THE ROLE OF SOCIAL ENVIRONMENT ON MORPHINE RESPONSE IN ADOLESCENT MICE

A Dissertation

by

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ABSTRACT

Drug abuse is strongly influenced by socio-environmental factors. Also, social environment is one of the most important predictors for adolescent drug use. In this dissertation, I examined how social housing conditions affect morphine reward, dependence, and antinociception. I also explored possible mechanisms that may underlie these responses. Mice were group-housed in one of two conditions referred to as 'only' and 'mixed'. In the only condition, all mice in the cage receive the same treatment and are physically and visually separated from mice that receive different treatments (i.e., saline only and morphine only). Mice in the mixed condition were housed together with mice that received a different treatment- (i.e., morphine cage-mate mice are housed with drug naïve mice and saline cage-mate mice are housed with morphine-treated mice).

Being housed with drug-naive mice conferred a protective effect for the rewarding properties of morphine, as well as the occurrence of withdrawal symptoms. Similarly, while it did not prevent the development of tolerance to morphine analgesia, it did reduce the persistence of this tolerance. Moreover, it provided protection from morphine-induced hyperalgesia. Additionally, some of the neurobiological mechanisms underlying this protection were identified. Inhibiting the sensation of social grooming in morphine cage-mates blocked the protective effect of being housed with drug-naïve animals. Additionally, increased expression of vasopressin mRNA was observed in the striatum of morphine only animals, while being housed with drug-naïve mice protected against this effect. In line with this finding, antagonizing V1b receptors blocked the

development of morphine reward in morphine only animals. Lastly, I identified various genes with increased expression levels in striatum of morphine only animals, but not morphine cage-mates. This makes them potential targets for future studies aiming to reveal the underlying molecular mechanisms involved in the protective effects of being housed with drug-naïve animals. These studies further support the notion that social conditions alter the propensity for developing opioid addiction, and can be used in the development of more efficacious behavioral and pharmacological treatments for adolescent opioid addicts.

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Contributors

This work was supervised by a dissertation committee consisting of Professor Shoshana Eitan [advisor], Professor Paul Wellman, and Professor Stephen Maren of the Department of Psychology and Professor Michael Smotherman of the Department of Biology.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Addiction is a very pervasive and debilitating disease. More than 20% of Americans have used an illicit drug, with many escalating to abuse (National Institutes of Health, 2015). Drug addiction is a complex and misunderstood disorder with a wide variety of consequences. Among these consequences are problems with health, finances, and social relationships. Aside from these personal consequences, addiction also has negative consequences for society. The estimated annual costs for addiction in the United States, which include crime-related costs and costs for health and productivity, is greater than $600 billion.

Addiction is most often defined by compulsive seeking and use of a drug of abuse. Therefore, some individuals may use a drug and not become addicted. Unfortunately, many do. There are multiple theories for why some people become addicts while others do not. It has been suggested that many people maintain drug use because of the drug's rewarding properties, or to avoid withdrawal (Koob, 1992a, 1992b; Koob, Stinus, et al., 1989; Koob, Wall, & Bloom, 1989). Koob and colleagues argue that addiction involves the transition from an impulsive to a compulsive state. In other words, while initial use of a drug is due to its positive reinforcing properties, addiction arises because of the onset of negative reinforcement. Another prominent theory, proposed by Robinson and Berridge (1993), argues that some users become addicts because of the ability of drugs to produce enduring changes in areas of the brain that are

implicated in natural reward, and that mediate motivation to seek reward. Once these areas have become 'hijacked', they become increasingly sensitive to drugs and related stimuli, thus leaving the addict very motivated to seek out these stimuli. This has been termed incentive salience-, or incentive sensitization (Robinson & Berridge, 1993). Indeed, drugs of abuse often cause chemical, and structural, alterations in the brain of the addict that make abstaining extremely difficult. Most notably, these occur in the mesocorticolimbic pathway, and involve regions integral in dopaminergic transmission (Hyman & Malenka, 2001).

The mesocorticolimbic pathway includes the ventral tegmental area (VTA), nucleus accumbens (NAcc), and prefrontal cortex (PFC). Not all drugs of abuse impact the brain in the exact same way; however, the contribution of these regions was initially observed with psychostimulants, and the evidence for their involvement with psychostimulant addiction is convincing. The involvement of the mesocorticolimbic pathway in incentive sensitization has been expanded to include other drugs of abuse. The classic example of the acute reinforcing effects of drugs of abuse involves dopaminergic neurons projecting from the VTA to the NAcc. Upon acute ingestion of a drug, activation of this circuit leads to pleasurable, rewarding effects that become associated with the drug. Schultz (2001) showed that, in primates that learned to associate a cue with food, dopamine levels were increased when they were presented with the cue, but not the food. Therefore, dopamine may also be involved in the anticipation to obtain a drug, and not just in its rewarding effects. Often individuals who

become addicted no longer report rewarding effects of a drug, but rather withdrawal, which may act as a negative reinforcer.

As previously stated, addiction is characterized by the uncontrollable preoccupation and craving for a drug. Once a person becomes addicted to a drug, several consequences may arise. For example, there are many medical consequences of drug use, including cardiovascular and lung disease, stroke, and cancer. Addicts may also be more prone to illness. Addicts may also abandon their jobs and ignore their families/friends. Unfortunately, this leads to a lack of social support, which may further propel the addict towards a drug (or multiple drugs).

Because of the enormous burden on society it creates, it is of great societal interest to investigate addiction, in order to potentially determine the neural mechanisms that underlie it, so that better treatments and interventions may be developed to treat addiction. While addiction can develop at any age, adolescents are in a stage which makes them likely to experiment with drugs, which may increase the likelihood of the development of addiction (Adriani et al., 2004; Parker & Bradshaw, 2015). Adolescence is a period of profound developmental changes. Among these changes are increases in risk-taking and novelty-seeking behaviors, which occur in both human adolescents and non-human animals (Spear, 2000). Additionally, several physical and cognitive changes occur that may increase the adolescent's susceptibility to delinquent behaviors, including drug experimentation. Indeed, adolescence is when most people initiate drug use, as statistics show that many addicts report initial drug use between the ages of 12 and 14 (National Survey on Drug Use and Health, 2008). Moreover, abuse of opioids, including

prescription pain medications and heroin, has increased dramatically in this population (Johnston et al., 2015), with many more beginning to use every day. In fact, the percent of adolescents seeking treatment for heroin dependence has increased from 16.6% to 25.8% in the past decade, and from 15.5% to 34.5% for prescription opioids (Johnston et al., 2015). Currently, there are limited treatment options for adolescent opioid addicts. The use of opioid maintenance treatments is usually limited to severe cases in this age group. Thus, most treatment regimens for adolescent opioid addicts involve brief detoxification, coupled with therapy in outpatient settings (Fiellin, 2008; Subramaniam, Fishman, & Woody, 2009). Unfortunately, this approach is often not effective in reducing relapse. It has been shown that social support can also be a factor that aids in the cessation of use of both licit and illicit drugs (Kelly et al., 2008; Klimas et al., 2014; Luchenski et al., 2015; McCutcheon, Luke, & Lessov-Schlaggar, 2016), while lack of social support may increase an adolescent's likelihood of engaging in drug use (Bardo, Neisewander, & Kelly, 2013). Similarly, peer support, operationalized as social activity in a 12-step program, was shown to increase the length of abstinence in adolescents with, and without, social anxiety disorder (Pagano et al., 2015). Nevertheless, more effective treatments need to be developed for adolescent opioid users.

The brain undergoes several modifications during adolescence, and it is increasingly recognized that these changes may contribute to adolescent abuse liability. Among these developmental modifications are increases in cerebral white matter during adolescence (Giedd et al., 1999; Groeschel et al., 2010). The results of these studies indicate that there are white matter changes in areas that are implicated in drug craving,

seeking, and relapse (for review see, (Van den Oever et al., 2010). Furthermore, the immaturity of the adolescent prefrontal cortex in adolescents may contribute to an increased susceptibility to drug use. Another modification during adolescence that is relevant to drug use is the remodeling of the density and distribution of dopamine receptors in the nucleus accumbens (Laviola, Pascucci, & Pieretti, 2001), which may result in higher sensitivity to rewards. Indeed, adolescents display heightened activation of this area in response to reward-related cues, particularly during reward anticipation (Bjork et al., 2004; Ernst et al., 2005; Geier et al., 2010). Lastly, these modifications in the mesocorticolimbic pathway may result in greater salience of social cues in this age group (A. R. Smith et al., 2015; Mark A. Smith & Pitts, 2014; van Kerkhof et al., 2013).

Social interaction has been reported to activate this pathway (van Kerkhof et al., 2013). Adolescent rodents interact more with their peers, and exhibit greater affiliative behaviors than other age groups, including social play and huddling (Spear, 2000; Vanderschuren, Niesink, & Van Ree, 1997). Moreover, adolescents, but not adults, demonstrate riskier behavior, and less cognitive control, in the presence of peers (Chein et al., 2011; Gardner & Steinberg, 2005). This increase in risky behavior is accompanied by higher preferences for immediate rewards when in the presence of peers (O'Brien et al., 2011). Interestingly, Chein et al. (2011) found that adolescents showed heightened activity in the ventral striatum and orbitofrontal cortex – areas implicated in reward – in the presence of peers, along with decreased activation of lateral prefrontal cortex – an area involved in cognitive control. These patterns of activation likely underlie the fact that adolescents engage in riskier behavior in the presence of peers. Lastly, social

influences are their most powerful during adolescence, and decline with age (Kendler et al., 2008).

Prior research shows that drug use initiation is often influenced by social environment (Bardo et al., 2013; Chassin, Hussong, & Beltran, 2009). In the clinical literature, neighborhood environment seems to contribute to the initiation of using drugs - as individuals living in an area rife with drug use may be more likely to use illicit drugs, including cocaine and heroin (Crum, Lillie-Blanton, & Anthony, 1996; Karriker-Jaffe, 2011). It was also shown that heroin overdose fatalities were higher in lower-income neighborhoods than in higher-income neighborhoods (Cerda et al., 2013). Moreover, individuals living in poorer neighborhoods may not develop beneficial relationships, as it has been suggested that people in lower-income neighborhoods may feel discouraged from developing relationships that might prevent drug use in those neighborhoods (Cohen, Farley, & Mason, 2003). Availability of drugs by interacting with peers who use drugs highly correlates with one's own drug use behavior (Gorsuch & Butler, 1976), and problem behavior is often determined by proximal peer influences (Patterson, DeBaryshe, & Ramsey, 1989). Moreover, according to some studies, the use of opioids by peers is influential by itself and not only as the source for the opioids (Luthar et al., 1992; Yarnold, 1996). Even the mere perception of opioid abuse by peers, results in increased initiation of and escalation in opioid use (McDougall et al., 2014).

Similarly, the animal literature suggests that, in adolescent rodents, the drug history of social partners may influence opioid and stimulant taking and seeking behavior (H. Chen et al., 2011; Hunt, Holloway, & Scordalakes, 2001; M. A. Smith,

Lacy, & Strickland, 2014). In line with human and rodent literature, my laboratory demonstrated that housing conditions can alter the behavioral sensitization to morphine in adolescent rodents. Specifically, housing with drug-naïve animals reduces the development of morphine locomotor sensitization (Hofford et al., 2012). Rodent models represent efficacious paradigms for studying human drug use, abuse and dependence. These models provide scientists with the ability to control for multiple variables that cannot be controlled for in human subjects, such as initial age of drug exposure, dosage, and duration of drug exposure. Although there are many limitations in the extrapolation from animal models to human addiction, the use of animal models provides valuable information and there are a variety of paradigms that have been developed in animals for assessing drug use (Tzschentke, 2007). Furthermore, animal models have allowed for invasive and in-depth exploration of the mechanisms underlying adolescent drug use, including the important brain regions that might be involved. Therefore, the goal of the present research is to evaluate the influence of social environment on morphine sensitivity in adolescent mice. Specifically, I sought to learn more about the influences of social environment on the development of morphine reward and dependence, as well as the effects of social housing conditions on the antinociceptive properties of opioids. Moreover, I explored the underlying molecular mechanisms for these effects.

CHAPTER II

GENERAL METHODS

2.1 Subjects

Adolescent C57BL/6 male mice were used for the experiments. Mice were purchased from Envigo (Houston, TX), or bred in-house (Chapter VI), and acclimated to the colony for a minimum of 5 days before the start of experiments. They received food and water *ad libitum* and were housed on a 12:00 hour light/dark cycle with the lights on at 7:30am and off at 7:30pm. All procedures were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals after receiving the approval of Texas A&M University's Institutional Animal Care and Use Committee.

2.2 Housing Conditions

All mice were group-housed, 4 per cage. As depicted in Figure 1, mice were housed in one of 3 housing conditions: morphine only, mixed, and saline only conditions. In morphine only cages, all mice received repeated treatments with morphine, while in saline only cages, all mice received repeated treatments with saline. However, in mixed cages, half of the animals received saline (referred to as saline cage-mates) and half received morphine (referred to as morphine cage-mates).

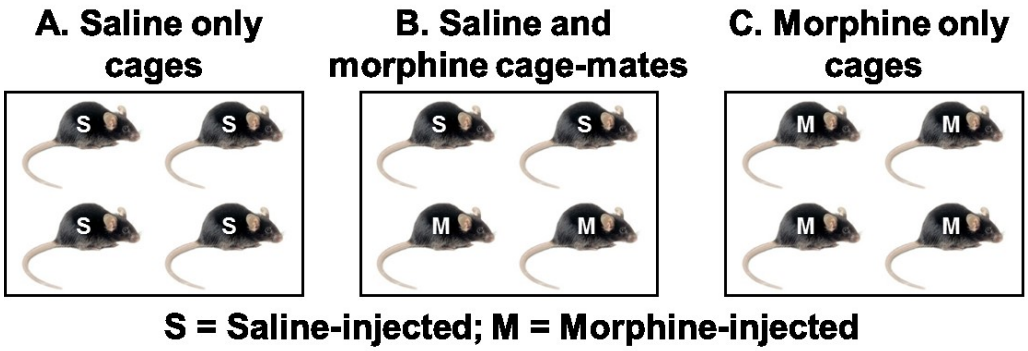

Figure 1. Schematic of social housing conditions.

2.3 Drugs

Morphine sulfate and sodium pentobarbital (10 ml/kg) were purchased from Sigma-Aldrich (St. Louis, MO). The doses represent salt concentrations. For experiment 4, clozapine-N-oxide was purchased from Sigma-Aldrich, as well.

CHAPTER III

EXPERIMENT 1: EFFECT OF SOCIAL ENVIRONMENT ON THE ACQUISITION AND EXTINCTION OF MORPHINE CONDITIONED PLACE PREFERENCE IN ADOLESCENT MICE*

3.1 Introduction

As discussed in Chapter 1, an issue that many adolescent opioid addicts report is a lack of social support, which includes feeling a lack of social attachment. Similarly, in rodents, a lack of social interaction is aversive. In a series of experiments, Panksepp and coworkers showed that a withdrawal syndrome induced by social isolation produces symptoms similar to opioid withdrawal (Panksepp, 1980). Panksepp and colleagues (1978) examined whether opioids could alleviate social distress, defined as a brief period of social isolation, in young puppies. Indeed, opioid treatment alleviates social distress in a variety of animal species including chicks and guinea pigs (Panksepp, Herman, et al., 1978; Panksepp, Vilberg, et al., 1978). This is contrasted by the fact that social play is rewarding, and activates reward processes in the striatum (van Kerkhof et al., 2013). Similarly, social affiliation and interaction can be rewarding as a social partner is sufficient to produce CPP, with the most robust socially-induced CPP being shown by adolescent males (Douglas, Varlinskaya, & Spear, 2004; Thiel, Okun, & Neisewander,

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*Reprinted from Drug and Alcohol Dependence, 142, M.L. Shawn Bates, Michael A. Emery, Paul J. Wellman & Shoshana Eitan, Social housing conditions influence morphine dependence and the extinction of morphine place preference in adolescent mice, 283-289, Copyright (2014), with permission from Elsevier.

2008). Moreover, social interaction decreases levels of phosphorylated p38, a member of mitogen-activated protein kinase family that has been shown to be activated by drugs of abuse and during moderate levels of stress (Salti et al., 2015).

Data from my lab shows that being housed with drug-naïve animals seems to have a protective effect on future morphine reward in adolescent males. Specifically, morphine only adolescents develop a markedly higher degree of locomotor sensitization as compared to morphine cage-mates (Hofford et al., 2012). Sensitization is an indirect measure of drug reinforcement, and is often used as an initial assessment. However, a more direct measure, conditioned place preference, or CPP, corroborates these findings. Cole et al. (2013) studied how social environment affects the sensitization of morphine reward using CPP. In these experiments, a short CPP paradigm was used in order to explore the differences in the acquisition of morphine reward between morphine only and morphine cage-mate adolescent mice. Animals previously exposed to morphine in their home cages acquire morphine CPP more readily than drug-naïve animals (Gaiardi et al., 1991; Shippenberg, Heidbreder, & Lefevour, 1996). Using a similar paradigm, my lab found that morphine-treated mice housed only with other morphine-treated mice (morphine only) acquired CPP after only one conditioning session. That was established across a variety of doses (10, 20, & 40 mg/kg). In contrast, morphine-treated mice that were housed with drug-naïve ones (morphine cage-mates) acquired a significantly lessened degree of reward after a single conditioning session.

