figure 14. Mean (±SEM) frequency of (A) pouncing, (B) pinning, and (C) boxing during the first social pairing in Experiment 2. * $p < 0.05$ vs saline. n = 4 per treatment group.

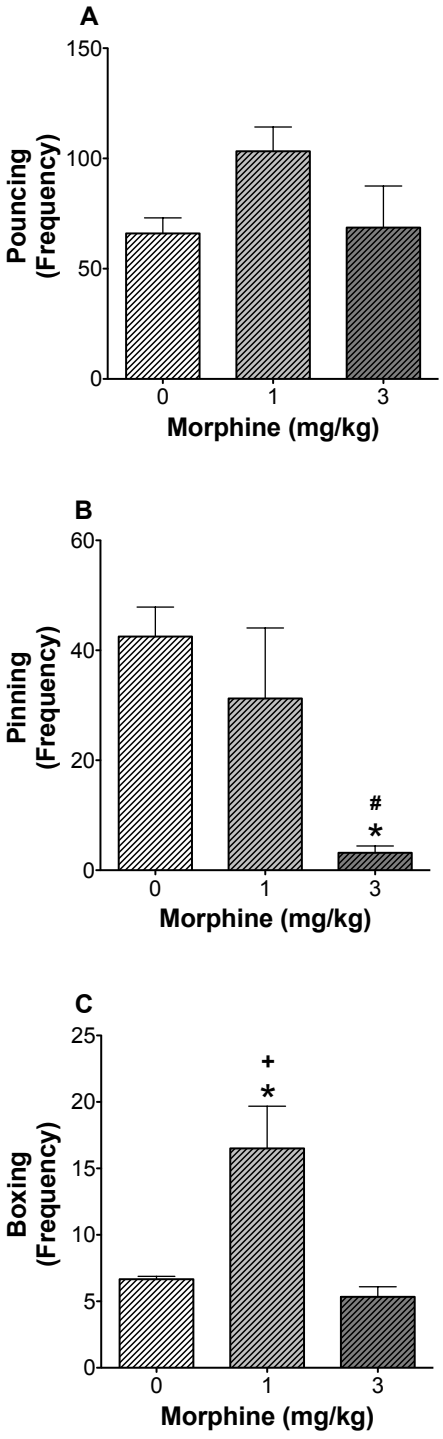

Figure 15. Mean (±SEM) frequency of (A) pouncing, (B) pinning, and (C) boxing during the fourth social pairing in Experiment 2. * $p < 0.05$ vs saline. # $p < 0.05$ vs 1 mg/kg morphine. + $p < 0.05$ vs 3 mg/kg morphine. n = 4-6 per treatment group.

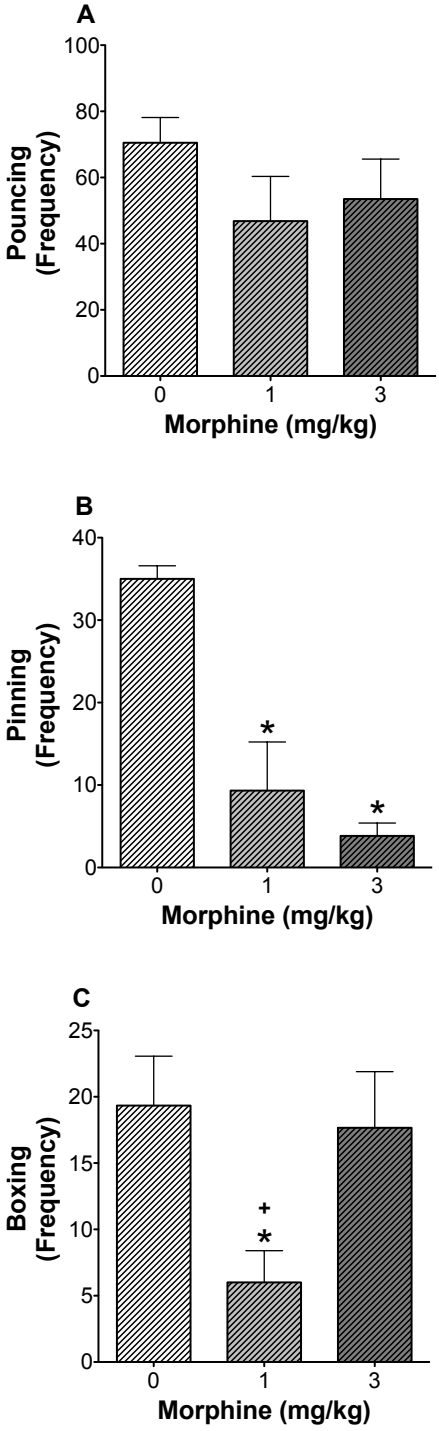

Figure 16. Mean (±SEM) frequency of (A) pouncing, (B) pinning, and (C) boxing during the eighth social pairing in Experiment 2. * $p < 0.05$ vs saline. + $p < 0.05$ vs 3 mg/kg morphine. n = 6 per treatment group.

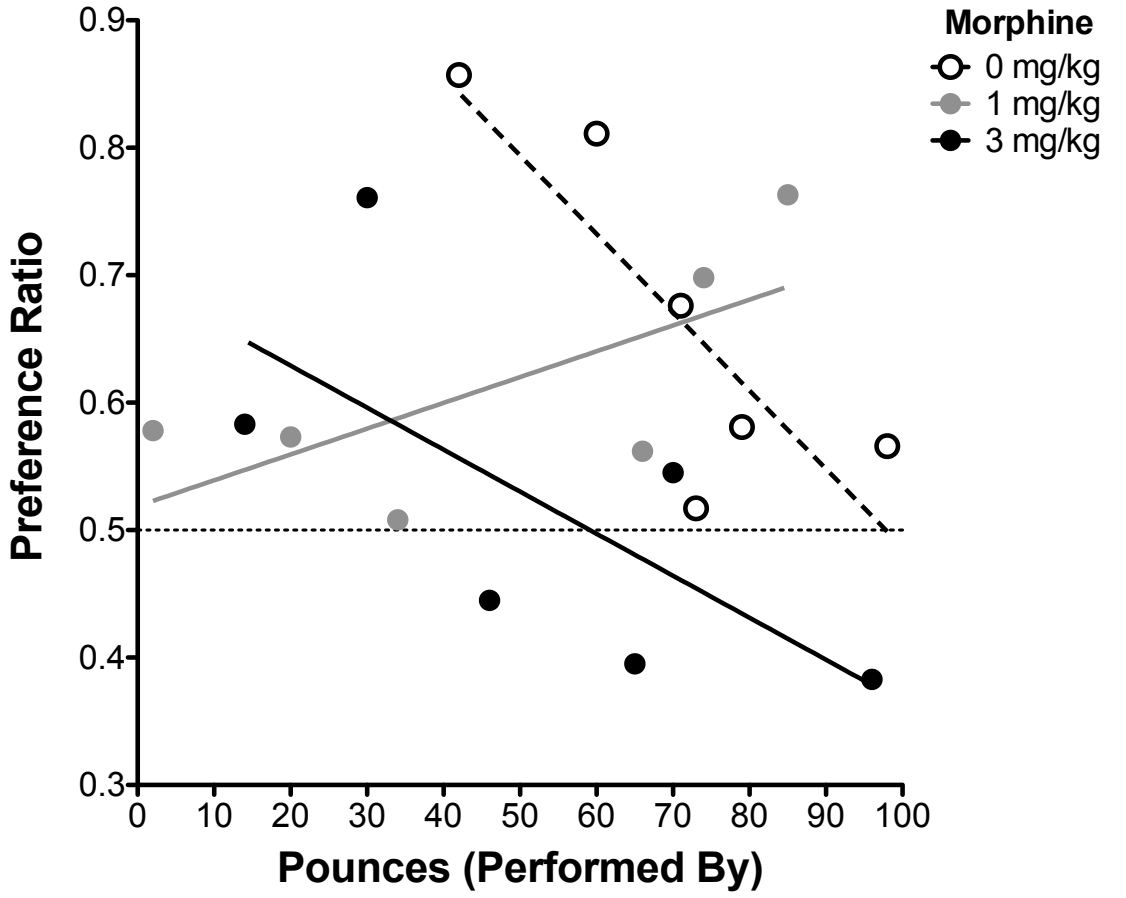

Figure 17. Correlation between pounces performed by a rat to their social peer during the 8th social pairing and CPP preference ratio during the second post-conditioning test session (thin dashed line indicates equal preference for both compartments) for rats that received saline (open circles, thick dashed line), 1 mg/kg morphine (gray closed circles, gray solid line), and 3 mg/kg morphine (black closed circles, black solid line). n = 6 per treatment group.

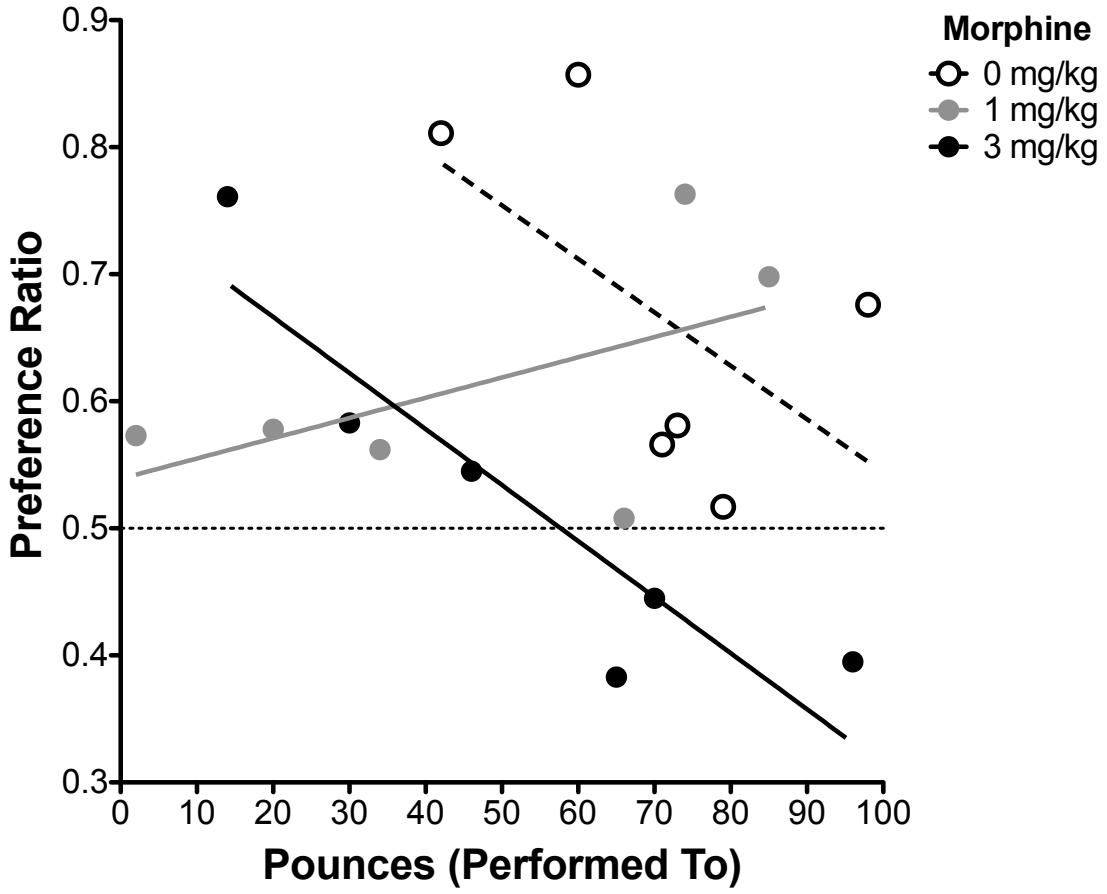

Figure 18. Correlation between pounces performed to a rat to their social peer during the 8th social pairing and CPP preference ratio during the second post-conditioning test session (thin dashed line indicates equal preference for both compartments) for rats that received saline (open circles, thick dashed line), 1 mg/kg morphine (gray closed circles, gray solid line), and 3 mg/kg morphine (black closed circles, black solid line). n = 6 per treatment group.

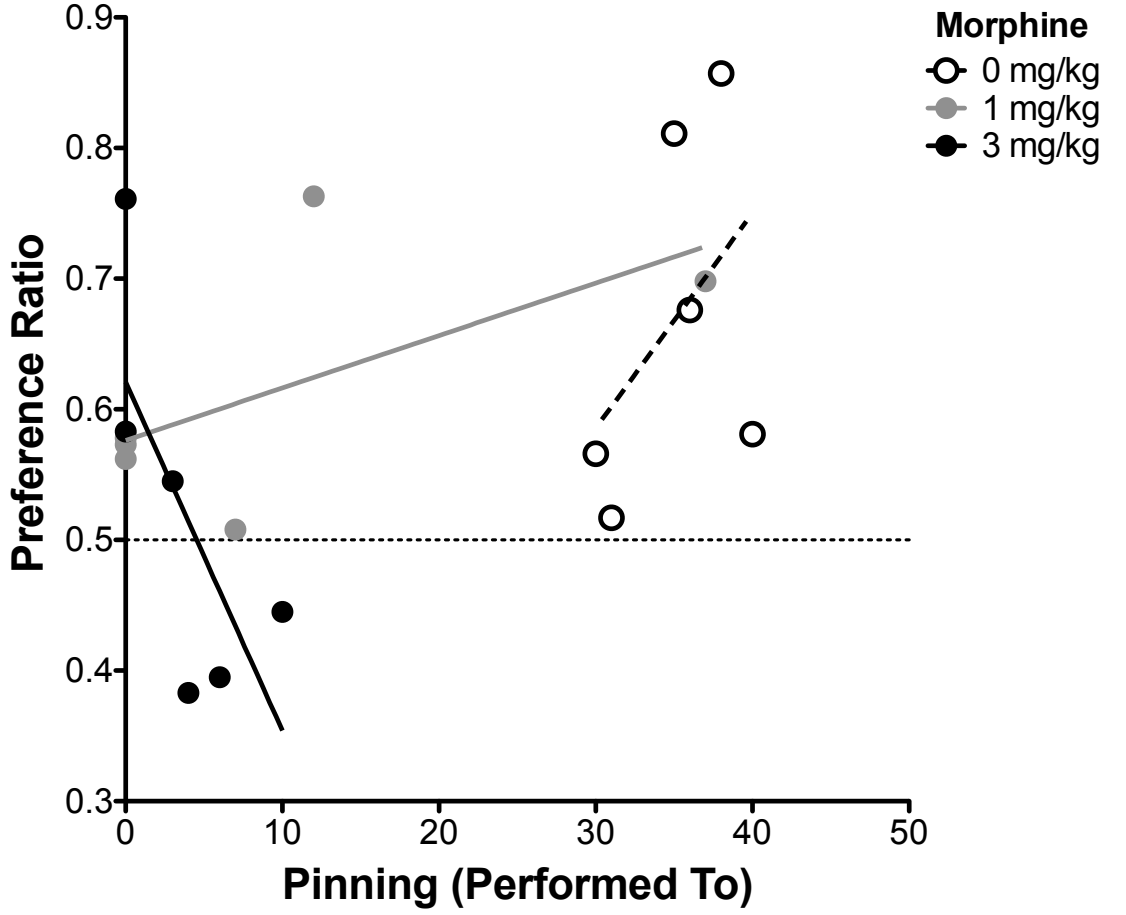

Figure 19. Correlation between pinning performed to a rat to their social peer during the 8th social pairing and CPP preference ratio during the second post-conditioning test session (thin dashed line indicates equal preference for both compartments) for rats that received saline (open circles, thick dashed line), 1 mg/kg morphine (gray closed circles, gray solid line), and 3 mg/kg morphine (black closed circles, black solid line). n = 6 per treatment group.

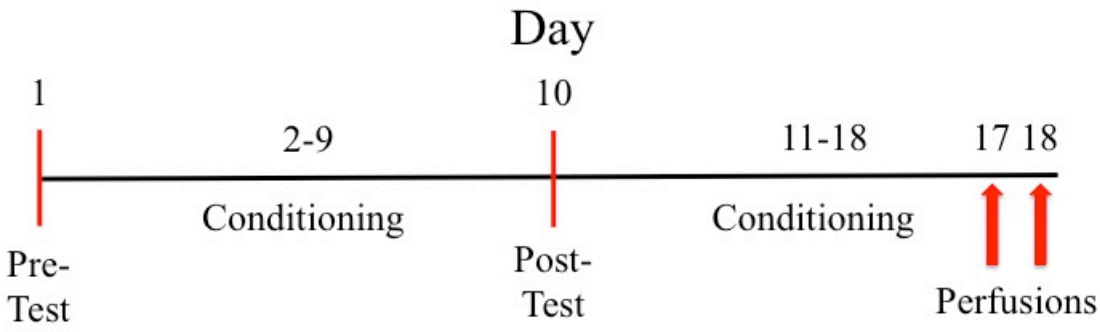

Figure 20. Timeline for Experiment 3.