The CPP acquisition paradigm is not very quantitative, and a common method to establish a more quantitative measure of the difference in reward acquisition is to study

the rate of CPP extinction. The rate of extinction refers to the length of time required in order to attenuate the conditioned place preference (Kaplan & Coyle, 1998; X. Ma, Zhang, & Yu, 2012; Self & Choi, 2004). In other words, if a drug-related memory is more established, it should take more time to extinguish it. Extinction is a process in which a conditioned response gradually diminishes over time as an animal learns to uncouple a response from a stimulus (Peters, Kalivas, & Quirk, 2009; Quirk & Mueller, 2008). It is believed that it leads to the formation of a new, inhibitory memory that becomes expressed instead of the previous memory (Gantt, 1927; X. Ma et al., 2012). In terms of CPP, the memory of the morphine reward is the stimulus that motivates animals to seek out the morphine-paired chamber (Bardo, Miller, & Neisewander, 1984; Blander et al., 1984; Mucha & Iversen, 1984; Mucha et al., 1982; van der Kooy et al., 1982). Therefore, animals with memories that are less robust will most likely extinguish an acquired place preference more quickly than those with a strong memory. In order to extend the findings of Cole et al. (2013), in this experiment, I examined whether social environment affects the acquisition and extinction of morphine place preference using a longer, classical, CPP paradigm.

3.2 Method

3.2.1 Animals

Adolescent, male mice were used in this experiment. There were 32-40 animals per group. Only morphine cage mate and morphine only animals were studied. The saline cage-mates of the morphine-treated animals remained in their home cages, and

treated with saline, for the entire duration of the study. Saline only and saline cage-mate animals were not studied for the acquisition and extinction of morphine CPP, because the acquisition of CPP requires multiple administrations of morphine, and this by default would eliminate them from being a saline only or saline cage mate conditions.

3.2.2 Pretreatment Regimen

Mice were pretreated once daily with saline or 20 mg/kg morphine (10 mg/mL, s.c.) at 9:00AM for six days. This dose represents the salt concentration.

3.2.3 Conditioned Place Preference Apparatus

Morphine CPP was assessed in eight apparatuses. Each apparatus contained three 20x20x30.5 cm square chambers. Two of the chambers were used for conditioning, and contained distinct olfactory and visual (cow/lemon vs. checker/almond) cues, and the third chamber was neutral (containing no distinct cues). The olfactory scents (200µl) were applied to filter paper that was placed in the top corner of each chamber 5 minutes before the beginning of the session.

These apparatuses were located inside a Photobeam Activity System (San Diego Instruments, San Diego, CA), which allowed for the automated assessment of time spent in each chamber, as well as location throughout test sessions. The system was located in a soundproof room with dim light, and a continuously operating 40 dB white noise generator.

On habituation, test, and extinction sessions, mice were placed in the neutral chamber and allowed free access to all three chambers, while on conditioning days, they were confined to one of the chambers. Mice were habituated to the test room for 30 minutes prior to being individually placed in an apparatus, which was thoroughly cleaned with 70% ethanol and water, and completely dried between sessions.

3.2.4 Morphine CPP Experimental Design

Three days following the final dose of morphine pretreatment, CPP acquisition began. This involved a "biased" CPP design (Tzschentke, 2007). During habituation, mice were placed in the neutral chamber of the CPP apparatus, and allowed to explore the apparatus in its entirety for 30 minutes. This provided the baseline preference, which was used to determine subsequent conditioning chambers. Conditioning began the day after habituation, and lasted for 4 or 8 days, for 60 minutes each day. Animals were injected with saline (10 ml/kg, s.c.), and confined to the initially preferred compartment in the morning (9:00AM), and injected with morphine (5 mg/kg) and confined to their initially non-preferred compartment, in the afternoon (2:00 PM). After the first 4 days of conditioning, animals underwent a test session, which was identical to the habituation session. If place preference was not learned, defined as a significant increase in time spent in the morphine-paired chamber, animals underwent another 4 days of conditioning that were identical to the first 4.

Extinction sessions began the day after the last acquisition test. Animals that developed place preference were given once daily extinction sessions until they achieved

extinction. These sessions were similar to the habituation and test sessions. The animals were considered to have reached extinction when they displayed three consecutive days of no preference for the morphine-paired chamber.

3.2.5 Statistical Analysis

Conditioned place preference was determined as the difference in the time spent in the morphine-paired chamber and the time spent in the saline-paired chamber. In other words, a difference score was calculated between how long the animal was in the morphine-paired chamber on test day, and how much time it spent in the saline-paired chamber on test day. Differences in preference were analyzed using a split-plot ANOVA with a between-group factor of housing and a within-group factor of time. Extinction criterion was defined as the first days of 3 consecutive days in which a mouse did not display preference for the morphine-paired chamber. Survival scores for the number of mice per treatment condition that met criterion using the Kaplan-Meier survival estimation were computed. Differences between the experimental groups were analyzed using the Breslow (Generalized Wilcoxon) test. Differences with p-values of less than 0.05 were considered statistically significant.

3.3 Results

3.3.1 Social housing conditions' effect on acquisition of morphine CPP

On habituation day, both morphine only (-7.14 ± 1.8s) and morphine cage-mate (-9.25 ± 1.4s) mice prefer the saline-paired chamber, and there were no significant

differences between the experimental groups ($t(70) = -0.90$, *NS*). Morphine only animals acquired CPP faster than morphine cage-mates (Breslow Generalized Wilcoxon: $\chi 2\ (1) = 7.33$, $p<0.01$). All 32 morphine only animals acquired CPP after 4 conditioning sessions. For the morphine cage-mates, 19 of the animals acquired morphine CPP after 4 conditioning sessions and 21 acquired CPP after 8 conditioning sessions. This is consistent with previous studies from the lab, which showed that CPP was acquired more quickly by morphine only animals. Twelve morphine only animals (27.3%) and 16 morphine cage-mate animals (28.6%) did not acquire CPP and were omitted from the study. Although rate of acquisition differed, morphine cage-mate (340.18 ± 34.3) and morphine only (346.99 ± 37.8) mice show comparable levels of CPP acquisition during the conditioning phase of the experiment ($t(70) = -0.13$, *NS*). Moreover, comparable levels of CPP were observed in morphine cage-mates who acquired after 4 (369.68 ± 53.6) and 8 (313.50 ± 44.2) conditioning trials ($t(38) = -0.81$, *NS*).

3.3.2 Social housing conditions' effect on morphine CPP extinction

All morphine cage-mate animals were subjected to 30 minutes daily extinction sessions. For the morphine only mice, 15 of them were subjected to 30 min extinction sessions and 17 of them were subjected to 60 min extinction sessions. After 40 days of daily 30-minute extinction sessions, I did not observe a significant change in the overall preference scores for the morphine-paired chamber in the morphine-only group (Figure 2), and only 40% of these animals reached extinction criteria (Figure 3). However, morphine only animals were able to extinguish their CPP after using a more robust

extinction procedure. When 60-minute extinction sessions were applied, 64.7% of the morphine only animals did reach extinction criteria (Figure 3), and exhibited an overall loss in the preference for the morphine-paired chamber.

In stark contrast with the morphine only group, morphine cage-mate animals readily extinguished their CPP for the morphine-paired chamber when exposed to 30-minute daily extinction sessions. Using a repeated-measures ANOVA, I found significant differences between morphine only and morphine cage-mates in their CPP extinction. There was a main effect of housing ($F(1, 56) = 5.27$, $p < 0.05$) and time ($F(39, 2067) = 1.825$, $p < 0.001$), and a significant interaction between time and housing (order 5: $F(1,56) = 4.05$, order 33: $F(1, 56) = 5.823$, $p < 0.05$). Moreover, 72.5% of the morphine cage-mates extinguished CPP during the 40 days of 30-minute extinction sessions (Figure 3). A Kaplan-Meier survival analysis showed that morphine only mice reached extinction criteria significantly slower than morphine cage-mates when both had 30-minute daily extinction sessions (Breslow (Generalized Wilcoxon): $\chi2(1) = 8.46$, $p < 0.01$). There were no significant differences in the extinction rate between morphine cage-mates that acquired CPP after 4 or 8 conditioning sessions ($\chi2(1) = 0.51$, *NS*). Interestingly, morphine only animals extinguished morphine CPP more slowly than both of the morphine cage-mate groups: animals that acquired after 4 ($\chi2(1) = 11.49$, $p<0.001$) or 8 ($\chi2(1) = 4.62$, $p<0.05$) sessions.

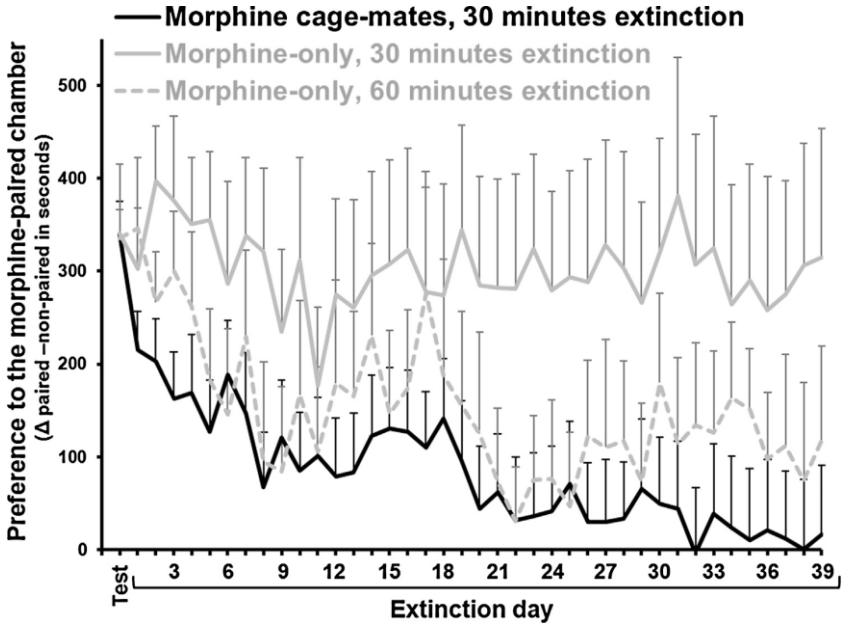

Figure 2. The mean preference for the morphine-paired chamber. Morphine cage-mates (black line) subjected to 30 min daily extinction procedure and in the morphine only groups subjected to 30 min (gray line) or 60 min (dotted gray line) daily extinction sessions. Results are presented as mean ± SEM.

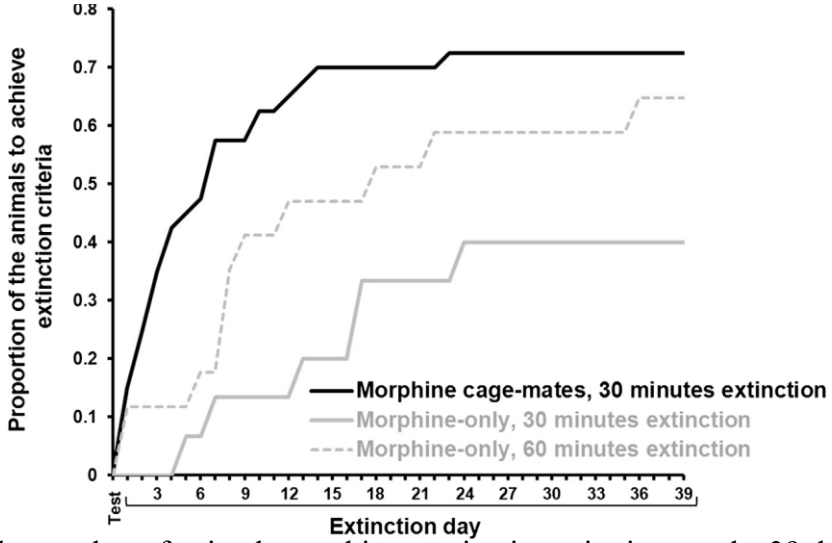

Figure 3. The number of animals to achieve extinction criteria over the 39 days of extinction procedure in the morphine cage mates (black line) subjected to 30 min daily extinction procedure and in the morphine only groups subjected to 30 min (gray line) or 60 min (dotted gray line) daily extinction sessions.

3.4 Discussion

Extinction of a drug-paired memory is essentially extinction of the association between the interoceptive sensation caused by the drug and the external cues associated with it. It has previously been shown that prior administration of morphine leads to sensitization of its rewarding properties (Gaiardi et al., 1991; Lett, 1989; Shippenberg et al., 1996). Sensitization involves an increase in a specific drug effect following repeated use. Thus, an animal previously administered morphine is expected to be sensitized to morphine, and this would lead to a longer time to extinguish a morphine place preference. In my experiments, the morphine only animals extinguish morphine place preference much more slowly than morphine cage-mates. This suggests that the morphine cage-mates are less sensitized to the rewarding properties of morphine as compared to the morphine only mice. Thus, the present study extends and supports my previous study demonstrating the ability of social housing conditions to modulate the rewarding properties of morphine in adolescent mice. Specifically, my previous study demonstrated that exposure to drug-naïve animals slows the acquisition rate of morphine CPP (Cole et al., 2013).

I detected fluctuations in place preference across days in some of the animals tested. These fluctuations are likely due to the fact that extinction testing lasted for a long period of time, and some animals may have experienced a spontaneous recovery of

their place preference. Spontaneous recovery is a phenomenon in classical, Pavlovian conditioning paradigms in which an extinguished response may resurface (Rescorla, 2004). Spontaneous recovery can occur for multiple conditioning paradigms, and has been shown to occur in cocaine seeking (Di Ciano & Everitt, 2004; Peters, LaLumiere, & Kalivas, 2008) and for morphine place preference (X. Ma et al., 2012; Sakoori & Murphy, 2005). It is possible that the lack of overall change in the mean preference to the morphine-paired chamber in the morphine only group is, at least partially, explained by spontaneous recovery. This will suggest lower rates of spontaneous recovery in the morphine cage-mates, in which overall change in the mean preference to the morphine-paired chamber was observed. However, I did not experimentally test this. Further manipulations should be done in the future to determine if spontaneous recovery does occur in my place preference paradigm, and to see if differences would exist among housing conditions. Alternatively, the lack of change in overall mean morphine preference in the morphine only animals might be due to strengthening of the preference to the morphine-paired chamber in some of the animals that did not extinguish. This may also explain the increase in variance in the groups across days. Nevertheless, morphine only animals did extinguish after longer extinction sessions.

Importantly, animals remaining drug-naïve for the entire duration of the study (i.e. mice that were never injected with morphine, only saline, even in the CPP apparatus) obviously could not acquire morphine CPP, and thus, could not be tested for extinction. Moreover, CPP itself (i.e., administration of morphine during CPP acquisition) will unavoidably transform any drug-naïve cage into a cage that corresponds

to one of my morphine social housing conditions. For similar reasons, the saline-injected animals housed with the morphine cage-mates remained both undisturbed in their home cages and were drug-naïve for the entire duration of the study. Thus, social housing conditions were not changed by CPP testing, and the drug-naïve mice continue to provide the social housing conditions for their morphine cage-mates for the duration of the study.

The results of this experiment demonstrate that social housing conditions can affect the extinction of morphine CPP. Because CPP extinction is related to drug craving and the strength of the memory of morphine reward, the present results also indicate that the memory of morphine reward is stronger in the morphine only animals, as compared to the morphine cage-mates. Nevertheless, I have only examined reward using social housing conditions. However, the dependence and withdrawal symptoms caused by opioids are a major issue that keeps addicts from abstaining. Therefore, in the following experiment, I examined the occurrence of spontaneous withdrawal symptoms, and if social environment affected them.

CHAPTER IV

EXPERIMENT 2: EFFECT OF SOCIAL ENVIRONMENT ON MORPHINE DEPENDENCE IN ADOLESCENT MICE*

4.1 Introduction

In the context of human addiction, drug withdrawal refers to the noxious symptoms that occur after addicts attempt to abstain from using. It has been suggested that many people maintain drug use because of the drug's rewarding properties, or to avoid withdrawal (Koob, 1992a; Koob, Wall, et al., 1989). Koob and colleagues argue that addiction involves the transition from an impulsive to a compulsive state. In other words, while initial use of a drug is due to its positive reinforcing properties, addiction arises because of the onset of negative reinforcement. Indeed, both non-medical and medical users of opioids often report the reasons for continued use is in order to stave off withdrawal symptoms (Weiss et al., 2014). Therefore, preventing withdrawal symptoms was considered to be more important for continued use than pain relief (in medical users). Moreover, as previously stated, social support may help to ameliorate withdrawal (Harocopos, Allen, & Paone, 2016).

Withdrawal symptoms are also present in animal models. The occurrences of these symptoms that arise following the cessation of drug administration serve as an

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indicator for dependence and addiction. Typically, withdrawal occurs upon cessation of chronic use of a drug, although some substances like alcohol, may induce withdrawal after single use, or can occur even during use. Withdrawal states in rodents present as a variety of symptoms. Repetitive jumping, or escape attempts, during withdrawal is correlated with dependence, as drug-naïve animals do not jump spontaneously, when placed in a "confined" space (Schulz & Herz, 1977). Withdrawal from several substances also activates the HPA axis, and produces elevated corticosterone levels, and gene expression of CRF in the nucleus accumbens (Brown & Russell, 2004; Collier, 1980; Hyman, 1993; Koob, 1992a; Malin et al., 1992; Manik & Katz, 1984). Withdrawal has also been shown to modulate affective states in the rodent, and produce increases in anxiety and depression-associated behaviors including anhedonia, or decreased pleasure from a previously natural reward, and increased aggression.