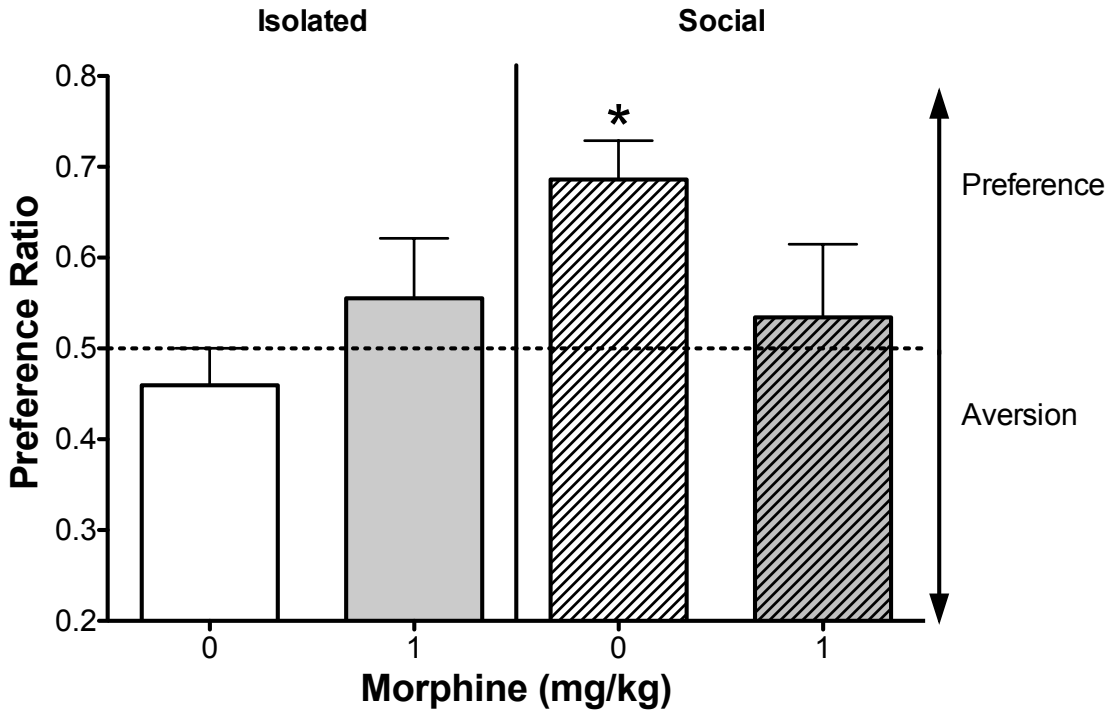

Figure 21. Preference ratio for isolated and socially paired adolescent males following morphine and/or social interaction in Experiment 3. Bar represents mean (±SEM) preference ratio, with the dashed line indicating equal preference for both compartments. Asterisk (*) represents a difference relative to saline controls, $p < 0.05$. n = 6 per treatment group.

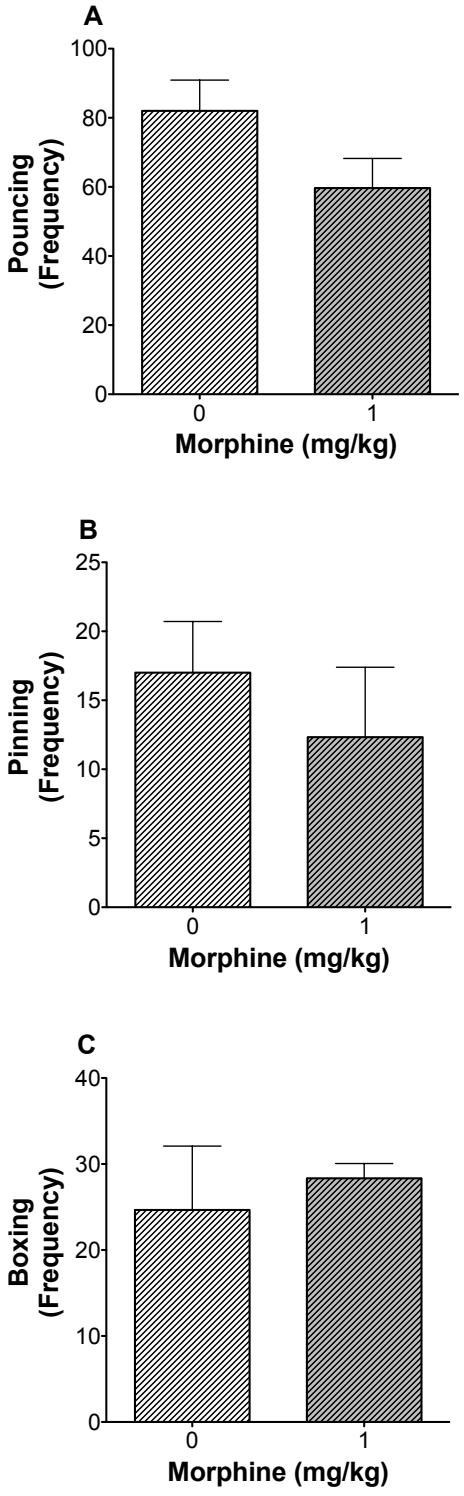

Figure 22. Mean (±SEM) frequency of (A) pouncing, (B) pinning, and (C) boxing during the first social pairing in Experiment 3. n = 6 per treatment group.

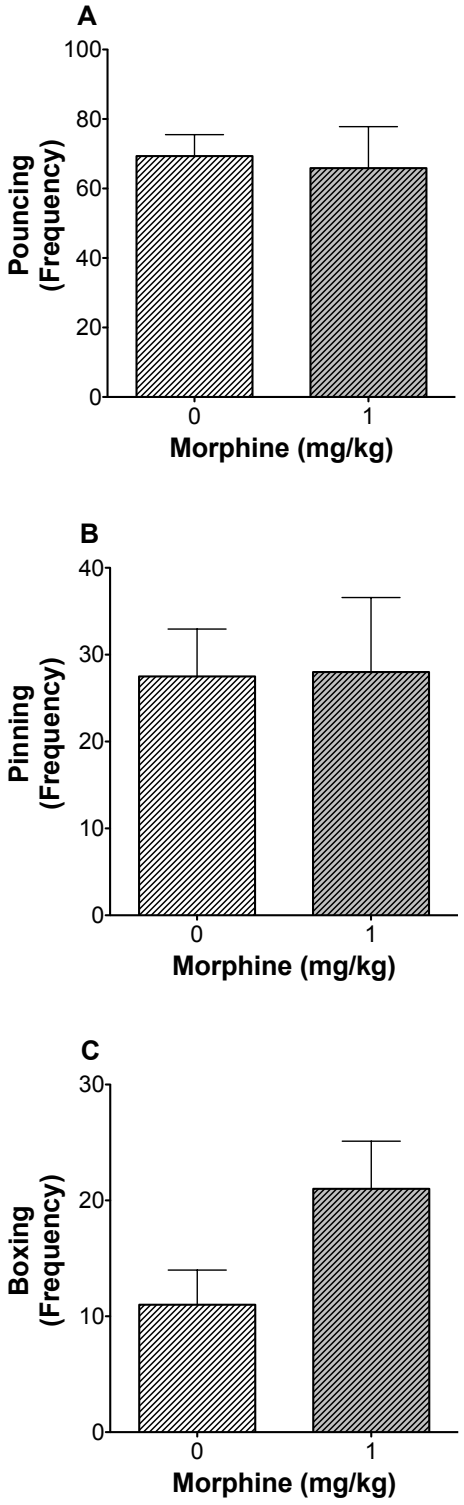

Figure 23. Mean (±SEM) frequency of (A) pouncing, (B) pinning, and (C) boxing during the fourth social pairing in Experiment 3. n = 6 per treatment group.

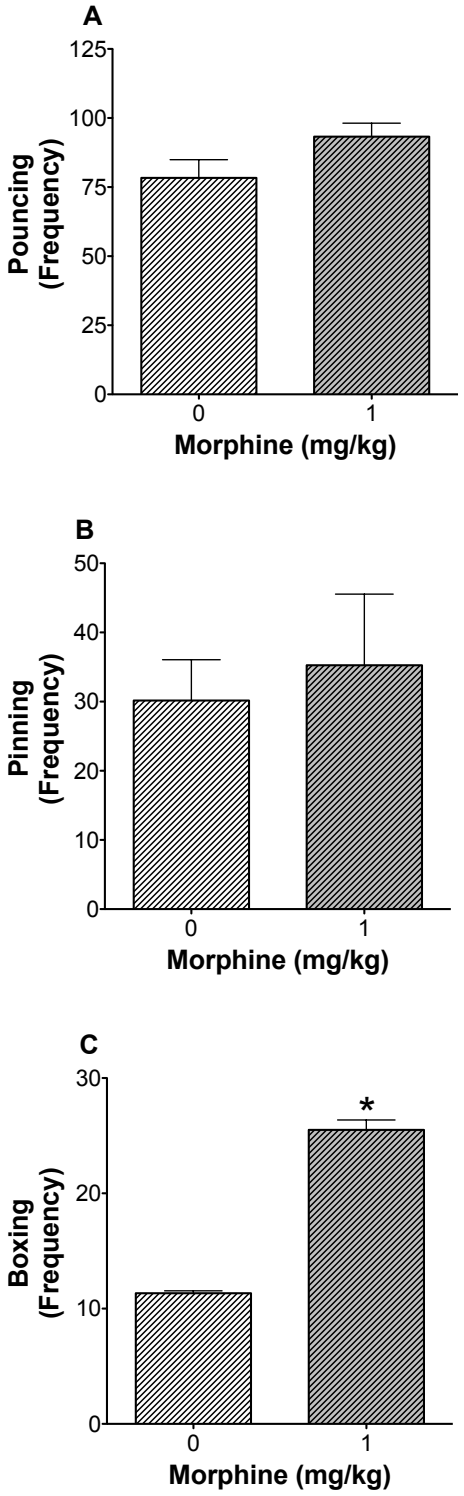

Figure 24. Mean (±SEM) frequency of (A) pouncing, (B) pinning, and (C) boxing during the eighth social pairing in Experiment 3. n = 4-6 per treatment group.

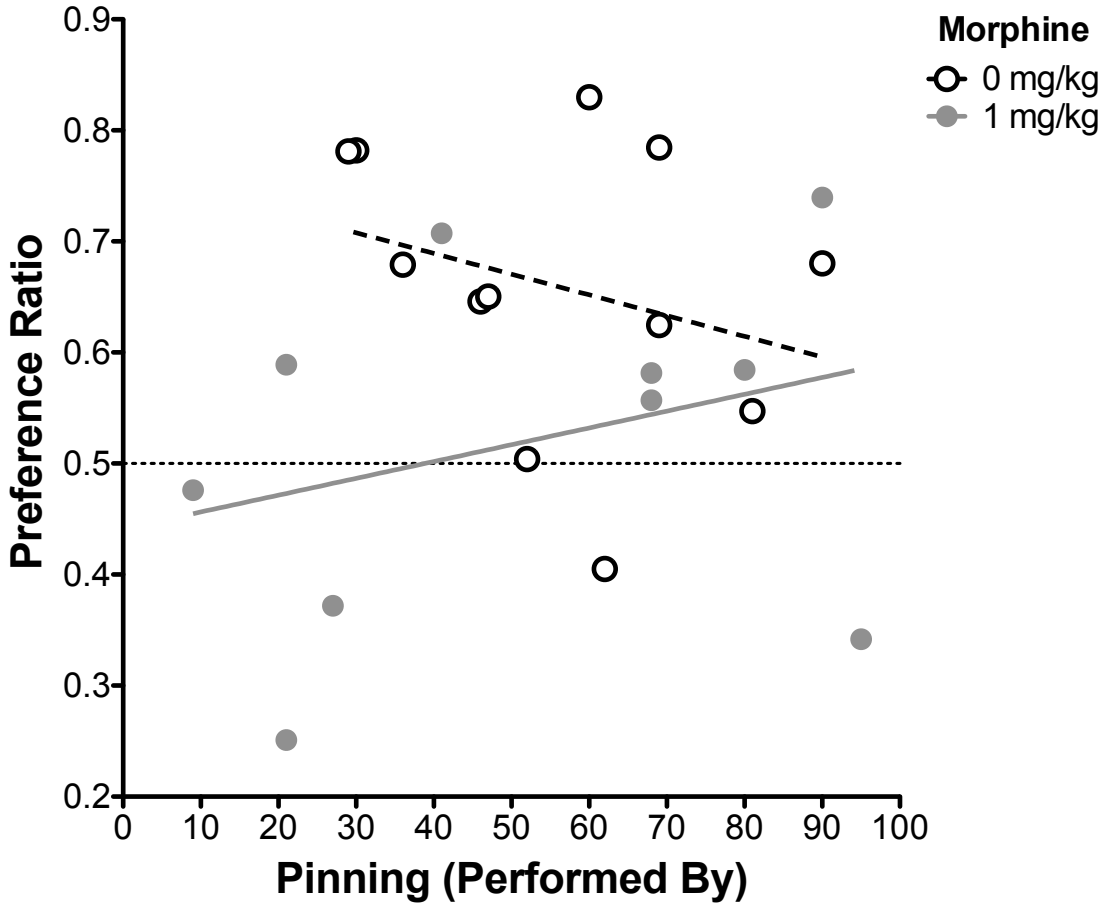

Figure 25. Correlation between pinning performed by a rat by their social peer during the 4th social pairing and CPP preference ratio during the first post-conditioning test session (thin dashed line indicates equal preference for both compartments) for rats that received saline (open circles, thick dashed line) and 1 mg/kg morphine (gray closed circles, gray solid line). n = 12 per treatment group.

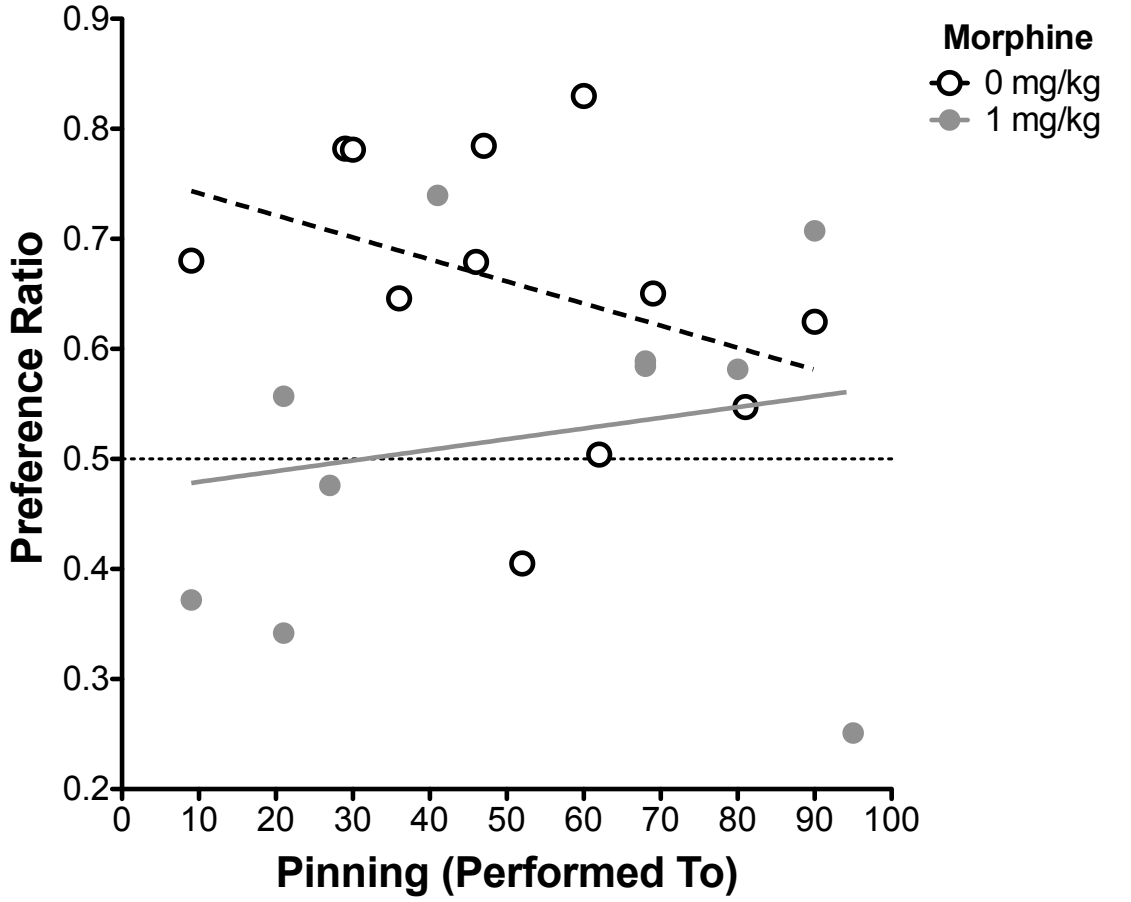

Figure 26. Correlation between pinning performed to a rat by their social peer during the 4th social pairing and CPP preference ratio during the first post-conditioning test session (thin dashed line indicates equal preference for both compartments) for rats that received saline (open circles, thick dashed line) and 1 mg/kg morphine (gray closed circles, gray solid line). n = 12 per treatment group.