In rodent models, social environment has been reported to modulate withdrawal. Adolescent and adult rats that were housed in isolation for 30 days showed fewer withdrawal symptoms than those that were pair-housed (Broseta et al., 2005; Coudereau et al., 1997). Data from my lab show that social conditions can affect plasma testosterone levels, as saline-treated animals housed with morphine-treated ones (i.e., saline cage-mates) showed decreased testosterone as compared to saline only mice (Hofford, Wellman, & Eitan, 2011). Opioids have been shown to modulate testosterone levels, as opioid treatment produces androgen-deficiency syndrome (O'Rourke & Wosnitzer, 2016). Nevertheless, the saline cage-mates were not administered morphine, which suggests that their testosterone levels were affected by the morphine-treatment

animals in their social environment. Lastly, social stress was shown to produce a state of opioid dependence, thus further supporting a role for social environment on opioid withdrawal (Chaijale et al., 2013).

Because opioid withdrawal is a major issue, and because social environment has been shown to affect it, I monitored withdrawal symptoms in this experiment. Because previous data in my lab suggests that morphine may produce more severe behavioral alterations in morphine only animals as compared to all other groups, I hypothesized that morphine only animals would display more repetitive jumping during withdrawal than any of the other groups.

4.2 Methods

4.2.1 Subjects

Adolescent, male mice were used in this experiment. There were 32-40 animals per group as outlined above.

4.2.2 Pretreatment Regimen

Mice were treated once daily for six days with saline or 20 mg/kg morphine (10 mg/mL, s.c.) at 9:00AM. This dose represents the salt concentration.

4.2.3 Spontaneous Withdrawal Symptoms

Withdrawal symptoms were tested 4, 8, 24, and 48 hours following the final morphine injection. They were individually placed in Plexiglas cylinders (37 cm tall x

14.5 cm in diameter) and videotaped for 30 minutes. The videotapes were then scored for jumping behavior by observers who were blind to treatment.

4.2.4 Statistical Analysis

The differences in number of jumps between the morphine cage-mates and morphine only animals were analyzed using a split-plot ANOVA (SPSS Statistics 18, Somers, NY) with a between-group factor of housing and a within-group factor of time (4, 8, 24, and 48 hours). Post hoc contrasts between each treatment group were conducted using Bonferroni post hoc procedure.

4.3 Results

The number of jumps at 4, 8, 24, and 48 hours following the final morphine administration were assessed, and are presented in Figure 4. Using a split-plot ANOVA, I found a main effect of housing ($F(1, 94) = 4.17$, $p < 0.05$), a main effect of time ($F(3, 282) = 15.27$, $p < 0.0001$), and a significant interaction between housing and time ($F(3, 282) = 2.93$, $p < 0.05$). A Bonferroni post-hoc analysis showed that morphine cage-mates jumped significantly less than morphine only animals at the 24-hour time point ($p<0.05$).

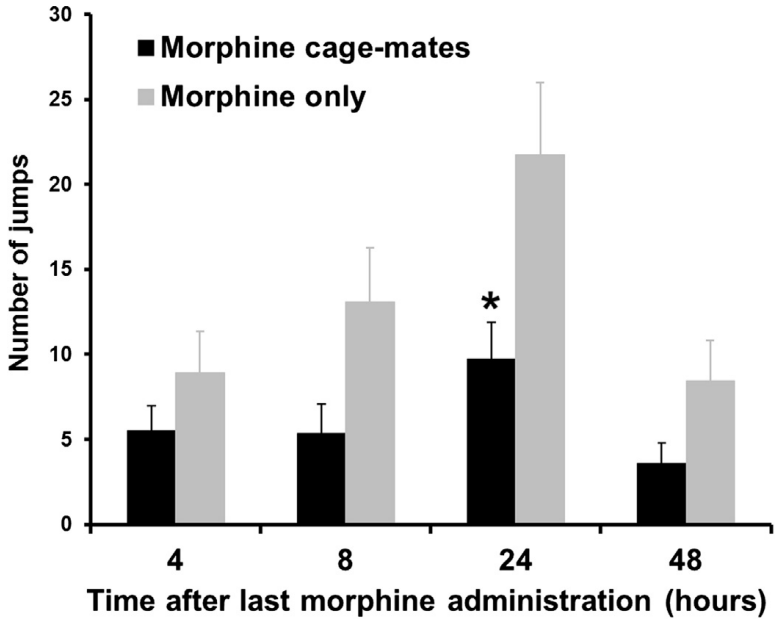

Figure 4. The number of jumps in the morphine cage-mates (black bars) and morphine only (gray bars) animals 4, 8, 24, and 48 h after the last administration of morphine in their home cages. *Indicates significant difference ($p < 0.05$) from morphine only animals. Results are presented as mean ± SEM.

4.4 Discussion

Social housing conditions affected somatic withdrawal symptoms. Morphine only animals exhibited significantly more jumping than the morphine cage-mates. Repetitive jumping during opioid withdrawal is correlated with opioid dependence, as the literature (Schulz & Herz, 1977) and my previous studies using identical testing conditions (Hodgson et al., 2008) demonstrate that drug-naïve animals do not jump spontaneously when placed in a "confined" space. In fact, there are a variety of somatic withdrawal symptoms that may be displayed by a mouse, including wet dog shakes, paw tremors, and teeth chattering, but jumping is the most pronounced of these symptoms

(Stevens & Klemm, 1979). Different patterns of social interaction, such as increased aggression, as measured by increases in threat and attack behaviors towards conspecifics, may also occur during periods of opioid withdrawal (Kantak & Miczek, 1986, 1988). Given that this study examined the withdrawal syndrome in a spontaneous (non-induced) model, following once daily administration of 20 mg/kg morphine, I did not observe a significant amount of wet dog shakes, paw tremors, and teeth chattering. Future studies, using higher doses of morphine, or a naloxone-precipitated model, should examine the effects of social housing on the display of other withdrawal syndromes.

The social housing conditions tested in this study might modulate morphine dependence and reward because of differences in the quality of social interaction between the morphine-treated animals and between the drug-naïve animals and their morphine cage-mates. Endogenous opioids play an important role in social play and social reward (Panksepp et al., 1980; Trezza, Baarendse, & Vanderschuren, 2010). Morphine decreases social investigation in mice (Kennedy et al., 2011; Landauer & Balster, 1982), and opioid withdrawal increases social aggression (Kantak & Miczek, 1986, 1988). Thus, it may be that morphine-treated animals are less engaged with their cage-mates and are more aggressive with one another during withdrawal. This might resemble a form of social inaccessibility existing in a non-supportive environment. Animals that are socially isolated are more prone to develop drug dependence and to display signs of drug reward and drug seeking (Bowling & Bardo, 1994; El Rawas et al., 2009; Starosciak et al., 2012; Zakharova et al., 2009). In contrast, the drug-naïve animals might provide constant tactile feedback (Vrontou et al., 2013), or some other form of

beneficial interaction, to their morphine cage-mates. This would result in the morphine cage-mates living in a housing condition that resembles a more supportive environment.

Supportive social interactions were demonstrated to play a role in cessation of opioid use in human adolescents (Hyucksun Shin, 2012; Vaughn et al., 2012). Moreover, animals that are housed in enriched environments demonstrate attenuated reward to drugs of abuse including heroin, ecstasy, and methamphetamine (El Rawas et al., 2009; Starosciak et al., 2012; Zakharova et al., 2009). It is possible that the housing conditions in which the morphine cage-mates live in represent an environment that is more socially enriched as compared to the housing conditions of the morphine only mice.

The previous experiments focus on morphine reward and dependence. One factor that contributes to the possibility of developing addiction is the chronic use of morphine to treat pain. Therefore, the following experiment examines whether or not the paradoxical pain symptoms that are concomitant with morphine use, and morphine analgesia, might be affected by social environment.

CHAPTER V

EXPERIMENT 3: EFFECT OF SOCIAL ENVIRONMENT ON THE ANTINOCICEPTIVE PROPERTIES OF MORPHINE *

5.1 Introduction

Opioids are commonly prescribed for treating moderate-to-severe pain, and their popularity is related to their potent analgesic properties (Furlan et al., 2006; Kalso et al., 2004; Volkow et al., 2011).The ability of opioids to produce pain relief (analgesia or antinociception) has been well-documented. However, chronic opioid use, both recreationally and under physician supervision, is complicated by the development of antinociceptive tolerance and opioid-induced hyperalgesia (Angst & Clark, 2006; L. Chen, Sein, et al., 2014; Jamison & Mao, 2015; Mao, 2002).

Antinociceptive tolerance refers to desensitization of antinociceptive mechanisms, which usually requires dose escalation to achieve the original response. Opioid-induced hyperalgesia refers to abnormal pain sensitivity induced by opioids due to sensitization of nociceptive mechanisms. This abnormal pain sensitivity refers both to increased sensitivity to noxious stimuli (termed hyperalgesia), and to an increased painful response to previously non-noxious stimuli (termed allodynia). Heroin addicts describe sensations of hyperalgesia after chronic use (Carcoba et al., 2011). Moreover,

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patients on methadone maintenance therapy display hyperalgesic responses to cold-pressor pain (Compton, Charuvastra, & Ling, 2001). These adverse effects may also undermine the expected outcomes of opioid treatment. Moreover, clinical data have shown that hyperalgesia and allodynia may lead to other adverse outcomes, including negative mood states, such as depression, anger, and confusion (White, 2004). Therefore, developing a treatment that may combat these effects is of importance.

Rodent models have been used to examine opioid analgesia, and social enrichment has been shown to affect it. For example, rats housed in social isolation appear to be less sensitive to the antinociceptive effects of morphine in the tail shock, tail compression, and tail withdrawal tests (Kostowski et al., 1977; Panksepp, 1980; M. A. Smith et al., 2005). Interestingly, isolated mice exposed to opioids with lower efficacy for the μ opioid receptor, including buprenorphine, butorphal, and nalbuphine, showed diminished antinociception of these drugs (M. A. Smith et al., 2005). Also, when combined with morphine (an opiate with high μ opioid receptor efficacy), these drugs antagonized antinociceptive effects in isolated animals, but potentiated them in socially housed animals.

The experience of pain appears to be modulated by social environment (for review see (Morales-Rivera et al., 2014)). Social play deprivation and peer-rejection during adolescence decreased pain reactivity in rodents (P. Schneider, Hannusch, et al., 2014). Moreover, opioid antinociception is affected by social stress (Huhman et al., 1991; Miczek, Thompson, & Shuster, 1982; Rodgers & Hendrie, 1983). Additionally, the observation of a cage-mate in pain or the observation of a cage-mate who does not

experience pain increases or reduces, respectively, the responses to pain in their pain-inflicted cage-mates (Langford et al., 2006). Specifically, mice that were tested in groups of two, that were both treated with acetic acid, displayed greater pain sensitivity than mice that were tested in isolation, or when only one of the mice received acetic acid. Lastly, it was shown that being housed with alcohol-withdrawn animals increases pain sensitivity in alcohol-naïve ones (M. L. Smith et al., 2016). This suggests that pain mechanisms can be modified by social environment.

The previous studies focus on susceptibility to develop opioid dependence and reward in adolescents and whether or not it is dependent on the nature of social housing conditions. Given that social environment alters both opioid-induced behaviors and nociceptive mechanisms, in this study I examined the effect of social housing conditions on the development of both antinociceptive tolerance and opioid-induced hyperalgesia in adolescent mice. I hypothesized that morphine only animals would show elevated signs of antinociceptive tolerance and hyperalgesia, as compared to morphine cage-mates.

5.2 Methods

5.2.1 Animals

Adolescent, male mice were used in this experiment. There were 16-20 animals per group.

5.2.2 Pretreatment regimen

Mice were injected once daily (9:00AM) in their home cage for 6 consecutive days (experiment 1, tail withdrawal) or 14 consecutive days (experiment 2, allodynia and hyperalgesia) with 20 mg/kg of morphine or 0.9% saline (10 ml/kg). This dose represents the salt concentration.

5.2.3 Experiment 1 - Tail withdrawal

Morphine's ability to relieve pain was measured with the tail withdrawal assay. Animals were gently restrained and placed in a plastic conical tube, and had the distal half of their tail set in a 48ºC water bath. The length of time to withdraw the tail was recorded in seconds. Tail withdrawal was defined as a strong flexion of the entire tail from the water. Lastly, a maximum cutoff time of 60 seconds was set to prevent tail tissue damage.

The first portion of the experiment included a habituation session, followed by a baseline session 15 minutes later. Fifteen minutes after the baseline session, all mice were injected with morphine (10 mg/kg, 10 mL/kg). The latency to withdraw the tail was recorded 30 minutes following injection, and every subsequent 30 minutes thereafter for 5 hours. Between time points, mice were returned to their home cages. Animals were tested 24 hours following the final treatment, and then again at 7, 14, 21, and 28 days. For each time point, percent maximum possible effect (%MPE) was calculated using this formula:

$$\left(\frac{[withdrawal\ latency]\text{-}[baseline\ latency]}{[60s]\text{-}[baseline\ latency]}\right) \times 100$$

5.2.4 Experiment 2- Allodynia and Hyperalgesia

5.2.4.1 Mechanical allodynia

Mechanical allodynia was assessed using nylon von Frey filaments. Mice were placed in a Plexiglas cylinder on top of a mesh platform. The filaments were applied to the plantar surface of the hind paw – of both feet – until a withdrawal response was provoked. If no response is given, the next stiffer fiber was applied to the same paw until it evoked a response. However, if there was a response, a less stiffer fiber was applied until there was no longer a response. The final filament to produce a response was recorded, and scores were averaged across both feet.

5.2.4.2 Cold allodynia

Cold allodynia was assessed by applying acetone to the plantar surface of the hind paw of both feet. Mice were placed atop a mesh platform and once the animal withdrew its paw, the severity of response was recorded, and scores were averaged across both feet. I used a 4-point scale, which is depicted below:

Table 1. Scale for scoring of cold allodynia

Score	Type of Response
1	Slow, smooth paw withdrawal response
2	Rapid paw withdrawal response
3	Rapid paw withdrawal response; with flick
4	Rapid paw withdrawal response; with vocalization or licking

5.2.4.3 Hyperalgesia

The hot-plate test was utilized to assess analgesia. The temperature was maintained at 55° ± 1°C, and mice were put inside a Plexiglas cylinder on top of the plate. Licking of a hindpaw, or jumping out of the cylinder, was recorded as a response – latency to provoke one of these responses was recorded in seconds. Lastly, similar to the tail withdrawal assay, a latency time of 60 seconds was set to prevent tissue damage each trial.

5.2.4.4 Experimental Design

Animals were tested the day before morphine treatment began in order to establish baseline scores (PND 25). Testing order was as follows: mechanical allodynia → cold allodynia → hot-plate test. The next day, morphine treatment began (experiment day 1). Mice were tested again on experimental days 7, 14, 21, and 28. On each test day, they were given a pretest, injected with morphine (10 mg/kg, s.c.), and then given a posttest one hour later. Percent maximum possible effect was calculated as described in the tail withdrawal assay. Lastly, the development of hyperalgesia was calculated by computing the difference scores for the daily pretests and the baseline scores (on experimental day 0). Drug potency was calculated by computing the differences scores between the posttest and pretest scores.

5.2.5 Statistical Analysis

5.2.5.1 Tail withdrawal

The differences in baseline tail withdrawal latencies among the experimental groups on each testing day were analyzed using two-way ANOVA with between-group factors of treatment (morphine, saline) and housing (only, cage-mates). Then, for each mouse and for each time point, percent maximum possible effect (%MPE) was calculated using the above formula. The differences in %MPE between the different experimental groups on each day of tail withdrawal testing was analyzed using a split-plot ANOVA with between-group factors of treatment and housing and a within-group factor of time (every 30 min for 5 h). The differences in latencies (using log 10(x + 1) transformation) between the different experimental groups were analyzed similarly. Post hoc contrasts between each treatment group were conducted using Bonferroni procedure.

5.2.5.2 Allodynia and hyperalgesia

The differences between the weekly pre-treatment scores and the original baseline score were analyzed using a split-plot ANOVA with between-group factors of treatment (morphine, saline) and housing (only, cage-mates) and a within-group factor of time (week). Additionally, for the hot plate test, for each mouse and for each time point, percent maximum possible effect (%MPE) was calculated as explained above. Similar to the raw latencies (scores), the differences in %MPE between the different experimental groups were each analyzed using a split-plot ANOVA with between-group factors of

treatment and housing and a within-group factor of time. Post hoc contrasts between each treatment group were conducted using Bonferroni procedure.

5.3 Results

5.3.1 Tail withdrawal

No significant differences between groups in tail withdrawal latencies at baseline were seen. Using a two-way ANOVA, I found no main effect of treatment ($F(1,60) = 0.52$, *NS*), no main effect of housing ($F(1,60) = 0.80$, *NS*), and no treatment x housing interaction ($F(1,60) = 1.38$, *NS*). Additionally, both morphine only and morphine cage-mate animals developed similar degrees of antinociceptive tolerance (Figure 5). Repeated measures ANOVA revealed a main effect of time ($F(9, 540) = 126.46$, $p< 0.0001$), a main effect of treatment ($F(1, 60) = 33.58$, $p< 0.0001$), a significant interaction between time and housing ($F(9,540) = 3.23$, $p< 0.001$), and a significant interaction between time and treatment ($F(9, 540) = 14.35$, $p< 0.0001$). Bonferroni post hoc comparisons revealed a significantly reduced response to morphine in the morphine-treated animals as compared to the saline-injected animals (Figure 5A). However, no significant differences were observed between morphine only animals and morphine cage-mates.