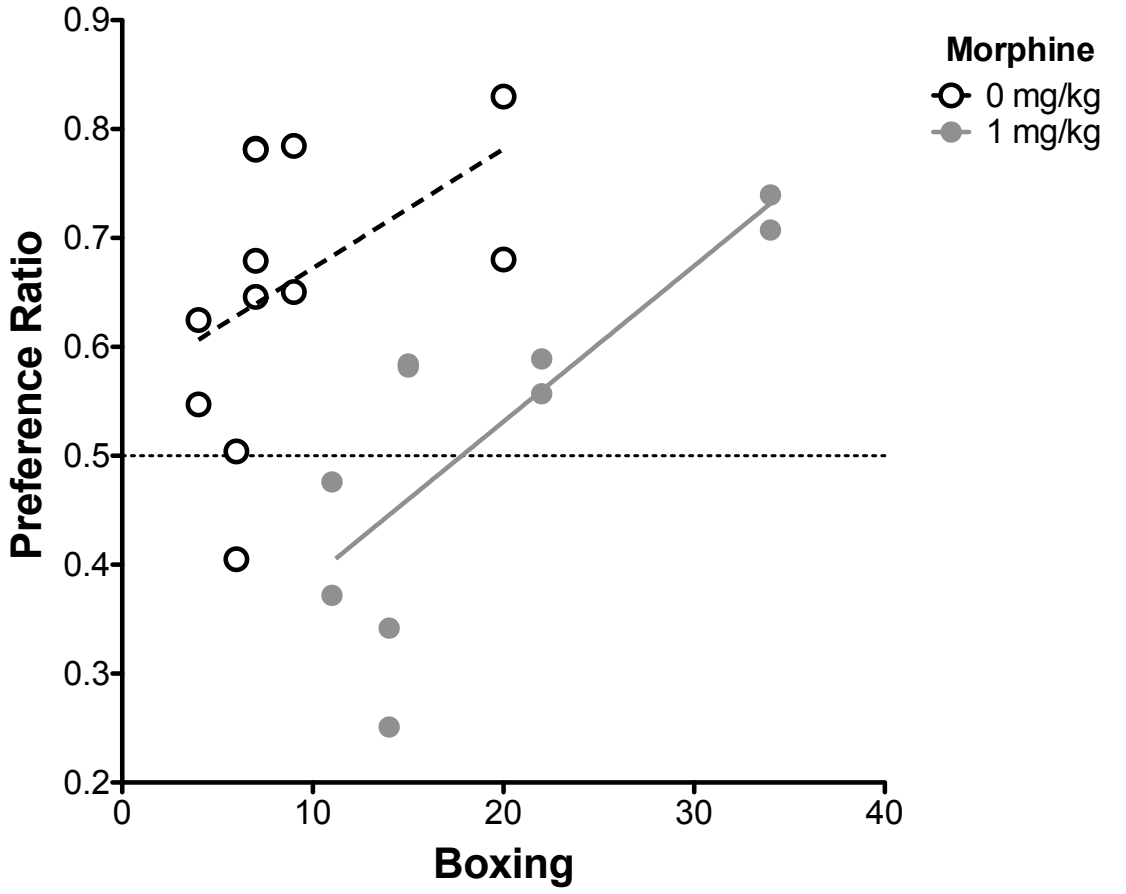

Figure 27. Correlation between boxing during the 4th social pairing and CPP preference ratio during the first post-conditioning test session (thin dashed line indicates equal preference for both compartments) for rats that received saline (open circles, thick dashed line) and 1 mg/kg morphine (gray closed circles, gray solid line). n = 12 per treatment group.

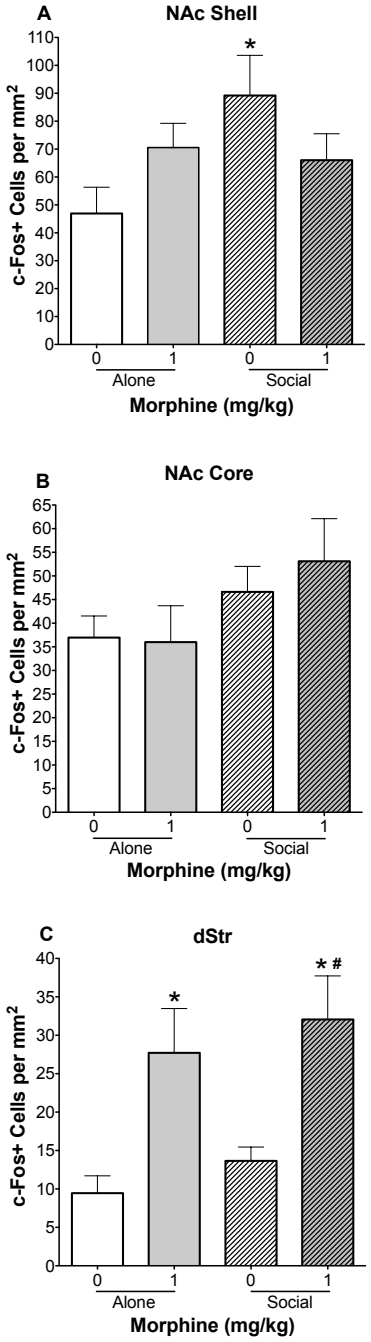

Figure 28. Immunofluorescence results from (A) NAc shell, (B) NAc core, and (C) dStr showing the mean (±SEM) number of cFos+ cells per mm^2 at 100 min after drug administration and/or social interaction. * $p < 0.05$ compared to saline control. # $p < 0.05$ compared to social group. n = 6 per treatment group.

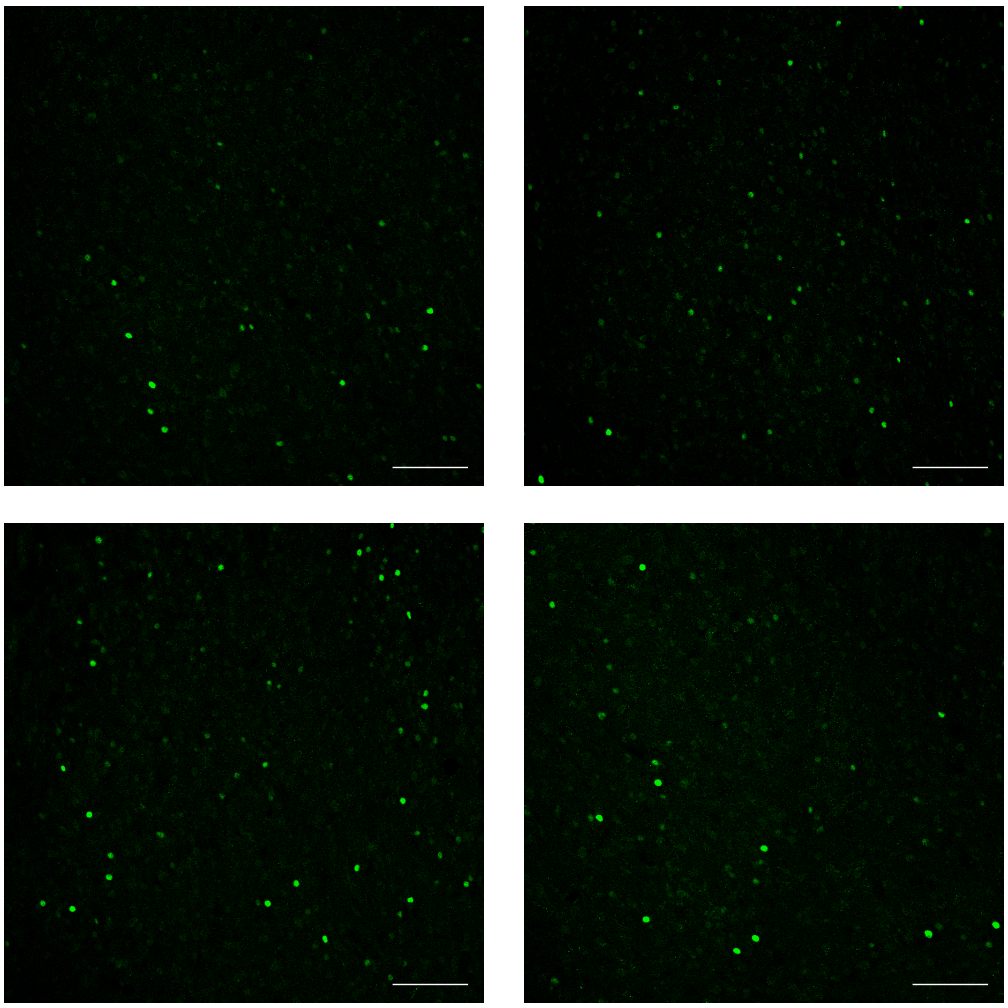

Figure 29. Confocal images showing representative fluorescent cells in the NAc shell expressing cFos. Going clockwise from the top left: saline, 1 mg/kg morphine, social, and 1 mg/kg morphine with social. n = 6 per treatment group. Scale bar represents 100 μm. Images were acquired with a 20x lens.

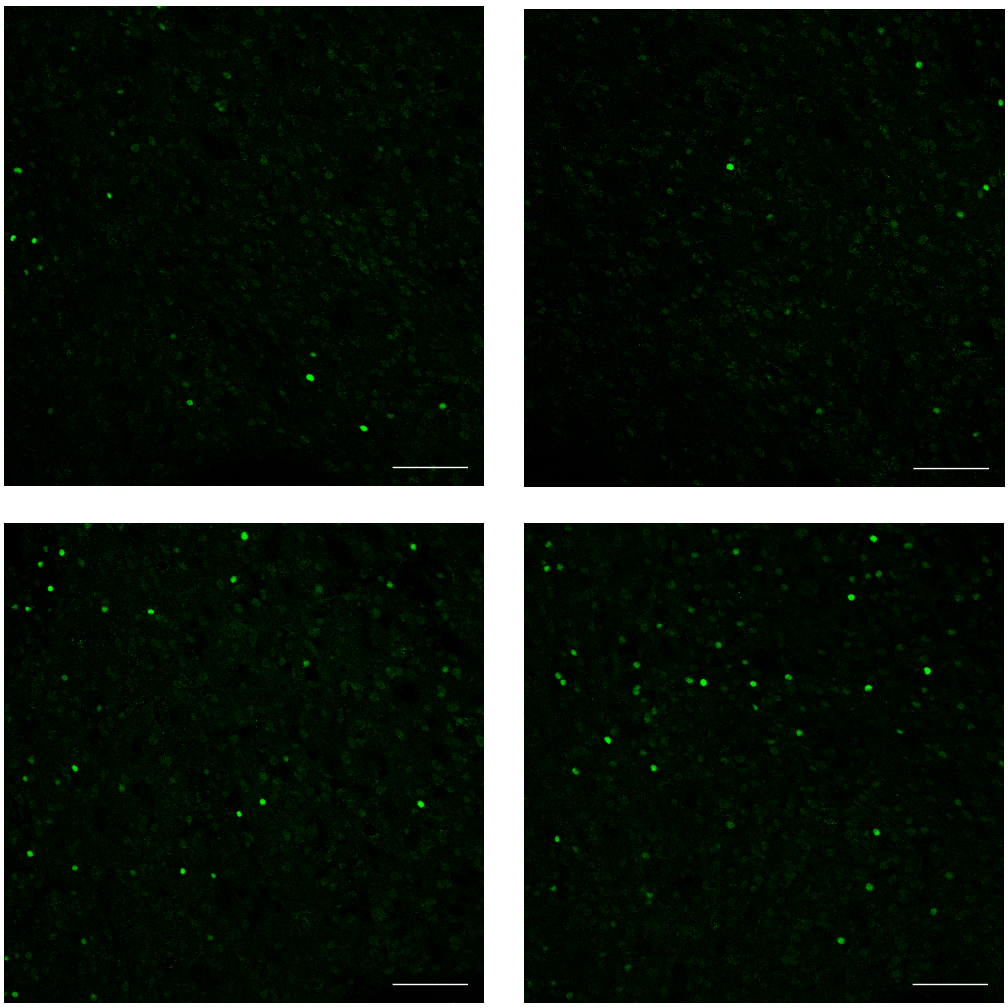

Figure 30. Confocal images showing representative fluorescent cells in the NAc core expressing cFos. Going clockwise from the top left: saline, 1 mg/kg morphine, social, and 1 mg/kg morphine with social. n = 6 per treatment group. Scale bar represents 100 μm. Images were acquired with a 20x lens.

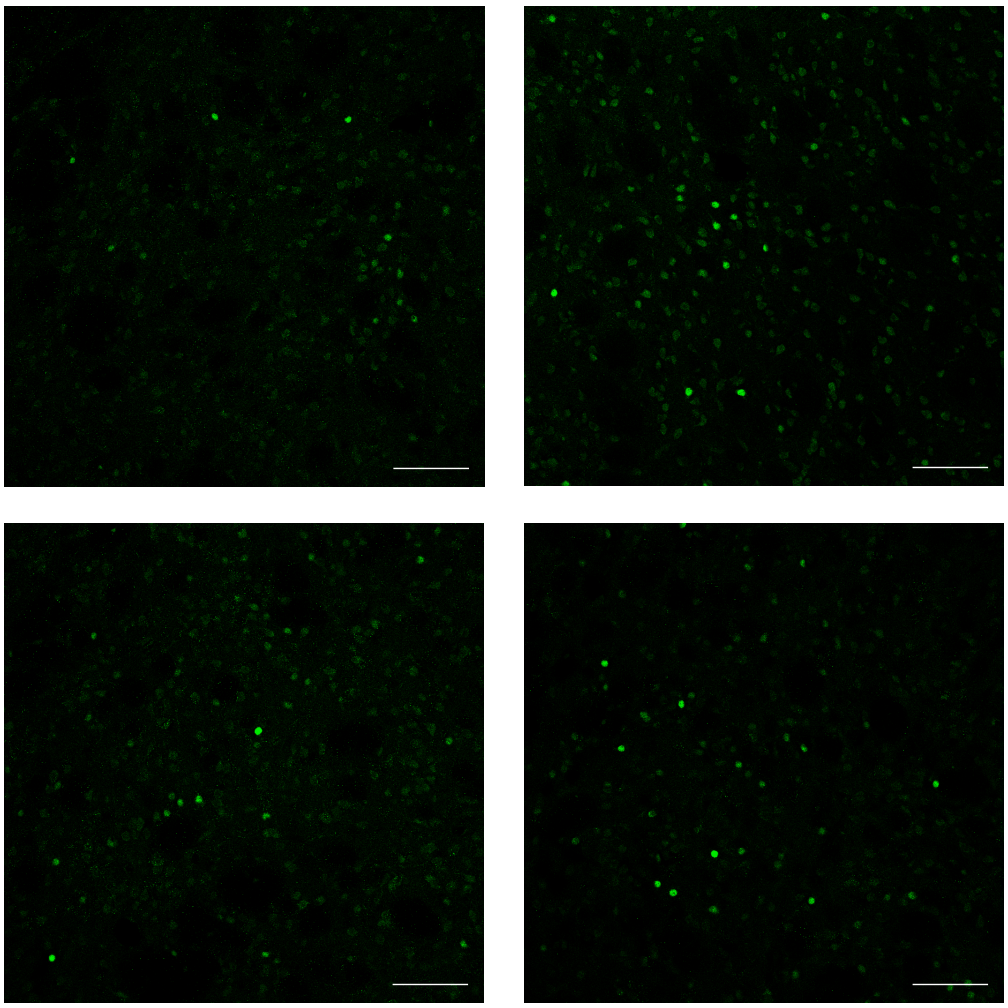

Figure 31. Confocal images showing representative fluorescent cells in the dStr expressing cFos. Going clockwise from the top left: saline, 1 mg/kg morphine, social, and 1 mg/kg morphine with social. n = 6 per treatment group. Scale bar represents 100 μm. Images were acquired with a 20x lens.

Table 1. Experimental groups for Experiment 1.

Compartment 1	Compartment 2
Saline	Saline
1 mg/kg Morphine	Saline
3 mg/kg Morphine	Saline
Saline + Social Interaction	Saline
1 mg/kg Morphine + Social Interaction	Saline
3 mg/kg Morphine + Social Interaction	Saline

Table 2. Experimental groups for Experiments 2 and 3.

Compartment 1	Compartment 2
Saline	Saline
1 mg/kg Morphine	Saline
Saline + Social Interaction	Saline
1 mg/kg Morphine + Social Interaction	Saline

Table 3. Intercepts and slopes correlating round 2 CPP preference ratios and social play behaviors during social pairing 8 in Experiment 2. * $p<0.05$ compared to saline control.

Behavior	Morphine (mg/kg)	Intercept (±SEM)	Slope (±SEM)
Pin – Did	0	0.724 ±0.503	-0.0016 ±0.014
	1	0.558 ±0.507	+0.0059 ±0.015
	3	0.585 ±0.508	-0.0173 ±0.02
Pounce – Did	0	1.101 ±0.165	-0.0061 ±0.002
	1	*0.519 ±0.18**	*+0.0020 ±0.003**
	3	*0.695 ±0.186**	-0.0032 ±0.003
Pin – Done To	0	0.091 ±0.461	+0.0165 ±0.013
	1	0.576 ±0.464	+0.0040 ±0.013
	3	*0.621 ±0.465**	-0.0266 ±0.019
Pounce – Done To	0	0.964 ±0.169	-0.0042 ±0.002
	1	*0.539 ±0.185**	+0.0016 ±0.003
	3	0.754 ±0.191	-0.0044 ±0.003
Boxing	0	0.784 ±0.105	-0.0059 ±0.005
	1	0.536 ±0.122	+0.0131 ±0.009
	3	0.728 ±0.138	-0.0119 ±0.006

Table 4. Intercepts and slopes correlating round 1 CPP preference ratios and social play behaviors during social pairing 4 in Experiments 2 and 3. * $p<0.05$ compared to saline control.