A week after discontinuation of morphine treatment, no significant differences in baseline tail withdrawal latencies were observed among the different experimental groups. Two-way ANOVA revealed no main effect of treatment ($F(1,60) = 0.07$, $p> 0.05$, *NS*), no main effect of housing ($F(1, 60) = 1.21$, $p> 0.05$, *NS*) and no interaction

between treatment and housing ($F(1, 60) = 0.00$, $p > 0.05$, NS). However, a significant difference in the magnitude of antinociceptive tolerance was observed between morphine only mice and morphine cage-mates. Specifically, antinociceptive tolerance was still observed in the morphine only animals, but not in the morphine cage-mates, as compared to saline-injected animals (Figure 5B). Repeated measures ANOVA revealed a main effect of time ($F(9, 540) = 96.9$, $p < 0.0001$), and a main effect of treatment ($F(1, 60) = 4.53$, $p < 0.05$), a significant interaction between time and housing ($F(9, 540) = 2.78$, $p < 0.01$), a significant interaction between time and treatment ($F(9, 540) = 3.71$, $p < 0.0001$), and a significant interaction between time, housing and treatment ($F(1, 540) = 3.20$, $p < 0.001$). Bonferroni post hoc comparison revealed a significantly reduced response to morphine in the morphine only animals as compared to morphine cage-mates and to the saline-injected animals (Figure 5B). No significant differences were observed between morphine cage-mates and the saline-injected animals.

Two and three weeks after discontinuation of morphine treatment, no significant differences in baseline tail withdrawal latencies were observed between the different experimental groups. Additionally, no significant differences in the response to morphine between the different experimental groups were observed at that time (Figures 5C & D). Similar results were obtained when the differences in latencies (i.e. the differences between post- and pre-morphine latencies) were analyzed.

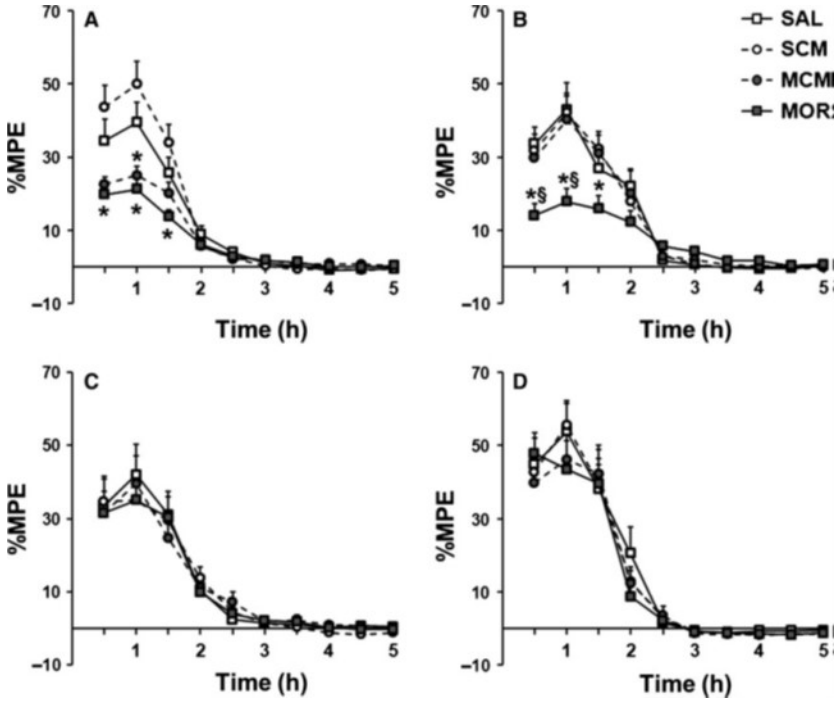

Figure 5. Results from the tail withdrawal test. Mice (n = 16/group) were treated for 6 days with morphine or saline. The differences in %maximum possible effect to withdraw their tails between the different experimental groups were recorded following 6 days of morphine or saline (A) as well as one (B) two (C) and three (D) weeks after discontinuation of morphine treatment. Mice were tested for withdrawal latencies at baseline and then every 30 min for 5 h post-morphine or saline injections. *indicates significant difference ($p < 0.05$) from saline only animals; §indicates significant difference ($p < 0.05$) from morphine cage-mate animals. SAL, saline only; SCM, saline cage-mates; MCM, morphine cage-mates; MOR, morphine only. Results are presented as mean ± SEM.

5.3.2 Mechanical Allodynia

Differences between weekly pre-treatment scores (in grams) and the original baseline score are presented in Figure 6A. Three-way ANOVA revealed a main effect of week ($F(1, 103) = 17.99$, $p<0.0001$) and significant interactions between week and treatment ($F(1, 103) = 10.12$, $p<0.01$). Tukey's HSD post-hoc comparison revealed significantly reduced pain reactivity in morphine only mice ($p<0.05$) and a trend for

reduced pain reactivity in morphine cage mates (p=0.113) at 3 weeks after discontinuation of morphine treatment as compared to the saline-injected mice.

The responses to saline (i.e., differences between post- and pre-saline scores in grams) are presented in Figure 6B. Three-way ANOVA revealed only a main effect of week (F(1, 32) =12.63, p<0.01). However, no significant differences were found among the different experimental groups.

The development of antinociceptive tolerance in the different experimental groups (i.e., the differences between post- and pre-morphine scores in grams) is presented in Figure 6C. Three-way ANOVA revealed a main effect of week (F(1, 67) =82.13, p<0.0001), a main effect of treatment (F(1, 67) =72.59, p<0.0001), a significant interaction between treatment and week (F(1, 67) =29.12, p<0.0001), and a significant interaction between treatment and housing (F(1, 67) =12.98, p<0.001).

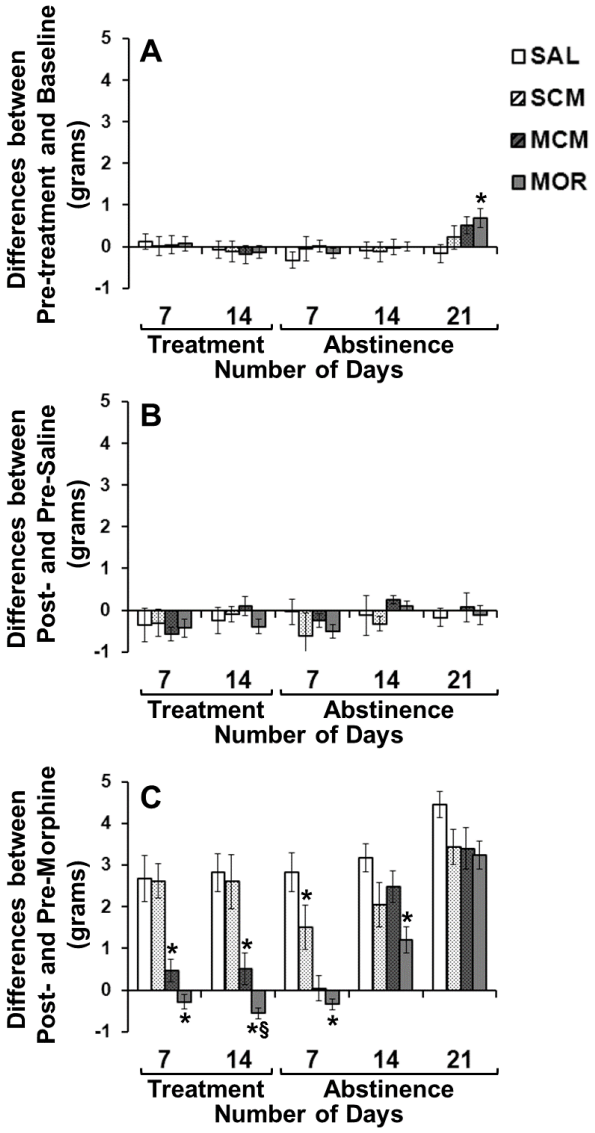

Figure 6. Results from the von Frey Filaments test. Mice (n=16-20/group) were treated for 14 days with morphine or saline. They were recorded weekly during morphine treatment and in the following 3 weeks of abstinence for: (A) the change in baseline scores (differences between weekly pre-treatment scores and the original baseline score in grams); (B) the response to saline (differences between post- and pre-saline scores in grams); and (C) the response to morphine (differences between post- and pre-morphine scores in grams). * indicates significant difference ($p < 0.05$) from saline only animals; § indicates significant difference ($p < 0.05$) from morphine cage-mate animals. SAL – Saline Only, SCM – Saline Cage-mates, MCM – Morphine Cage-mates, MOR – Morphine Only. Results are presented as mean ± SEM.

Tukey's HSD post-hoc comparison revealed a significant development of antinociceptive tolerance in both the morphine cage-mates and morphine only mice during the 14 consecutive days of morphine treatment ($p<0.001$). Notably, the magnitude of the antinociceptive tolerance was significantly more robust in the morphine only animals as compared to the morphine cage-mates ($p<0.05$). Additionally, in line with the results from the tail withdrawal test, antinociceptive tolerance was more persistent in the morphine only animals. In the morphine cage-mates, antinociceptive tolerance was observed one week ($p<0.0001$), but not 2 weeks ($p>0.05$, *NS*), after discontinuation of morphine treatment. In contrast, antinociceptive tolerance was observed both one week ($p<0.0001$) and 2 weeks ($p<0.01$) after discontinuation of morphine treatment in the morphine only group.

5.3.3 Cold Allodynia

Differences between weekly pre-treatment scores and the original baseline score are presented in Figure 7A. A Kruskal–Wallis test revealed significant differences among the experimental groups after 14 days of morphine treatment ($\chi^2=9.37$, df=3, $p<0.05$), but not at other time points (7 days of morphine treatment: $\chi^2=2.93$, df=3, $p>0.05$, *NS*; 7 days abstinence: $\chi^2=3.47$, df=3, $p>0.05$, *NS*; 14 days abstinence: $\chi^2=7.56$, df=3, $p>0.05$, *NS*; 21 days abstinence: $\chi^2=2.53$, df=3, $p>0.05$, *NS*). Specifically, saline only animals exhibited lower pain reactivity as compared with morphine cage-mates ($p<0.05$) and morphine only ($p<0.05$) animals. A trend was also observed for lower pain reactivity in the saline only animals as compared to the saline cage mates ($p=0.053$).

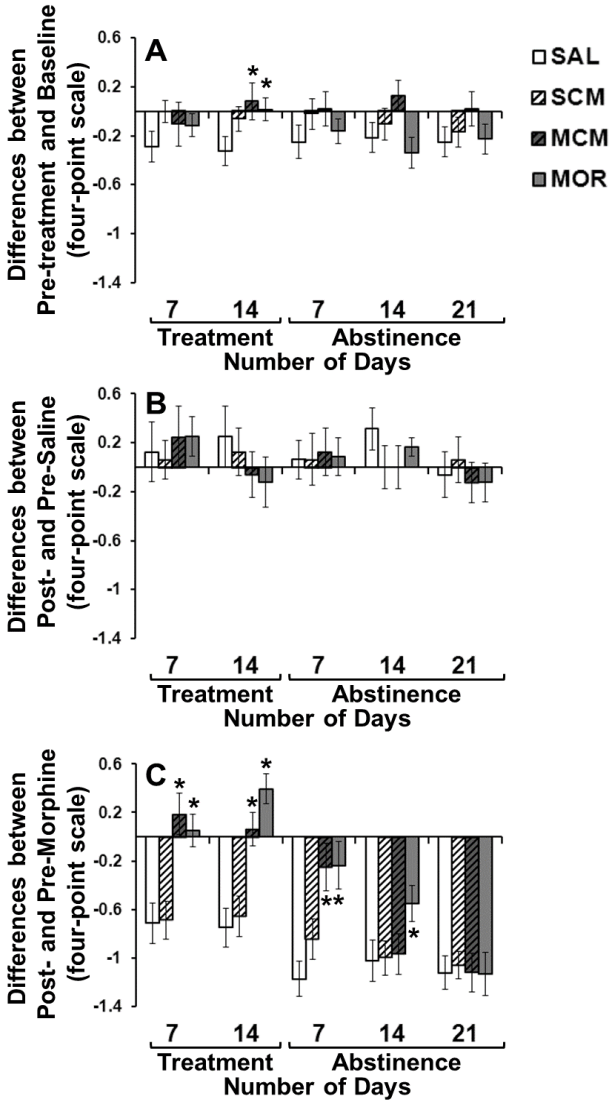

Figure 7. Results from the acetone test. Mice (n=16-20/group) were treated for 14 days with morphine or saline. They were recorded weekly during morphine treatment and in the following 3 weeks of abstinence for: (A) the change in baseline scores (differences between weekly pre-treatment scores and the original baseline score); (B) the response to saline (differences between post- and pre-saline scores); and (C) the response to morphine (differences between post- and pre-morphine scores). * indicates significant difference ($p < 0.05$) from saline only animals. SAL – Saline Only, SCM – Saline Cage-mates, MCM – Morphine Cage-mates, MOR – Morphine Only. Results are presented as mean ± SEM.

The responses to saline (i.e., differences between post- and pre-saline scores) are presented in Figure 7B. A Kruskal–Wallis test revealed no significant differences among the experimental groups at any time point tested (7 days of morphine treatment: χ^2=1.07, df=3, p>0.05, *NS*; 14 days of morphine treatment: χ^2=2.17, df=3, p>0.05, *NS*; 7 days abstinence: χ^2=0.08, df=3, p>0.05, *NS*; 14 days abstinence: χ^2=3.26, df=3, p>0.05, *NS*; 21 days abstinence: χ^2=0.34, df=3, p>0.05, *NS*).

The development of antinociceptive tolerance in the different experimental groups (i.e., the differences between post- and pre-morphine scores) is presented in Figure7C. A Kruskal–Wallis test revealed significant differences between the experimental groups during the 14 consecutive days of morphine treatment (7 days of morphine treatment: χ^2=21.87, df=3, p<0.0001; 14 days of morphine treatment: χ^2=29.50, df=3, p<0.0001) as well as 7 days following discontinuation of treatment (7 days abstinence: χ^2=17.88, df=3, p<0.0001). However, no differences were observed after 14 and 21 days of abstinence (14 days abstinence: χ^2=5.61, df=3, *NS*; 21 days abstinence: χ^2=0.40, df=3, *NS*). No significant differences were observed between the saline only and saline cage-mate animals at any time point (*NS*). A significant development of antinociceptive tolerance was observed in both the morphine cage-mates and morphine only mice during the 14 consecutive days of morphine treatment as well as 7 days following discontinuation of morphine treatment (p<0.01). Additionally, a significant amount of antinociceptive tolerance was still observed in the morphine only animals after 14 days of abstinence (p<0.05), but not in the morphine cage-mates (*NS*).

5.3.4 Hot plate test

The development of hyperalgesia in the different experimental groups (i.e. differences between weekly pre-treatment latencies and the original baseline latency) is presented in Figure 8A. Three-way ANOVA revealed a main effect of week ($F(1, 103)=13.20$, $p<0.0001$) and significant interactions between week and housing conditions ($F(1, 103) =6.54$, $p<0.05$), week and treatment ($F(1, 103) =10.49$, $p<0.01$) as well as week, housing, and treatment ($F(1, 103) =5.53$, $p<0.05$). As expected, Tukey's HSD post-hoc comparison revealed that hyperalgesia was not developed in the saline-injected mice. Significant levels of hyperalgesia developed solely in the morphine only group following 14 consecutive days of morphine treatment ($p<0.05$). A trend toward a hyperalgesic response was already observed following 7 consecutive days of morphine treatment, but did not reach statistical significance ($p=0.15$). The opioid-induced hyperalgesia observed in the morphine only animals was abolished one week after discontinuing morphine treatment. Notably, a significant difference was observed between the response of the morphine cage-mates and the morphine only mice ($p<0.05$). The morphine cage-mate animals did not develop significant levels of hyperalgesia at any time during the 14 days of morphine treatment or after discontinuation of morphine treatment. There were no significant differences between the pre-treatment responses of the morphine cage-mates and the saline-injected mice.