Behavior	Morphine (mg/kg)	Intercept (±SEM)	Slope (±SEM)
Pin – Did	0	0.875 ±0.101	-0.0061 ±0.003
	1	*0.499 ±0.124**	+0.0007 ±0.003
Pounce – Did	0	0.823 ±0.197	-0.0024 ±0.003
	1	0.490 ±0.237	+0.0004 ±0.003
Pin – Done To	0	0.907 ±0.096	-0.0071 ±0.003
	1	*0.512 ±0.118**	*+0.0003 ±0.003**
Pounce – Done To	0	0.802 ±0.196	-0.0021 ±0.003
	1	0.596 ±0.236	-0.0009 ±0.003
Boxing	0	0.563 ±0.064	+0.0109 ±0.006
	1	*0.246 ±0.110**	+0.0143 ±0.008

CHAPTER 4

General Discussion

These findings suggest that adolescents who use opioids may find the experience less rewarding when in the presence of social peers, compared to using alone. This may lead them to withdrawal from social situations. Because opioids and social interaction both engage the endogenous opioid system (Niesink and Van Ree, 1989; Vanderschuren et al., 1995; Meyer, 2005), these adolescents may begin to compensate for the lack of social interaction with more opioid abuse. This possibility is particularly startling given the high usage rate in the United States (SAMHSA, 2016). Alternatively, if the interaction is in the opposite direction, where social interaction is decreasing the rewarding value of morphine, initiation of opioid use in a social situation may actually decrease the likelihood that the individual will continue to misuse the drugs. Of course, both of these scenarios are assuming that the person was not originally prescribed opioids by a physician for pain and took them as prescribed.

The diminishing effects that repeated morphine has on activation of the NAc shell is in keeping with previous preclinical and clinical literature (Gardner, 2011). In contrast to repeated morphine, however, the effect of social interaction on NAc shell activity did not dissipate with repeated exposure. The ability for social stimuli to activate the NAc following repeated exposure has also been observed in clinical literature (Knutson et al., 2001; Knutson et al., 2005; Gasic et al., 2009; Dichter et al., 2012). Because social interaction is a natural reward, and required for human survival (Holt-Lunstad et al., 2010; Debarre et al., 2014), it makes logical sense that mammals would not show a decrease in neural activation of reward relevant brain regions across time.

Several key findings emerged from this dissertation. First, flooring has a large impact on social play behavior and, consequently, CPP. The CPP apparatus used in this dissertation was designed for adult rats, and therefore the spacing between the wire mesh and metal bars are too far apart for adolescent rats to maintain a stable stance. In support of this hypothesis, the frequency of pouncing and pinning were noticeably lower when animals were conditioned in compartments with the standard flooring (mean±SEM; 73.25±8.56 and 5.75±2.02) compared to the non-standard solid flooring (mean±SEM; 126.25±12.38 and 29.5±13.28). Therefore, we believe that social CPP results obtained from experiments that included the non-standard solid flooring (i.e. Experiments 2 and 3) are more valid than those from experiments that included the standard flooring (i.e. Experiment 1). As such, this section will primarily focus on the implications of results of Experiments 2 and 3.

A second key finding from this dissertation is that, with the procedures utilized here, rats conditioned with morphine showed no reliable CPP across multiple experiments using doses of either 1 or 3 mg/kg. While it was not hypothesized that 1 mg/kg morphine would produce CPP, previous research suggested that 3 mg/kg would (Bardo et al., 1995). One possible explanation for this contradictory finding is the age of the animals used in the current experiments. Most literature regarding morphine CPP in rodents has been conducted using adults, whereas this dissertation focused on adolescents. Given that adolescent and adult rats metabolize and respond to drugs differently (Campbell et al., 2000; Kennedy et al., 2011; Koek et al., 2012, 2014; Dannenhoffer et al., 2016; Wiley et al., 2008), it is also possible that there are differences

in the rewarding effects as well. These differences between age groups may also help to explain why the 0.3 mg/kg dose of morphine *did* produce CPP.

While 1 mg/kg morphine did not produce CPP in Experiment 3, its administration (both with and without social interaction) led to significant increases in cFos expression in dStr. Previous research has shown that medium spiny neurons in the dStr are involved in encoding locomotor relevant information (Yamin et al., 2013; Barbera et al., 2016), which could have been increased by the repeated administration of a low dose of morphine (Hofford et al., 2012; Li et al., 2014; Maisonneuve et al., 1992). It should be noted that flooring also had an impact on morphine CPP. While the non-standard flooring inserts were associated with morphine sensitization at the 1 mg/kg dose, the standard flooring inserts were associated with morphine sensitization at the 3 mg/kg dose.

In NAc core and shell, morphine alone did not alter cFos expression following either single or repeated administration at the doses used in this dissertation. This result was not surprising, given that previous literature has reported that doses required to observe an increase in cFos expression from morphine administration are significantly higher than those used in this dissertation (Hamlin et al., 2009; Liu et al., 1994).

A third key finding from this dissertation is that, with the procedures utilized here, social CPP was not reliably produced without extended conditioning trials and solid flooring. This finding contradicts previous literature, which has found that social CPP is produced with as few as 4 conditioning trials in adolescent male rats (Trezza et al., 2014). It is unlikely that session length was the cause of this discrepancy, as both shorter (Douglas et al., 2004) and longer (Weiss et al., 2015; Yates et al., 2013) session durations can produce robust social CPP in both adolescent and adult rats. While the non-standard

flooring increased the probability of producing social CPP (particularly following only the first round of conditioning), it is difficult to explain why social CPP was not observed with the standard flooring. One possible explanation is that the lighting was too bright. Most social CPP paradigms significantly dim the lights, because rodents are nocturnal animals and tend to seek shelter in bright light (Crawley, 1985). Due to the video recording system, the lighting was slightly brighter in order to be able to distinguish rats from one another during coding.

Also contradictory to previous findings, there was a negative correlation between social play and CPP in rats that received social interaction without morphine. Vanderschuren et al. (2016) reported that there was a positive correlation between pinning/pouncing and time spent in the social paired compartment in adolescent male rats. Unfortunately, the authors of the previous article did not provide in-depth methods to allow for direct comparison to the current results. Therefore, while differences in procedure and analyses may account for the opposing findings, we are unable to know for sure.

With regard to neural activation, another key finding was that animals exposed to social interaction showed differences in cFos expression, but this depended on whether exposure was acute or chronic. Rats that underwent a single social play pairing in Preliminary Experiment 2 had significant increases in cFos in NAc core, but not NAc shell. In contrast, after repeated play pairings in Experiment 3, rats had significant increases in NAc shell, but not NAc core. Increases in cFos expression within NAc following a single social play pairing have been previously reported (van Kerkhof et al.,

2014). To our knowledge, this dissertation is the first to investigate cFos expression following 16 conditioning trials of CPP.

It is of interest that different reward regions are activated with varying amounts of exposure to social interaction. NAc core, which was found to be activated following a single exposure, appears to be involved in motor function related to rewarding stimuli. Specifically, NAc core encodes motor programs, which can facilitate the acquisition of a reward in the future (Malenka et al., 2009). In contrast, NAc shell is more closely linked to the cognitive processing of rewarding stimuli, including "liking" and motivational salience (Baliki et al., 2013; Berridge and Kringelbach, 2015; Saddoris et al., 2015). Therefore, it is plausible that when the rat has relatively little experience with social play, that the NAc core may encode appropriate play behavior that is found to be rewarding, but as more play pairings occur, the NAc shell is recruited to inform the animals of previous experiences with the rewarding social play behaviors. This role of the NAc shell would be particularly important during CPP post-conditioning test sessions, which occur in the absence of any conditioned stimuli.

A final key finding of this dissertation is that social interaction decreases the rewarding value of morphine. This disconfirms our original hypothesis, which proposed that social interaction would lead to an enhancement of morphine CPP in male adolescent rats. Following 8 conditioning trials, neither 1 or 3 mg/kg morphine produced CPP when combined with social interaction. Furthermore, even after 16 conditioning trials, 1 and 3 mg/kg morphine in combination with social interaction still did not produce CPP. Interestingly, there was also a positive correlation between social play and preference for

the morphine and peer paired compartment. As mentioned above, a negative correlation was observed with social interaction alone.