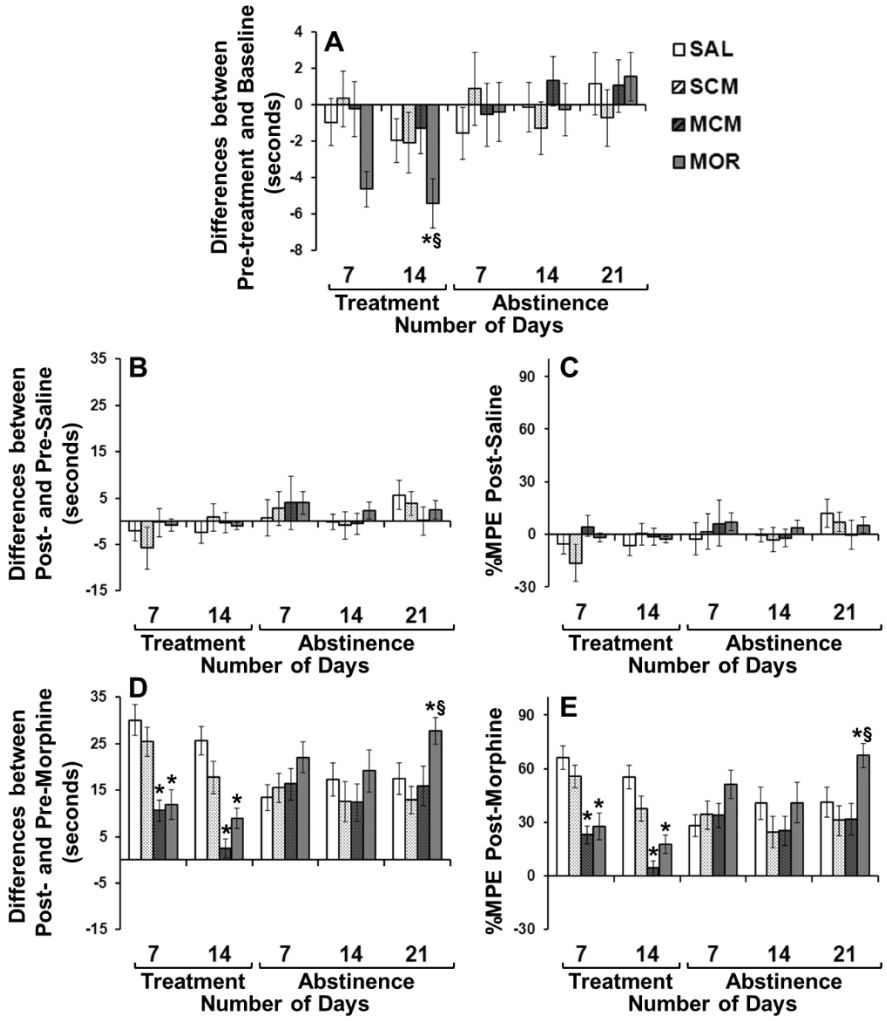

Figure 8. Results from the hot-plate test. Mice (n=16-20/group) were treated for 14 days with morphine or saline. They were recorded weekly during morphine treatment and in the following 3 weeks of abstinence for: (A) the change in baseline latencies (differences between weekly pre-treatment latencies and the original baseline latency in seconds); the response to saline (B & C) – (B) differences between post- and pre-saline latencies in seconds, (C) %MPE post-saline; and the response to morphine (D & E) – (D) differences between post- and pre-morphine latencies in seconds, (E) %MPE post-morphine. * indicates significant difference ($p < 0.05$) from saline only animals; § indicates significant difference ($p < 0.05$) from morphine cage-mate animals. SAL – Saline Only, SCM – Saline Cage-mates, MCM – Morphine Cage-mates, MOR – Morphine Only. Results are presented as mean ± SEM.

The responses to saline (the differences between post- and pre-saline latencies and %MPE post-saline) are presented in Figures 8B and 8C. Three-way ANOVA revealed a main effect of week (%MPE: $F(1, 32) = 6.93$, $p<0.05$; Latencies: $F(1, 32) = 7.42$, $p<0.01$) and a significant interaction between week and treatment (%MPE: $F(1, 32) = 4.20$, $p<0.05$). However, no significant differences were observed among the experimental groups in their response to saline.

The development of antinociceptive tolerance in the different experimental groups (i.e., the differences between post- and pre-morphine latencies and %MPE post-morphine) is presented in Figures 8D and 8E. Three-way ANOVA revealed a main effect of week (%MPE: $F(1, 67) = 6.52$, $p<0.05$), a main effect of housing (%MPE: $F(1, 67) = 11.59$, $p<0.001$), a main effect of treatment (%MPE: $F(1, 67) = 7.32$, $p<0.01$), and a significant interaction between week and treatment (%MPE: $F(1, 67) = 29.31$, $p<0.0001$; Latencies: $F(1, 67) = 18.29$, $p<0.0001$). As expected, Tukey's HSD post-hoc comparison revealed that antinociceptive tolerance was developed in the morphine cage-mates and morphine only mice ($p<0.05$ as compared to saline-injected mice). No significant differences were observed between the morphine cage-mates and morphine only mice at any time during the 14 days of morphine treatment or after discontinuation of morphine treatment. Following 14 days of morphine treatment, the antinociceptive tolerance in the morphine cage-mates visually appears to be stronger than in the morphine only mice, however not even a statistical trend was observed. Notably, 3 weeks after discontinuation of morphine treatment, morphine produced stronger

antinociceptive response in the morphine only mice as compared to the saline-injected ($p<0.05$) and morphine cage-mate mice ($p<0.05$).

5.4 Discussion

This study demonstrated that social housing conditions modulate the adaptive nociceptive responses to repeated opioid treatments in adolescent male mice. Specifically, both mice that received morphine while being housed with drug-naïve mice (i.e. morphine cage-mates) and mice that received morphine while being housed only with other morphine-injected animals (i.e. morphine only) developed antinociceptive tolerance. However, the magnitude of this antinociceptive tolerance was more robust and persistent in the morphine only group. Additionally, morphine only animals, but not morphine cage-mates, developed opioid-induced hyperalgesia. Thus, this study suggests that certain social environments might improve the effectiveness of long-term opioid treatment.

Multiple pain tests were used in this study, which differ in the modality of pain or type of receptors and sensory processes they examined (Berge, 2011; Le Bars, Gozariu, & Cadden, 2001). Notably, social housing conditions had different effects in the various tests. In this study, enhanced/prolonged antinociceptive tolerance was observed in the morphine only animals as compared to the morphine cage-mates in the tail withdrawal, von Frey, and acetone tests. Thus, these findings suggest that certain social conditions, such as housing with drug-naïve animals, can mitigate the development of both thermal and mechanical antinociceptive tolerance. However, no

differences were observed in the development of antinociceptive tolerance between morphine only and morphine cage mates in the hot-plate test. The contradictory findings might be due to the tail withdrawal response being mediated primarily by a simple spinal reflex, while the responses in the hot plate test involved supraspinal sensory processing (Dirksen et al., 1994; Piercey et al., 1981). Perhaps the development of morphine antinociceptive tolerance on spinal levels is more affected by social conditions than on supraspinal sensory processing. Alternatively, perhaps the existence of parallel overlapping supraspinal sensory processing involved in hot plate response as compared to simple spinal reflex mediating the tail withdrawal response, makes it more resilient for manipulations.

Interestingly, the morphine only animals exhibit an enhanced antinociceptive response in the hot plate test after 21 days of abstinence as compared to the other experimental groups. The morphine only animals were also the only experimental group that experienced thermal hyperalgesia in the hot plate test. Opioid-induced hyperalgesia was not observed in other experimental groups or in the morphine only animals in the other tests. Thus, I hypothesized that the enhanced antinociceptive response might represent a delayed opponent process response to alter baseline pain reactivity in this experimental group (Leknes & Tracey, 2008; Seymour et al., 2005). Further studies are required to examine this phenomenon.

The social housing conditions tested in this study might modulate opioid-induced tolerance and hyperalgesia because of differences in the quality of social interaction between the morphine-treated animals and between the drug-naïve animals and their

morphine cage-mates. Endogenous opioids play an important role in social play and social reward (Leknes & Tracey, 2008; Polakiewicz et al., 1998). Morphine decreases social investigation in mice (Kennedy et al., 2011; Landauer & Balster, 1982), and opioid withdrawal increases social aggression (Kantak & Miczek, 1986, 1988). Thus, it may be that morphine-treated animals are less engaged with their peers and are more aggressive with one another during withdrawal. This might resemble a form of social isolation existing in a non-supportive environment.

Animals that are socially isolated exhibit an enhanced pain sensitivity and enhanced opioid antinociceptive tolerance (H. C. Becker & Baros, 2006; Coudereau et al., 1997; Defeudis, Defeudis, & Somoza, 1976; Puglisi-Allegra & Oliverio, 1983). In contrast, the drug-naïve animals that the morphine cage-mates interact with might provide constant tactile feedback (Vrontou et al., 2013), or some other form of beneficial interaction, to their morphine cage-mates. This would result in the morphine cage-mates living in a housing condition that resembles a more supportive environment. Thus, it is possible that the housing conditions in which the morphine cage-mates live represent an environment that is more socially enriched as compared to the housing condition of the morphine only mice.

Environmental enrichment has been demonstrated to mitigate adaptive nociceptive responses (Berrocal et al., 2007; Rossi & Neubert, 2008; Tall, 2009; Vachon et al., 2013). Both the social and physical aspects of environmental enrichment were demonstrated to mitigate the magnitude and persistence of adaptive nociceptive processes (Gabriel et al., 2010). Moreover, animals that are housed in enriched

environments exhibit higher sensitivity to the antinociceptive properties of opioids (M. A. Smith, Bryant, & McClean, 2003; M. A. Smith et al., 2005). Future studies should further explore the nature of social interactions in the different housing conditions.

The results of the aforementioned studies suggest that exposure to drug-naïve animals mitigates the development of morphine dependence and reward in adolescent mice (Bates et al., 2014; Cole et al., 2013). Specifically, those studies demonstrate that morphine only mice developed stronger dependency on morphine than did the morphine cage-mates. Additionally, morphine is more rewarding to the morphine only mice than the morphine cage-mates, as morphine cage-mates are slower in acquiring morphine CPP and extinguish morphine CPP more readily than do morphine only mice. Although the processes underlying the development of tolerance and hyperalgesia are likely different than the processes underlying the development of abuse, in my studies social housing conditions modulate both the addictive and antinociceptive properties of opioids. It is possible that this could be explained by the effect of social environment on a common mediator which affects both processes underlying the different properties of opioids. This might suggest that patients who exhibit more pronounced development of opioid-induced tolerance and/or hyperalgesia, at least in certain pain modalities, might be individuals who are subjected to certain social conditions which also put them at higher risk for developing abuse. However, additional pre-clinical and clinical studies are necessary to confirm this prediction.

Opioids continue to be the most effective and commonly used drugs for managing moderate-to-severe pain (Volkow et al., 2011). The results suggest that certain

social conditions can minimize the magnitude of undesirable adaptive responses that develop following repeated opioid treatment. Thus, the present data represent an avenue that needs to be further elaborated, and could hold benefits for pain management. In the following experiment, I examine if social grooming – an affiliative behavior in the rodent – is important for conferring the protective effect observed in experiments 1-3.

CHAPTER VI

EXPERIMENT 4: ROLE OF SOCIAL GROOMING AND MRGPRB4+ NEURONS IN THE SOCIAL HOUSING EFFECT

6.1 Introduction

Adolescence is a stage in development with changes that are quite diverse. These changes may present themselves in many ways, including increases in risk-taking behaviors, and the possible initiation of drug use. For example, both the prefrontal cortex and the striatum, as well as the connection between them, have been implicated in adolescents' increase in risky behaviors, including drug experimentation (Cherner et al., 2010; DeWitt et al., 2015; Raznahan et al., 2014; Urosevic et al., 2014; Urosevic et al., 2015). Indeed, adolescence is when most people initiate drug use, as statistics show that many addicts report initial drug use between the ages of 12 and 14 (National Institutes of Health, 2015). Moreover, abuse of opioids, including prescription pain medications, has increased dramatically in this population (Johnston et al., 2015), with many more beginning to use every day. The percent of adolescents seeking treatment for prescription opioids has increased from 15.5% to 34.5% in the past decade, and from 16.6% to 25.8% for heroin dependence (Johnston et al., 2015).

A common motif in the adolescent addict's story is the feeling of social isolation and a lack of acceptance. Unfortunately, this feeling is not unfounded, as many addicts are shunned or ostracized because of their addiction, and report feeling low levels of social inclusion (Choi, DiNitto, & Marti, 2016). This lack of acceptance is particularly

troubling for adolescents, as social affiliation is most important during this stage of development. Nevertheless, the fortunate few that are able to find a social circle outside of other addicts report that social support can aid in the progression to abstinence (Johnson et al., 2016; Pagano et al., 2015). Moreover, helping others has been shown to be another predictor of a greater likelihood of abstaining from drug use (Pagano et al., 2004).

Similar to humans, social affiliation is also rewarding in rodents. Affiliative interactions in rodents include social grooming and social play, which are most prevalent in adolescence (Spear, 2000). Because of the importance and prevalence of social interaction in adolescence, it should stand that it would activate regions that underlie reward (Vanderschuren, Achterberg, & Trezza, 2016). Indeed, social grooming and social play activate portions of the classical reward circuitry in limbic and cortical regions (van Kerkhof et al., 2014). Social grooming and play also lead to activation of the endogenous μ-opioid system, and are thought to facilitate relationships, as they are typically observed between related or familiar conspecifics (Nummenmaa et al., 2016; Vanderschuren, Niesink, et al., 1995). Additionally, these behaviors are increased by administration of an opioid receptor agonist, morphine, and attenuated by an opioid receptor antagonist, naloxone, which further provides evidence for a role of the endogenous opioid system (Mitchem et al., 1999; Niesink & Van Ree, 1989).

Although the mechanisms of the pleasurable effects of grooming have been established, until recently, the precise mechanism that mediated grooming was unknown. In an elegant set of experiments, Vrontou et al. (2013) were able to show that grooming,

but not a noxious stimulus like pinching, is partially mediated by peripheral dorsal root ganglia sensory neurons expressing Mas-related G protein–coupled receptor B4 (MRGPRB4). Moreover, pharmacogenetic activation of MRGPRB4+ neurons, through the use of Designer Receptor Exclusively Activated by Designer Drug (DREADD) technology, induces conditioned place preference in adolescent mice (Vrontou et al., 2013).

The interaction between social affiliation (in the home cage) and the propensity to develop drug-related behaviors in adolescent rodents has been investigated. An interaction was observed between social reward and sub-threshold levels of cocaine and nicotine (i.e. levels of the drugs that by themselves could not induced conditioned place preference) (Thiel et al., 2008; Thiel et al., 2009). However, group housing in home cages decreases cocaine CPP in adolescent rats, when levels of drug sufficient to establish CPP by itself were examined (Zakharova et al., 2009). Similarly, a complex interaction was observed between social enrichment in their home cages, social reward, and the establishment of morphine reward in adolescent mice (Kennedy et al., 2012).

As demonstrated in earlier chapters, social housing conditions alter the rewarding properties of morphine during adolescence. Specifically, exposure to drug-naïve animals attenuates morphine dependence, as well as the acquisition of a conditioned place preference (CPP) to morphine (Bates et al., 2014; Cole et al., 2013). Moreover, exposure to drug-naïve animals also expedites the extinction of morphine CPP. Using DREADD technology, I examined whether activation of MRGPRB4+ neurons in morphine only mice (i.e., mice housed only with other morphine-treated mice) affects

morphine withdrawal symptoms, and the time needed to extinguish morphine CPP. Conversely, I examined if, in morphine cage-mates (i.e., mice treated with morphine in the presence of drug-naïve animals), inactivation of these neurons produces the opposite effect. Therefore, I may be able to decipher whether or not grooming, through its underlying physiological mechanisms, is a potential channel that mediates the social housing effect.

6.2 Methods

6.2.1 Animals

All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at Texas A&M University. Transgenic mice in which the entire open reading frame of the MRGPRB4 gene was replaced by an mtdTomato-2A-NLSCre-Frt-PGK-Neomycin-FRT cassette (i.e., transgenic mice carrying Cre under the control of the MRGPRB4 promotor) were purchased from Jackson lab (MRGPRB4-Cre mice; B6N.129S1-*Mrgprb4*$^{tm3(Cre)And}$/J; Bar Harbor, ME, USA). Mice were bred in house and housed with food and water *ad lib* in a temperature-controlled (21+/- 2°C, humidity 45%) vivarium with a 12-hour light/12-hour dark cycle (lights on at 7:30 AM and off at 7:30PM).

6.2.2 Injection of Adeno-associated viral vectors

Following the procedure established by Anderson and colleagues (Vrontou et al., 2013), on PND2, mice were intraperitoneally injected with Cre-dependent AAV-hSyn-hM4D (viral vector carrying Gi-coupled receptor), Cre-dependent AAV-hSyn-hM3D (viral vector carrying Gq-coupled receptor), or Cre-dependent AAV-hSyn (control viral vector). Specifically, mice were removed from their cage and briefly placed on an ice bath with their heads facing up, until they appeared anesthetized (4-5 minutes). Pups were then gently held, and the abdomen was cleaned with an alcohol pad. A syringe (insulin syringe, 0.3 cm^3, 8mm length, 31G needle) was used to inject the AAV8 virus intraperitoneally, which was obtained from UNC Viral Vector core. The pups were then covered with nestlet and gently set on a heating pad until they began to move. Following this, they were returned to their dam, and observed to ensure that they were healthy.

6.2.3 CNO and Morphine treatment regimen

Starting on PND 28, mice were injected twice daily (8:30AM and 6:00PM) in their home cage with 1 mg/kg of clozapine-N-oxide (CNO) or vehicle (intraperitoneally, Sigma-Aldrich, St. Louis, MO, USA). Thirty minutes after the morning injection (9:00AM), mice were injected for 6 consecutive days with 20 mg/kg of morphine sulfate (subcutaneously, Sigma-Aldrich, St. Louis, MO, USA) (Bates et al., 2014). This dose represents the salt concentration. The drug-naïve cage mates were injected with saline (10 ml/kg).

6.2.4 Spontaneous Withdrawal Symptoms

All morphine-injected animals were tested 4, 8, 24, and 48 hours following the final morphine injection (Bates et al., 2014). They were individually placed in Plexiglas cylinders (37 cm tall x 14.5 cm in diameter) and were videotaped for 30 minutes. The videotapes were scored for jumping behavior by observers who were blind to experimental group assignments.

6.2.5 Conditioned Place Preference

This involved a biased CPP design. During habituation, mice were placed in the neutral chamber of the CPP apparatus, and allowed to explore the entirety of it for 30 minutes. This provided the baseline preference, which was used to decide subsequent conditioning chambers. Conditioning began the day after habituation, and lasted for 4 or 8 days, for 60 minutes each day. Animals were injected with saline (10 ml/kg, s.c.), and confined to the initially preferred compartment in the morning (9:00AM), and morphine (5 mg/kg), and confined to their initially non-preferred compartment, in the afternoon (2:00 PM). After the first 4 days of conditioning, animals underwent a test session, which was identical to the habituation session. However, unlike Chapter III, animals underwent another 4 days of conditioning regardless of whether or not they acquired place preference in the first 4 days of conditioning.

Extinction sessions began the day after the last acquisition test. Animals that developed place preference were given once daily extinction sessions until they achieved extinction. These sessions were similar to the habituation and test sessions. The animals

were considered to have reached extinction when they displayed three consecutive days of no preference for the morphine-paired chamber.

6.2.6 Experimental Design

On PND2, mice were i.p. injected with one of 3 Cre-dependent Adeno-associated viral vectors (Cre-dependent AAV-hSyn-hM4D, Cre-dependent AAV-hSyn-hM3D, or Cre-dependent AAV-hSyn). On PND21, mice were weaned and housed 4 per cage where all animals in each cage carry the same viral vector. Starting at PND28, mice were injected with morphine or saline for 6 days. For the cages of mice carrying the Cre-dependent AAV-hSyn-hM4D, 2 mice in each cage received morphine (i.e., morphine cage-mates) and 2 mice received saline. Of these morphine cage-mate mice, some received CNO (hM4D-CNO) and some received vehicle (hM4D-Veh). I also included control cages with mice carrying the Cre-dependent AAV-hSyn where 2 mice received morphine (i.e., morphine cage-mates) and 2 mice received saline. The morphine cage-mate mice received CNO (CON-CNO). For the cages of mice carrying the Cre-dependent AAV-hSyn-hM3D, all 4 mice in each cage received morphine (i.e., morphine only). Of these morphine only mice, some received CNO (hM3D-CNO) and some received vehicle (hM3D-Veh). I also included control mice carrying the Cre-dependent AAV-hSyn where all 4 mice in the cage received morphine (i.e., morphine only) and CNO (CON-CNO). All morphine-treated mice were examined for spontaneous withdrawal symptoms 4, 8, 24, and 48 hours following the final morphine injection.

Similar to Chapter III, three days following the final dose of morphine pretreatment, CPP acquisition began. Following the second test session, extinction sessions began and was ran until animals reached the extinction criteria described above. Extinction sessions lasted for 30 minutes for the first 15 days, and 60 minutes for the second 15 days. This is because I have previously seen (Bates et al., 2014) that even morphine only animals will extinguish using extinction sessions that last for 60 minutes. If an animal did not reach the extinction criteria after 30 days, the extinction sessions were ended.

6.2.7 Statistical Analysis

6.2.7.1 Spontaneous Withdrawal Symptoms

The effect of inhibiting or activating MRGPRB4+ neurons on the number of jumps following the cessation of morphine administration was analyzed using a split-plot ANOVA (SPSS Statistics 18, Somers, NY) with a between-group factor of experimental group (hM4D-CNO, hM4D-Veh, CNO-CNO or hM3D-CNO, hM3D-Veh, CNO-CNO) and a within-group factor of time (4, 8, 24, and 48 hours). The total number of jumps across the entire 4 time periods tested was also analyzed using a one-way ANOVA with a between-group factor of the experimental group. Post hoc contrasts between each treatment group were computed using Bonferroni post hoc procedure. Differences with p-values of less than 0.05 were deemed statistically significant. Results are presented as mean ± SEM.

6.2.7.2 Conditioned Place Preference

Conditioned place preference was determined as the difference in the time spent in the morphine-paired chamber on the test day, and time spent in the morphine-paired chamber on the baseline sessions. In other words, a difference score was calculated between how long the animal was in the morphine-paired chamber on test day, and how much time it spent in the saline-paired chamber on test day. In order to reduce variance within each time point, the raw data underwent a log transformation. Differences in preference were analyzed using a split-plot ANOVA (SPSS Statistics 18, Somers, NY) with a between-group factor of experimental group (hM4D-CNO, hM4D-Veh, CNO-CNO or hM3D-CNO, hM3D-Veh, CNO-CNO) and a within-group factor of time. Extinction criterion was defined as the first days of 3 consecutive days in which a mouse did not display preference for the morphine-paired chamber. Survival scores for the number of mice per treatment condition that met criterion using the Kaplan-Meier survival estimation were computed. Differences between the experimental groups were analyzed using the Breslow (Generalized Wilcoxon) test. Differences with p-values of less than 0.05 were considered statistically significant.

6.3 Results

6.3.1 The effect of inhibiting the activity of sensory MRGPRB4-expressing neurons on withdrawal-precipitated jumping behaviors in morphine cage-mate animals

Morphine cage-mate animals, in which the activity of sensory MRGPRB4-expressing neurons was inhibited by CNO (hM4D-CNO, n=14), displayed significantly

more jumps following the cessation of morphine administration as compared to two varieties of control morphine cage-mate animals - morphine cage-mate mice carrying the AAV-hM4D but that did not receive CNO (hM4D-Veh, n=10), and morphine cage-mate animals carrying a control AAV and receiving CNO (CON-CNO, n=15) (Figure 9A). Number of jumps between the various experimental groups were recorded and compared at 4, 8, 24, and 48 hours after the last morphine injection. Two-way repeated ANOVA revealed a significant main effect of experimental group ($F(2, 36) = 8.90$, $p < 0.001$), a significant main effect of time ($F(1, 36) = 10.52$, $p < 0.01$), and a significant interaction between experimental group and time ($F(2, 36) = 3.28$, $p < 0.05$). Similarly, one-way ANOVA revealed a significant main effect of experimental group ($F(2, 36) = 8.90$, $p < 0.001$) for the total number of jumps across the 4 time periods tested. Bonferroni post-hoc comparison revealed that the hM4D-CNO group displayed significantly higher number of jumps as compared to the control groups.

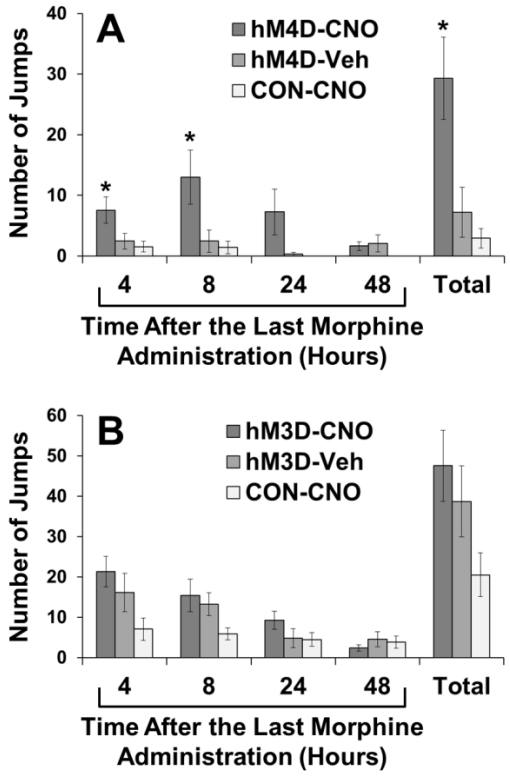

Figure 9. The number of jumping behaviors displayed. (A) Morphine-treated MRGPRB4-Cre mice that were transfected with hM4D or control AAV, injected with CNO or vehicle, and housed as morphine cage-mates. (B) Morphine-treated MRGPRB4-Cre mice that were transfected with hM3D or control AAV, injected with CNO or vehicle, and housed as morphine only mice. * indicates significant difference ($p < 0.05$) from control groups. Results are presented as mean ± SEM.

6.3.2 The effect of activating the activity of sensory MRGPRB4-expressing neurons on withdrawal-precipitated jumping behaviors in morphine only animals

Morphine only animals in which the activity of sensory MRGPRB4-expressing neurons was stimulated by CNO (hM3D-CNO, n=25) did not significantly differ in the number of jumps displayed following the cessation of morphine administration as compared to two kinds of control morphine only animals - morphine only mice carrying

the AAV-hM3D but do not receive CNO (hM3D-Veh, n=19), and morphine only animals carrying a control AAV and receiving CNO (CON-CNO, n=19) (Figure 9B). Number of jumps between the various experimental groups were recorded and compared at 4, 8, 24, and 48 hours after the last morphine injection. Two-way repeated-measures ANOVA revealed a significant main effect of time ($F(1, 60) = 27.08$, $p < 0.0001$), but no significant main effect of experimental group ($F(2, 60) = 1.98$, $p = 0.15$, *NS*), and no significant interaction between experimental group and time ($F(1, 60) = 2.96$, $p = 0.06$, *NS*). Similarly, one-way ANOVA revealed no significant main effect of experimental group ($F(2, 60) = 1.91$, $p = 0.16$, *NS*) for the total number of jumps across the 4 periods tested.

6.3.3 The effect of inhibiting the activity of sensory MRGPRB4-expressing neurons on conditioned place preference in morphine cage-mate animals

Mice that expressed hM4D and were treated with CNO (hM4D-CNO) (247.78 ± 40.65), mice that expressed hM4D and were treated with vehicle (hM4D-Veh) (224.35 ± 35.26), and mice that expressed mCherry and were treated with CNO (CON-CNO) (230.04 ± 30.84) show comparable levels of CPP acquisition during the conditioning phase of the experiment ($F(2,26) = 0.039$, *NS*). There were also no differences in the percentages of the animals that acquired CPP (Kruskal-Wallis: $\chi 2\ (2) = 0.69$, *NS*). Moreover, ten hM4D-CNO mice (47%), four hM4D-Veh (36%), and six CON-CNO (35%) animals did not acquire CPP and were omitted from the study.

After acquisition was established, animals underwent the CPP extinction procedure. There was a significant main effect of the time it took for the animals to no longer display a preference for the morphine-paired chamber ($F(9,306) = 5.14$, $p<0.001$), which suggests that all groups did eventually extinguish (Figure 10). However, there was not a significant effect of group ($F(2,34) = 1.25$, *NS*), nor was there a significant interaction between time and group ($F(18,306) = 1.13$, *NS*).

A Kaplan-Meier survival analysis showed that there were no differences among the groups in the rate at which they reached extinction criteria (Breslow (Generalized Wilcoxon): $\chi 2(2) = 1.13$, *NS*) (Figure 11). Also, after 30 days of daily extinction sessions, 45.5% of animals in the hM4D-CNO group reached extinction, while 42.9% of animals in the hM4D-Veh group reached extinction criteria. Lastly, 36.4% of animals in the CON-CNO group displayed extinction.

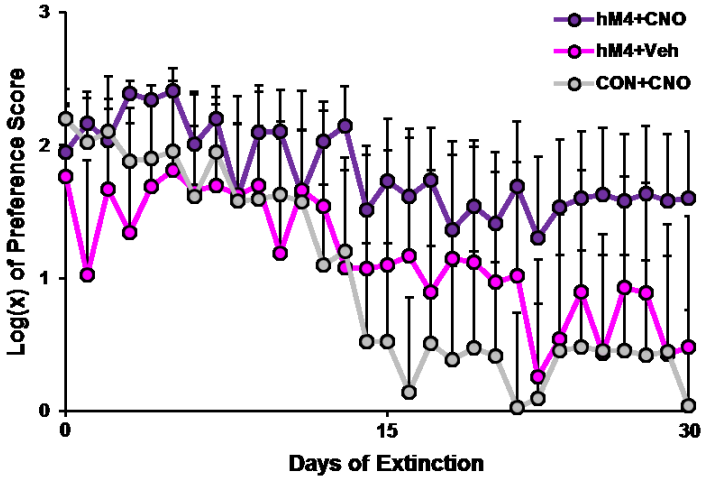

Figure 10. The log transformation of the mean preference for the morphine-paired chamber in animals housed as morphine cage-mates that had activity at MRGPRB4+ neurons inhibited. hM4-CNO animals (dark purple line), hM4-Veh (magenta line), and CON-CNO (gray line) subjected to up to 30 daily extinction sessions. Sessions lasted for 30 min. on days 1-15, and 60 min. on days 16-30. Results are presented as mean ± SEM.

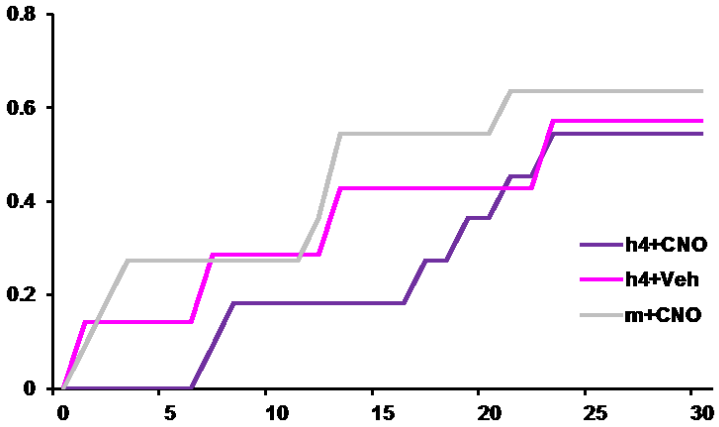

Figure 11. The number of animals, and the rate, to achieve extinction criteria over the 30 days of extinction procedure in the morphine cage mates that had activity at MRGPRB4+ neurons inhibited. hM4-CNO animals (dark purple line), hM4-Veh (magenta line), and CON-CNO (gray line).

6.3.4 The effect of activating the activity of sensory MRGPRB4-expressing neurons on conditioned place preference in morphine only animals

Mice that expressed the hM3D virus and were treated with CNO (hM3D-CNO) (198.5 ± 17.38), mice that expressed the hM3D virus and were treated with vehicle (hM3D-Veh) (293.2 ± 28.6), and mice that expressed the mCherry virus and were treated with CNO (CON-CNO) (257.7 ± 35.6) show a significant difference in levels of CPP acquisition during the conditioning phase of the experiment ($F(2,37) = 3.83$, $p<0.05$). This was due to the finding that acquisition in the hM3D-CNO group was significantly lower than the hM3D-Veh group ($p<0.05$). Also, there was a trend for

significance between the hM3D-CNO group and the CNO-CON group (p=0.15). However, there was no difference between the hM3D-CNO and the CON-CNO groups (*NS*). There were also no differences among the experimental groups in the percentages of the animals that acquired CPP (Kruskal-Wallis: $\chi 2$ (2) = 2.31, *NS*). Thirteen hM3D-CNO mice (45%), eight hM3D-Veh (40%), and seven CON-CNO (35%) animals did not acquire CPP and were omitted from the study.

After acquisition was established, the animals underwent the CPP extinction procedure. There was a significant main effect of the time it took for the animals to no longer display a preference for the morphine-paired chamber (F(9,333) = 8.76, p<0.001), which suggests that all groups did eventually extinguish (Figure 12). There was also a significant effect of group (F(2,37) = 3.83, p<0.05). Similarly, there was a significant interaction between time and group (F(18,333) = 2.05, p<0.01). This significant time × group interaction was driven by the decreased rate of acquisition in hM3D-CNO animals, as well as the significant difference (F(2,37) = 3.94, p<0.05) at the first timepoint between the hM3D-CNO and the CON-CNO groups (p<0.05). No other significant differences were observed between the experimental groups at any time point.

A Kaplan-Meier survival analysis showed that there were no differences among the groups in the rate at which they reach extinction criteria after daily extinction sessions (Breslow (Generalized Wilcoxon): $\chi 2$(2) = 0.47, *NS*) (Figure 13). Also, after 30 days of daily extinction sessions, 43.8% of animals in the hM3D-CNO group reached

extinction, while 41.7% of animals in the hM3D-Veh group reached extinction criteria. Lastly, 38.5% of animals in the CON-CNO group displayed extinction.

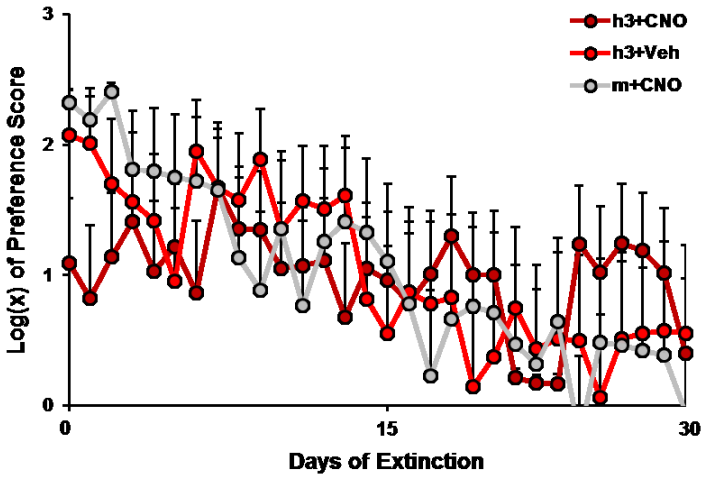

Figure 12. The log transformation of the mean preference for the morphine-paired chamber in animals housed as morphine only that had activity at MRGPRB4+ neurons activated. hM3-CNO animals (dark red line), hM3-Veh (red line), and CON-CNO (gray line) subjected to 30 daily extinction sessions. Sessions lasted for 30 min. on days 1-15, and 60 min. on days 16-30. Results are presented as mean ± SEM.