Not only did social interaction decrease morphine CPP, another interesting finding from this dissertation was that social CPP was blunted when combined with morphine. This is evident in Experiment 3 after 8 conditioning trials. The morphine-induced decrease in social CPP was not directly related to a decrease in social play behavior because 1 mg/kg morphine blunted social CPP in Experiment 3 without altering either pouncing or pinning (see Figures 22-24). However, similar to the ability of morphine to blunt social CPP, we found parallel evidence from the cFos results that morphine also blunted the social-induced increase in neural activity in NAc shell. Since this pattern of cFos expression results was obtained only in NAc shell, and not in either NAc core or dStr, these results suggest that the NAc shell may serve as a critical node of overlap between morphine and social reward. This is consistent with a host of literature identifying the NAc shell as a critical region involved in reward processing (Trezza et al., 2011; Saddoris et al., 2013; Sugam et al., 2014).

Also contrary to our hypothesis and previous literature (Blanco-Gandia et al., 2015; Trezza et al., 2014), 1 mg/kg morphine did not increase pinning and pouncing following the first administration. We originally hypothesized that this was due to the spacing between metal bars in the standard flooring. However, when a more solid floor was implemented, a morphine-induced decrease in social play behaviors persisted. To our knowledge, no one has previously observed changes in play behavior following morphine administration in a CPP compartment. Most research on social interaction utilizes apparatus closer in size to a locomotor chamber (Achterberg et al., 2016; Trezza

et al., 2008; Vanderschuren et al., 1995), which is significantly larger than a CPP compartment. Therefore, the size of the apparatus may have an enormous impact on how morphine effects play behaviors in adolescent male rats.

While the decrease in social play was contradictory to our hypothesis, the behavioral results are similar to those observed with stimulants. Stimulants decrease social play (Achterberg et al., 2014), but there can be a synergistic effect on CPP when the two stimuli are paired together (Thiel et al., 2008, 2009). While stimulants are known to increase locomotor activity, this effect can also occur with morphine under certain administration schedules. In particular, robust sensitization can occur with repeated administration of morphine at low doses (Maisonneuve et al., 1992; Li et al., 2003; Hofford et al., 2012). Thus, while we did not measure locomotor in these experiments, it is possible that sensitization may have occurred with 1 mg/kg morphine, thus decreasing play behaviors.

Limitations

There are several limitations with the experiments of this dissertation. A main limitation is the fact that dose-dependent decreases in social play behavior were observed, which is contradictory to what was expected based upon previous literature (Blanco-Gandia et al., 2015; Trezza et al., 2014). This may be due to a number of reasons. First, most play literature uses apparatus that are larger, and more akin to locomotor chambers (Achterberg et al., 2016; Trezza et al., 2008; Vanderschuren et al., 1995). Because this is the first study to investigate the effects of morphine and social

play behavior using CPP that coded play behavior, predictions of effects of morphine on play behavior were based upon literature using the larger chambers.

Second, flooring had a significant impact on play behavior and, alternatively, CPP. While we attempted to provide solid flooring for rats in each of the compartments, we still ran into difficulties. Some rats discovered a way to uproot the inserted mats. This lead to play behavior that was impossible to code, as well as a decrease in the amount of social interaction that occurred between the social peers. It is possible that the addition of solid, plastic flooring that is more permanently placed into the compartments may alleviate this issue.

Third, while a 20-min duration was chosen for CPP conditioning sessions, based on previous literature for both morphine and social CPP, this time may not have been optimal to produce robust CPP for either reinforcer. Our laboratory has previously shown robust social CPP with the use of a 30-min trial (Yates et al., 2013; Weiss et al., 2015), but we had no experience with our particular chambers using a shorter time point. Furthermore, the doses of morphine used were relatively low, and were well below the therapeutic threshold. Because of this, there was less literature on which to base a decision for an appropriate session time. Although the main decision was made based on a meta-analysis published from our lab (Bardo et al., 1995), the data used for the analysis was from literature that used adult subjects.

Fourth, there was a lack of replicability across the CPP experiments. The most likely reason for this phenomenon rests with the use of low threshold doses of morphine. While higher doses (10 mg/kg) would be expected to have produced more robust CPP, the purpose of these experiments was to determine if social interaction could enhance

CPP, and thus low threshold doses were intentionally selected. In addition to using a higher morphine dose, it is possible that more robust social CPP would have been obtained if a new partner was used on each conditioning trial, rather than the same partner as used in the completed experiments. Again, however, since the purpose of the experiments was to determine if morphine and social interaction produced synergistic effects, it would have not been ideal to select procedures that produced maximal CPP.

Finally, the decision to include the 1 mg/kg morphine dose in Experiment 3 was based upon multiple regression analyses of data with a relatively low number of subjects. Traditionally, most correlational analyses are done with a large number of subjects (Bujang and Adnan, 2016). Therefore, while the dose was chosen by both scientific analyses and interest, a larger number of subjects would have been more appropriate for such an analysis.

Other limitations include low subject number and the use of males only. As mentioned previously, a larger number of subjects would have allowed for more statistical power in correlational analyses, including the ability to analyze play behavior within dyads, in addition to individuals. However, the number of subjects that were used in this dissertation is typical for CPP studies (Yates et al., 2013; Weiss et al., 2015).

Adolescent males were used exclusively in this dissertation because previous literature has shown that they find social interaction more rewarding than any other sex or age group (Douglas et al., 2004). Female rats also do not engage in as much rough and tumble play as males, and instead show more exploratory behavior (Auger and Olesen, 2009). Because of this, social interaction literature focuses almost exclusively on adolescent males. However, drug use and social interaction are not limited to males, and

there are significant sex differences in morphine sensitivity. Following acute morphine exposure, males have a greater antinociceptive response, compared to females, and tolerance more quickly develops in males than females (Craft et al., 1999). Others have reported that, following chronic morphine exposure, females show increased locomotion and time spent in the open, while males show behavior similar to saline controls (Zhan et al., 2015). These results suggest that while males develop tolerance to morphine's effects, that females develop sensitization.

Females also self-administer significantly greater amounts of morphine and heroin than their male counterparts in a FR operant conditioning paradigm (Cicero et al., 2003). In the same study, females also had a higher break point during a progressive ratio schedule, with the highest break point in females being more than double that observed in males. This suggests that females find morphine more rewarding than males.

Because of the drastic sex differences in both morphine response and social play behavior, it is important to note that the results of this dissertation cannot be generalized beyond adolescent males.

Clinical Implications and Future Directions

The results of this dissertation are relevant for current clinical research. Adolescents who begin to use drugs of abuse recreationally are likely to be among social peers and begin at low doses (Bahr et al., 2005; Kandel and Logan, 1984). The results of this dissertation support the idea that adolescents find social interaction to be rewarding and this interaction may modify the drug experience. In addition, once humans begin to use drugs chronically, drugs intake tends to escalate, while social interaction tends to

decrease (Lesscher and Vanderschuren, 2012; Piazza and Deroche-Gamonet, 2013; Preller et al., 2014). Results from this dissertation with the 3 mg/kg dose of morphine supports the idea that chronic exposure to higher doses of morphine diminishes the rewarding effects of the drug in the presence of social peers. In addition, we found evidence that the rewarding effect of social interaction is reduced by morphine, suggesting a reciprocal interaction between social and drug reward.

While we found the interaction of morphine and social play to be unrewarding, the data from this dissertation cannot confirm the direction of this effect. Therefore, from a clinical perspective, it is important for physicians to assess the changes in social behavior of adolescents following administration of opioids. While it is possible that social interaction decreases the rewarding value of morphine, which may lead to decreased risk of the abuse of prescription opioids, it is also possible that morphine decreases the rewarding value of social interaction. This second possibility could lead to social withdrawal and increased drug use, which could be detrimental to patients. In order to better understand the direction of this effect, future research should more directly manipulate changes in social environments and interaction, as well as morphine dose.

Future research should also include investigation of neural mechanisms underlying the effect of social interaction on the rewarding value of higher doses of morphine. Results of this dissertation suggest that there may be an inverse relationship between the rewarding value of morphine alone and morphine combined with social interaction. In addition, all of the doses used in this dissertation were below the therapeutic threshold for analgesia (~10 mg/kg; Abbott et al., 1982). Therefore, it would be interesting to determine if social interaction alters morphine analgesia. Furthermore,

this dissertation focused on adolescent male rats. Future research should examine whether the same results are obtained in adolescent females, as well as adult males and females. Finally, these experiments focused on effects of morphine and social interaction in combination. It would be of interest to investigate what would occur if the two rewarding stimuli were compared against each other in different sides of the CPP apparatus. Such information would provide evidence about the potential utility of social interaction to compete with opioid taking behavior.

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Publications

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