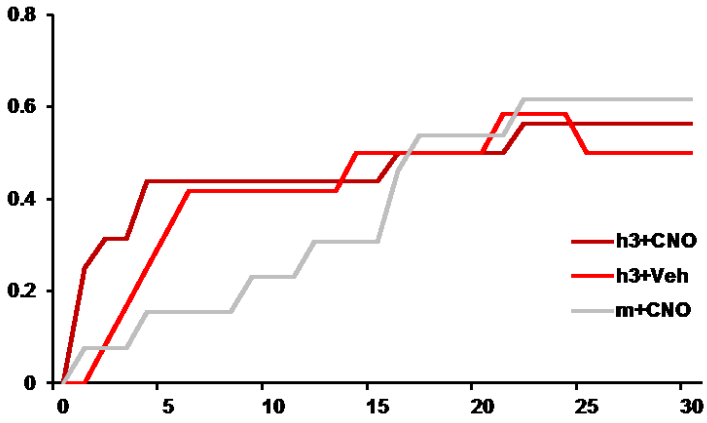

Figure 13. The number of animals, and the rate, to achieve extinction criteria over the 30 days of extinction procedure in the morphine only animals that had activity at MRGPRB4+ neurons activated. hM3-CNO animals (dark red line), hM3-Veh (red line), and CON-CNO (gray line).

6.4 Discussion

This study demonstrated that social grooming modulates the role of social housing conditions on morphine dependence. Specifically, MRGPRB4-Cre mice that were transfected with Gi-DREADD, treated with CNO, and housed as morphine cage-mates (hM4D-CNO) showed more withdrawal symptoms than did control mice housed in the same condition. These results suggest that reducing the sensation of social grooming by decreasing activity at MRGPRB4+ neurons has the ability to affect the previously observed social housing effect. As jumping behavior has been shown to indicate morphine dependence, these data show that CNO-treated mice developed stronger morphine dependence than control mice.

In this study, I observed a trend in which morphine cage-mate MRGPRB4-Cre mice transfected with Gi-DREADD, treated with CNO took longer to extinguish

morphine CPP than control animals. The time to extinguish a place preference has been associated with increased drug reward (Kaplan & Coyle, 1998; X. Ma et al., 2012; Self & Choi, 2004) and more robust memories of the cues that are associated with drug addiction. However, this observation did not reach statistical significance. My previous results demonstrate that mice that received morphine while being housed with drug-naïve mice (i.e. morphine cage-mates) express less jumping behavior and extinguish CPP more rapidly than mice that receive morphine but are exposed only to other morphine-injected animals (morphine only). Therefore, the findings in this study provide evidence for a role of social grooming in, at least some of, the protective effect of being housed with drug-naïve mice.

In this experiment, I also examined the effect of activating MRGPRB4+ neurons in animals housed in the morphine only condition. Activation of MRGPRB4+ reduced acquisition of CPP in the hM3D-CNO group. This may be because activation of these neurons was found to be rewarding in young animals (Vrontou et al., 2013). Moreover, social grooming and social play have been shown to be rewarding in adolescent animals (van Kerkhof et al., 2013). However, contrary to my hypothesis, these animals did not show any differences in withdrawal symptoms, or the extinction of CPP, as compared to the controls. It was expected that activation of the sensation of grooming would increase the rate of extinction of morphine CPP. However, I chronically administered CNO twice daily. There may have been excessive stimulation to these animals that was similar to social crowding, a form of social stress (Beery & Kaufer, 2015). This involves increasing the number of animals relative to the available cage area. It can be

accomplished by housing animals in smaller cages, by increasing the number of animals per cage, or by a combination of both. Similar to the reaction to social stress resulting from isolation, social stress as a result of social crowding was also suggested to increase the release of endogenous opioid peptides. Specifically, rodents show aversion to food presented in association with the administration of opioid antagonists, such as naloxone. Social crowding enhanced these aversive properties of naloxone, suggesting that social crowding increases endogenous opioid tone (Pilcher & Jones, 1981). Therefore, chronic activation of MRGPRB4+ neurons may have been an aversive stimulus that leads to a heightened response to opioids, which resulted in the lack of differences between control and treatment mice. Because this is the first to examine how MRGPRB4+ neuronal activity contributes to opioid reward, there is not much literature on this subject.

In my previous paper on morphine dependence and the extinction of CPP (Bates et al., 2014), I argued that housing conditions might modulate morphine dependence and reward because of differences in the quality of social interaction between the morphine-treated animals and between the drug-naïve animals and their morphine cage-mates. In this experiment, I attempted to study this by modulating activity at neurons expressing receptors known to be involved in social grooming. Grooming of a conspecific is a form of positive, affiliative social interaction in mice. Moreover, oxytocin enhances grooming behavior in mice, and this effect may be dependent on dopaminergic activity in the nucleus accumbens (Filippo Drago, Caldwell, et al., 1986; F. Drago, Pedersen, et al., 1986), suggesting that grooming activates reward circuitry and that oxytocin may play a role in grooming behavior. Oxytocin induces profound prosocial effects in both humans

and non-human animals, including increases in the salience of social stimuli, as well as increases in the time spent interacting with conspecifics in a social interaction test (Domes et al., 2007; Lukas et al., 2011). Conversely, oxytocin and oxytocin-receptor (OT-R) knockout mice show deficits in social recognition (Choleris et al., 2006; Ferguson et al., 2000; Takayanagi et al., 2005). Moreover, systemic administration of oxytocin has been shown to attenuate symptoms associated with withdrawal from opioids, including heroin and morphine (e.g. jumping and piloerection) (Kovacs, Borthaiser, & Telegdy, 1985; Kovacs, Horvath, et al., 1985; Kovacs et al., 1984; Kovacs & Marko, 1993). Similarly, systemic, as well as local nucleus accumbens and hippocampus, injections of oxytocin decreased the acquisition and maintenance of heroin self-administration in heroin-dependent adult animals (Ibragimov et al., 1987; Szabo et al., 1985). Therefore, it is possible that social grooming induces oxytocin, which, in the morphine cage-mates, helps to ameliorate the effects of receiving chronic opioids. By blocking the sensation of social grooming, oxytocin activity may have been blocked. Future studies should examine the role that oxytocin plays in the social housing effect, and how social grooming affects oxytocin. Moreover, oxytocin should be measured using MRGPRG4-Cre mice to see if blocking the sensation of social grooming produces differences in oxytocin levels.

It is known that early social environment plays a role in the propensity to abuse drugs (Bardo et al., 2013). Moreover, it has been shown that social support can also be a factor that aids in the cessation of use of both licit and illicit drugs (Kelly et al., 2008; Klimas et al., 2014; Luchenski et al., 2015; McCutcheon et al., 2016). One form of

affiliative social behavior in the rodent is through social grooming. In this experiment, I attempted to manipulate the sensation of social grooming in adolescent mice. Blocking this sensation might reduce the protective effect of being housed with drug-naïve mice. Adolescent opioid abuse is on the incline, and current treatments are not effective in reducing rates of relapse. Thus, the present data represent an avenue that needs to be further elaborated, and could hold benefits for the treatment of human addiction. In the following experiment, I examine the role that vasopressin may have in the social housing effect, as it has been implicated in pair-bonding and social interaction.

CHAPTER VII

EXPERIMENT 5: VASOPRESSIN'S ROLE IN THE SOCIAL HOUSING EFFECT

7.1 Introduction

Vasopressin is synthesized in the hypothalamus, and is secreted from the posterior pituitary into the blood stream, and into the central nervous system (Barberis & Tribollet, 1996). It can also contain arginine, and this form is referred to as arginine-vasopressin (AVP). Peripherally, vasopressin's main role is to facilitate the reabsorption of water into the kidney, and to induce contraction of the smooth muscle that surrounds arteries. When released into the brain, AVP's effects are manifold, including activation of corticotropin-releasing factor (CRF). It produces these distinct effects by its actions on three receptors, V1a (primarily liver, smooth muscle, and brain), V1b (pituitary and various locations in the brain), and V2 (primarily in the kidney). It is suggested that vasopressin may act as a neuromodulator in brain regions that are implicated in addiction, including the mesolimbic pathway, as AVP binding sites have been located in this region (Buijs, 1983; de Kloet et al., 1985).

The AVP system is important to drug addiction research for a variety of reasons. AVP release is triggered in response to stress and activation of V1b receptors produce ACTH secretion in the anterior pituitary. Importantly, vasopressin has also been shown to play a role in social cognition, recognition, and affiliation (Insel, 2010). AVP is expressed in regions that have been implicated in social anxiety, and its actions are associated with male-typical social behaviors, including pair bonding, courtship,

intermale aggression, and scent marking (Lim & Young, 2006). Also, increasing the number of vasopressin receptors in the brain increases social interaction in adult rats (Landgraf et al., 2003). It seems that AVP is involved in pairing stress and anxiety with social cues. Therefore, AVP is a suitable candidate for exploring the role that social environment plays in addiction.

There is developing consensus that AVP has a role in opioid-induced drug-taking and drug-seeking. Treatment with AVP facilitates the development of morphine physical dependence, and acute treatment with morphine decreases plasma levels of AVP (Van Ree & De Wied, 1977c; van Wimersma Greidanus et al., 1979). Acute treatment with morphine does not alter AVP levels in the hippocampus, while morphine dependent mice display decreased levels of AVP in this area (Kovacs et al., 1987). It appears that opioid history is also important concerning AVP. Regarding the findings in the hippocampus, the decreased levels of AVP likely represent the development of morphine tolerance, as vasopressin receptors in the hippocampus have been shown to mediate this effect (Su et al., 1998).

AVP decreases intravenous self-administration of heroin when administered both systemically, and through an intracerebroventricular injection (van Ree, Burbach-Bloemarts, & Wallace, 1988; van Ree & de Wied, 1977a, 1977b). Moreover, removing AVP with anti-vasopressin serum facilitated heroin self-administration (Van Ree & De Wied, 1977c). An increase in AVP mRNA in the amygdala was observed in the early, but not the late, withdrawal phase following heroin administration (Zhou et al., 2008). This was accompanied by increased levels of plasma corticosterone, suggesting a

relationship between AVP and stress during opioid withdrawal. The distinction between early and late withdrawal in Zhou et al. (2008) was 12 hours and 10 days, respectively. This was an important distinction to examine because, the most salient states of spontaneous withdrawal are observed within the first 24-hour period in rodents (Mucha, Gritti, & Kim, 1986). This finding is consistent with previous results showing HPA axis activation during early opioid withdrawal. Moreover, blockade of the AVP V1b receptor decreased both the stress and heroin-primed reinstatement of heroin self-administration (Zhou et al., 2008). This might suggest that stress may be involved in reinstatement, but it is important to note that AVP mRNA in the amygdala was only increased in heroin-dependent, but not heroin-naïve animals. Therefore, there is an interconnection between a stressor's ability to produce heroin-seeking and previous heroin experience. Also, if taken together with Kovacs et al. (1987)'s findings, it seems that, concerning heroin, AVP is only produced during withdrawal.

Previously, it was found that morphine only animals acquire morphine CPP more readily than morphine cage-mates (Cole et al., 2013). Because of AVP's role in social relationships, as well as its ability to modulate opioid-induced processes, I sought to explore a possible role for AVP in the observed social housing effect. I assessed the acquisition of morphine CPP after blockade of AVP receptor V1b, as well as mRNA expression of AVP in the striatum among the various groups.

7.2 Methods

7.2.1 Animals

All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee. The mice used were outlined in Chapter II. Briefly, adolescent C57BL/6 male mice were purchased from Envigo (Houston, TX) and acclimated to the colony for a minimum of 5 days before the start of experiments.

7.2.2 Housing conditions

Mice were housed in conditions as outlined in Chapter II. Briefly, they were housed as saline only (SAL), saline cage-mates (SCM), morphine cage-mates (MCM), or morphine only (MOR).

7.2.3 Morphine pretreatment regimen

In the real-time PCR study, mice were injected with 20 mg/kg morphine (10 ml/kg, s.c.) or saline once daily for 14 days. This dose represents the salt concentration. Mice were then euthanized and their dorsal striatum were dissected 24 hours following the final injection of morphine. In the behavioral study, similar to previous studies (Cole et al., 2013; Hodgson et al., 2010), mice were injected twice daily (9:00 am and 5:00 pm) in their home cage for 6 consecutive days with increasing doses of morphine (10–40 mg/kg, 10 ml/kg, s.c.) or saline for a total of 12 injections. Specifically, on days 1 and 2, the mice were injected with 10 mg/kg morphine or saline. On days 3 and 4, they were

injected with 20 mg/kg morphine or saline. On days 5 and 6, they were injected with 40 mg/kg morphine or saline. Morphine sulfate was purchased from Sigma (St. Louis, MO, USA).

7.2.4 RNA preparation and qPCR

Total RNA was isolated from homogenates of the dorsal striatum using the miRNAeasy Lipid Tissue mini kit (Qiagen, Valencia, CA) according to assay instructions and total RNA was quantified using a Nanodrop 1000 spectrophotometer (ThermoScientific, Waltham, MA). Extracted RNA was transformed into cDNA using supplies from New England Biolabs (Ipswich, MA) according to assay instructions.

qRT-PCR was used to measure gene expression results. The PCR primer for AVP was designed using NCBI Reference Sequences, and was designed to have a Tm of ~ 60ºC. Quantitative RT-PCR was conducted using SYBR® Green JumpStart ReadyMix (Sigma-Aldrich, Missouri, MO) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). AVP primer (Table 2) was purchased from Eurofins mwg Operon (Huntsville, AL). Differences among groups were calculated using the ΔΔCt method.

Table 2. AVP Primer

Gene:	Sequence:
AVP	F: 5'-TCCGTGGATTCTGCCAAGC-3' R: 5'-AAGTTTATTTTCCATGCTGTAGGG-3'

7.2.5 CPP apparatus

Morphine CPP was conducted in a set of eight identical apparatuses. The specifications of the apparatus are described in Chapter III. On habituation and test sessions, two doors allowed free access between the three chambers, while on conditioning days, animals were confined to one of two chambers. Also, on habituation and test sessions, the animals were placed directly into the neutral chamber. Each day, mice were habituated to the testing room for 30 minutes prior to being placed in the apparatus. The CPP apparatus was thoroughly cleaned with 70% ethanol and water and completely dried between mice.

7.2.6 Morphine CPP experimental design

Seven days following the final home cage treatment dose of morphine or saline (i.e. experimental day 13), mice (n=10-20 per experimental group) were placed in the neutral chamber and permitted free access to the entire apparatus for 30 minutes. The time spent in each of the chambers was recorded to measure any initial bias. Animals that spent more than 70% of the time in one chamber were eliminated from the study. The following 2 days, each animal had two conditioning sessions, one session per day. A biased design was used for this study wherein, for each mouse, the chamber that was less preferred in the initial recording was assigned to be the drug-paired chamber for that mouse. For one conditioning session, animals were injected with 20 mg/kg morphine or saline and were confined for 60 minutes to the less preferred conditioning chamber. For the other conditioning session, animals were injected with saline and were confined for

60 minutes to the other chamber. Animals from each treatment group were randomly assigned to receive a morphine conditioning session on experimental day 14 or 15, ensuring that from each treatment group and conditioning dose half of the animals received the morphine conditioning session on experimental day 14, and the other half received the morphine conditioning session on day 15. The following day (i.e. experimental day 16), mice in a drug-free state were allowed to freely explore the apparatus for 30 minutes. The time spent in each chamber was recorded to the nearest second.

7.2.7 Data Analysis

The differences in gene expression between groups were analyzed using a two-way ANOVA (SPSS Statistics 18, Somers, NY) with between-group factors of housing and treatment for each gene. Post hoc contrasts between each treatment group were conducted using the Bonferroni procedure.

Conditioned place preferences are reported as the differences in the time spent in a compartment between post- and pre-conditioning sessions. The data was than subjected to a log10 transformation (Kirk, 1968) and analyzed using a two-way ANOVA with the conditioning (morphine vs. saline) and AVP-V1b receptor antagonism (SSR149415 vs. vehicle) as variables. Post hoc contrasts between each treatment group were computed using the Bonferroni procedure. Differences with p-values of less than 0.05 were deemed statistically significant.

7.3 Results

7.3.1 Housing with drug-naïve animals protect against morphine-induced increase in AVP expression

I used qPCR to examine AVP mRNA expression in the striatum following repeated administration of morphine in animals that were differentially housed. AVP expression in animals housed in the morphine only condition was much higher than all other groups (Figure 14). A two-way ANOVA revealed a significant main effect of treatment ($F(1,34) = 4.24$, $p < 0.05$). Moreover, there was a strong trend for a main effect of housing ($F(1,34) = 3.96$, $p = 0.055$), as well as a significant interaction between housing and treatment ($F(1,34) = 3.75$, $p = 0.06$). Bonferroni post hoc analyses demonstrated that morphine only mice exhibit higher gene expression than animals housed as morphine cage-mates (morphine only vs. morphine cage-mates, $p = 0.035$). They also exhibit higher expression of AVP than saline-treated animals (morphine only vs. saline only, $p = 0.038$; morphine only vs. saline cage-mates, $p = 0.034$).

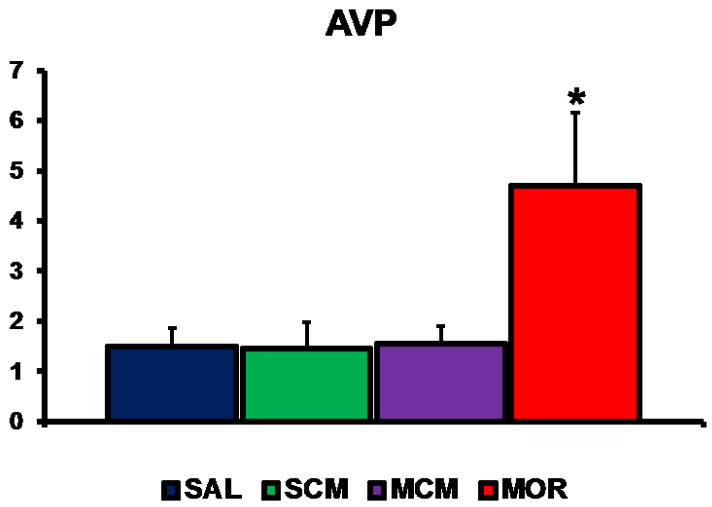

Figure 14. Gene expression of AVP in the striatum. Saline only, n = 9; saline cage mates, n = 9; morphine cage-mates, n = 10; and morphine only, n = 10. * indicates a significant difference from the other experimental groups. Results are represented as means ± SEM.

7.3.2 Inhibition of AVP-V1b receptor inhibits the development of morphine CPP in morphine only animals

SSR149415 inhibited the development of morphine CPP in morphine only animals (Figure 15A). Two-way ANOVA revealed a significant main effect of conditioning ($F(1, 56) = 7.93$, $p < 0.01$). There was no significant main effect of AVP-V1b receptor antagonism ($F(1, 56) = 3.07$, $p = 0.085$), but there was a significant interaction between conditioning and AVP-V1b receptor antagonism ($F(1, 56) = 4.39$, $p < 0.05$). Bonferroni post-hoc comparison revealed that injection of SSR149415 30 minutes prior to the conditioning session did not alter the acquisition of CPP in animals conditioned only to saline (MOR-SSR-saline vs. MOR-vehicle-saline, *NS*). As expected, morphine only animals receiving vehicle and conditioned to morphine acquired

significant morphine CPP as compared to morphine only animals receiving vehicle that were conditioning only with saline (MOR-vehicle-morphine vs. MOR-vehicle-saline, $p< 0.01$). In contrast, morphine only animals receiving SSR149415 and conditioned to morphine did not acquire significant morphine CPP as compared to controls (MOR-SSR-morphine vs. MOR-vehicle-saline or MOR-SSR-saline, *NS*). Moreover, acquisition of morphine CPP was significantly reduced in MOR-SSR-morphine animals relative to MOR-vehicle-morphine animals ($p< 0.05$).

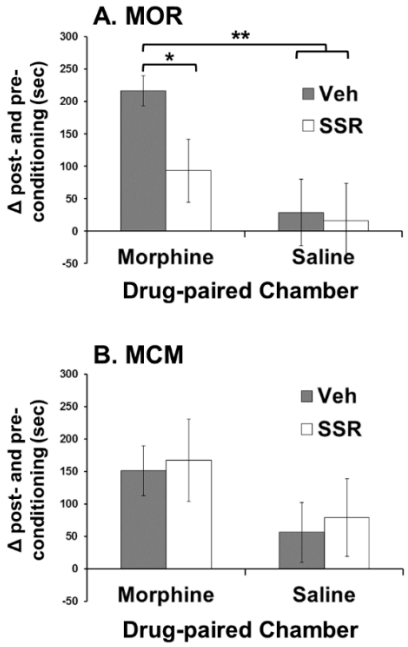

Figure 15. Mice were treated with morphine for 6 days and housed as morphine only (A) or morphine cage-mates (B). Subsequently, they were tested for the acquisition of CPP. They were conditioning in the drug-paired chamber with 20 mg/kg morphine or saline (controls). SSR149415 (SSR) or vehicle (Veh) were administered 30 minutes prior to the conditioning session. * indicates significant difference $p < 0.05$; ** indicates significant difference $p < 0.01$. from control groups. Results are presented as mean ± SEM.

7.3.3 Inhibition of AVP-V1b receptor did not alter the development of morphine CPP in morphine cage-mate animals

Morphine cage-mates acquired significant morphine CPP, when conditioning with morphine vs. saline (Figure15B). Two-way ANOVA revealed a significant main effect of conditioning ($F(1, 39) = 5.47$, $p < 0.05$). However, SSR149415 did not alter the acquisition of morphine CPP. Two-way ANOVA revealed no significant main effect of AVP-V1b receptor antagonism ($F(1, 39) = 0.18$, *NS*), and no significant interaction between conditioning and AVP-V1b receptor antagonism ($F(1, 39) = 0.53$, *NS*). Bonferroni post-hoc comparison revealed no significant different between the various experimental groups.

7.4 Discussion

In the present experiment, pharmacological antagonism of the AVP system via the V1b receptor resulted in decreased morphine reward in animals housed in the morphine only condition, but not animals housed as morphine cage-mates. Moreover, striatal AVP expression was increased in morphine only mice, but not in the morphine cage-mates. This suggests that housing with drug-naïve animals protects from morphine-induced increases in AVP expression. It provides evidence for vasopressin's role in the social housing effect.

As mentioned above, AVP has been shown to modulate opioid reward and dependence (van Ree et al., 1988; van Ree & de Wied, 1977b, 1977c). Moreover, antagonism of V1b receptor decreases heroin reinstatement (Zhou et al., 2008).

Therefore, my results align with previous findings that suggest vasopressin's role in opioid-induced reward and dependence.

Additionally, in line with the findings of the CPP experiment, I show that AVP gene expression in the striatum of morphine only animals was increased compared to all other groups. AVP mRNA was shown to be increased in the amygdala and hypothalamus during early heroin withdrawal – a time point that was similar to the time point used in this experiment (Zhou et al., 2008). Similarly, AVP was shown to be increased in the nucleus accumbens following chronic exposure to cocaine (Rodriguez-Borrero et al., 2010). Therefore, the present results are consistent with previous literature on AVP expression following chronic exposure to drugs of abuse.

The observed increase in expression of vasopressin could be due to augmented levels of stress (Tanaka, Versteeg, & De Wied, 1977). Morphine withdrawal increases corticosterone release and is stressful (Hofford et al., 2011). Moreover, vasopressin has been correlated with the increased stress that accompanies morphine withdrawal (Nunez et al., 2007). However, there were not any differences in corticosterone levels among groups during the time point that CPP was conducted in this study (Cole et al., 2013). Therefore, while it is unlikely that stress is the only reason that V1b antagonism decreased the acquisition of morphine CPP in morphine only mice, it may contribute to it.

The V1b antagonist, SSR419145, was administered systemically. Thus, its site of action is unable to be determined. While the results here suggest differences in striatal AVP expression levels, it is possible that the site of action for the V1b

antagonism is not the striatum. This is due to the fact that V1b expression is low in the striatum and other portions of the midbrain (Vaccari, Lolait, & Ostrowski, 1998). Moreover, V1b is abundantly expressed in many other areas of the brain, including the amygdala, which is involved in the stress response induced by vasopressin (Vaccari et al., 1998). Additionally, receptors for AVP exist both in the CNS and peripherally. Future studies should use local injections of V1b, and V1a, antagonists to measure their effect on morphine CPP.

Previously, it was shown that morphine only animals acquire morphine CPP after a single exposure to morphine, while morphine cage-mates do not. In this experiment, I found that antagonism of the vasopressin V1b receptor reduced the expression of morphine CPP in morphine only animals, but did not affect morphine cage-mates. Moreover, gene expression of AVP in the striatum was significantly higher in morphine only animals than any other group, including morphine cage-mates. Therefore, the results here demonstrate vasopressin's role in the social housing effect on acquisition of opioid reward. Vasopressin represents one possible neurobiological mechanism mediating the social housing effect on opioids' abuse liability. In the next experiment, I explore other possible mechanisms.

CHAPTER VIII

EXPERIMENT 6: SOCIO-ENVIRONMENTAL EFFECT ON GENE EXPRESSION IN THE ADOLESCENT MOUSE STRIATUM

8.1 Introduction

Morphine addiction involves many brain regions. Multiple theories propose that addiction involves the recruitment of brain regions implicated in natural reward and motivation to seek reward (Koob, 1992a; Robinson & Berridge, 1993). It is thought that after these areas become recruited, they become hypersensitive to drugs and related stimuli. One of the more notable regions involved in reward is the mesocorticolimbic pathway, which is integral in dopaminergic transmission, and includes the striatum, ventral tegmental area (VTA), and prefrontal cortex (PFC). Importantly, opioids activate opioid receptors located on GABAergic neurons in this pathway, thus activating dopaminergic neurons in the striatum to produce the effects associated with opioid use.

There is evidence showing that opioids alter gene expression in several brain regions implicated in addiction in adult rodents (Georges et al., 1999). Specifically, there are several studies suggesting alterations in the striatum (Jacobs et al., 2013; Korostynski et al., 2007; Ziolkowska et al., 2012). Nevertheless, there is a dearth of information in the literature regarding the effects of opioids, particularly morphine, on alterations in gene expression in the adolescent brain (Ellgren, Spano, & Hurd, 2007). For example, adolescent mice trained to self-administer oxycodone display differential patterns of gene expression than adults, in both the dorsal striatum and the hippocampus (Mayer-

Blackwell et al., 2014; Zhang et al., 2015). Interestingly, it was found that more genes were altered in the dorsal striatum of adolescents than adults, which may suggest a unique plasticity of adolescent brains to opioid exposure.

Moreover, despite the preponderance of behavioral evidence, little is known about the interaction between opioids and environment in the effects on gene expression. Therefore, in the current study, I examined the role of social environment on morphine-induced modulation of striatal gene expression, an area that has been shown to be involved in opioid reward and addiction. Given that housing with drug-naïve animals protects against the acquisition of opioid reward, I hypothesized that housing with drug-naïve animals will protect against morphine-induced modulation of striatal gene expression, resulting in significant differences in morphine-induced gene expression between morphine only and morphine cage-mate animals.

8.2 Methods

8.2.1 Animals

Adolescent, male mice were used in this experiment. There were 10 animals per group. Animals were housed according to the housing conditions outlined in the general methods: saline only, mixed cage (saline or morphine cage-mates), or morphine only.

8.2.2 Pretreatment Regimen

Mice were pretreated once daily with saline or 20 mg/kg morphine (10 mg/mL, s.c.) at 9:00AM for fourteen days. This dose represents the salt concentration.

8.2.3 Tissue Collection

Twenty-four hours following the final morphine injection, mice were deeply anesthetized with sodium pentobarbital (100 mg/kg, 10 mg/mL, i.p.). Brains were extracted, and flash-frozen, in a bath of 2-methylbutane cooled on dry ice. Dorsal striatum tissue was dissected out of the flash-frozen brains and stored at -80^{o}C until RNA extraction.

8.2.4 RNA Extraction

Total RNA was isolated from homogenates of the dorsal striatum using the miRNAeasy Lipid Tissue mini kit (Qiagen, Valencia, CA) according to assay instructions and total RNA was quantified using a Nanodrop 1000 spectrophotometer (ThermoScientific, Waltham, MA). Extracted RNA was transformed into cDNA using supplies from New England Biolabs (Ipswich, MA) according to assay instructions.

8.2.5 RNA-Seq

After the RNA was extracted, the samples were analyzed using high-throughput, next generation sequencing (RNA-Seq). The total RNA was submitted to AgriLife Genomics and Bioinformatics Service at Texas A&M University. Libraries were prepared for each sample by using the Illumina TruSeq RNA Sample Preparation Kit following the manufacturer's instructions. Data was sequenced on ten lanes of Illumina HiSeq 2500 sequencer. The study consisted of 40 total RNA – 10 samples per group.

Barcoding was used to multiplex biological replicates. Base calling and data preparations were completed using Illumina CASAVA software.

The data analysis started by quality control of the generated FASTQ files, using FastQC software (Andrews). Reads were aligned to the reference genome to locate the origin of each RNA fragment on the reference genome. The reference genome of mus musculus (mm10) and the gene annotation file was downloaded from UCSC and employ the Tuxedo protocol proposed by Trapnell et al. (2012).

8.2.6 Real-time PCR

qRT-PCR was used to measure gene expression results from the RNA-Seq. All PCR primers were designed using NCBI Reference Sequences, and were designed to have a Tm of ~ 60^{o}C. Quantitative RT-PCR was conducted using SYBR® Green JumpStart ReadyMix (Sigma-Aldrich, Missouri, MO) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). All primers were purchased from Eurofins mwg Operon (Huntsville, AL). Differences among groups were calculated using the ΔΔCt method. Sequences are in Table 3:

Table 3. Primers

Gene:	Sequence:
β-Actin	F: 5'-TCAAGATCATTGCTCCTCCTG-3' R: 5'-TGTAAAACGCAGCTCAGTAAC-3'
Bromodomain 3 (Brd3)	F: 5'-GGACTCAAACCCAGACGAGATT-3' R: 5'-TGTTGACAATGGTTTCCTCTGC-3'
Cyclin-dependent kinase 12 (Cdk12)	F: 5'-GTCCTCCAGCTATGAAAGGAG-3' R: 5'-GGATTTCCTACTGGGGAGTG-3'
Phosphatidic Acid-Preferring Phospholipase A1 (DDHD1)	F: 5'-GACTCCGCATTGGAACTGG-3' R: 5'-CGGTATCATGCTCGTGTTTG-3'

Table 3. Continued

Gene:	Sequence:
Coagulation factor V (F5)	F: 5'- CTGAATAGACGATGGCGTG-3' R: 5'- TTGTTGTGTTTGTGTGAGAGG-3'
Dopamine D1 receptor (D1R)	F: 5'- CCTGTTTTCTGTCCCTGCTTA-3' R: 5'- GACACAGCTAAAGAGATGACAAAGA -3'
Epidermal growth factor-like protein 7 (Egfl7)	F: 5'- ACCTACCGAACCATCTACCG-3' R: 5'- GCATATTGCTGCTCCACAAG-3'
Forkhead Box J2 (FoxJ2)	F: 5'-CCCAAGACTCAGCAGGATAC-3' R: 5'-AAGTCCCAGTCGAAGTCATC-3'
Geranylgeranyl transferase type-2 subunit beta (Rabggtg)	F: 5'- CTTTGATGGTGGATTTGGGTG-3' R: 5'- ATCTGGTAACTTCTCGGGTC-3'
Glucuronic Acid Epimerase (Glce)	F: 5'-GGTCCAGTATGACGGCTATG-3' R: 5'-AATGGCACACCTTCAACTCC-3'
Guanine nucleotide-binding protein-like 3 (Gnl3)	F: 5'- TGCTGACGATCAAGAAAATGG-3' R: 5'- AGATGGCTTACCTGCTGTTG-3'
Interleukin-1 receptor-associated kinase 1 (Irak1)	F: 5'- TGACCCAGAGGCAAAACTCC-3' R: 5'- CTTAGTTCCACAGAGCACCTCC-3'
Jagged 2 (Jag2)	F: 5'-GTCGTCATTCCCTTTCAGTTC-3' R: 5'-ATCTGGAGTGGTGTCATTGTC-3'
Klotho	F: 5'- CCCGAATGTCTATCTGTGG-3' R: 5'- CGAAGTAAGGTTATCTGAGGC-3'
Oxytocin receptor (OtR)	F: 5'- CTGTTCTCAACCATCCTCGG-3' R: 5'- AGGAGGGATGCAAACCAATC-3'
PDZ and LIM domain protein 7 (Pdlim7)	F: 5'- TCACTCTGGTGCCACAATAAAC-3' R: 5'- TAACCCTCATCCTCCTCCTGTC-3'
Phenylalanyl-TRNA Synthetase Alpha Subunit (FARSA)	F: 5'-TCTTCTTGGAGATGGGGTTCAC-3' R: 5'-GTTTCACGCGCTGGACATAG-3'
Phosphodiesterase 12 (Pde12)	F: 5'-ACCCATTAGCACAGGAGAAG-3' R: 5'-GAGACGAATGTATCCACCTTTTG-3'
Platelet Derived Growth Factor Receptor Alpha (Pdgfra)	F: 5'-TCCGGGTATCGGATTTTCTTTG-3' R: 5'-ATAAGAGCTGGCAGGAGATGAG-3'
Rotatin (Rttn)	F: 5'-TCTACTCAAGGGGTGGATAGC-3' R: 5'-CCATCTCTCCGCCACAATC-3'
Ubinuclein-2 (Ubn2)	F: 5'- ACCAATAAAACAAATGAGGAGGC-3' R: 5'- CAAACTTCTCTGCAACATCCC-3'
Zinc Finger Protein 518a (Zfp518a)	F: 5'-GGGGACCACGGCAGATAC-3' R: 5'-ACAACTTAATGCCTGGACAATG-3'
β-Arrestin 2	F: 5'-TACACACTGGACCCATCAC-3' R: 5'-ATTCACTCCTTGCGTTCAC-3'

8.2.7 Statistical Analysis

The differential gene expression analysis for each group in the RNA-Seq was performed between all the pairs using Cuffdiff 2, software included as part of Cufflinks. Cuffdiff 2 provides corrected p-values for multiple hypothesis testing usually called q-values. The genes were sorted based on the q-values and the False Discovery Rate (FDR).

The differences in gene expression between groups were analyzed using a two-way ANOVA (SPSS Statistics 18, Somers, NY) with between-group factors of housing and treatment for each gene. Post hoc contrasts between each treatment group were conducted using Bonferroni post hoc procedure.

8.3 Results

8.3.1 RNA-Seq

Over 1200 genes were altered in the morphine only animals (MOR) as compared to saline only animals. In contrast, only 121 genes were altered in the morphine cage-mates (MCM), as compared to saline only animals, and only 61 of the altered genes were common between the morphine only and morphine cage mate animals (Figure 16).

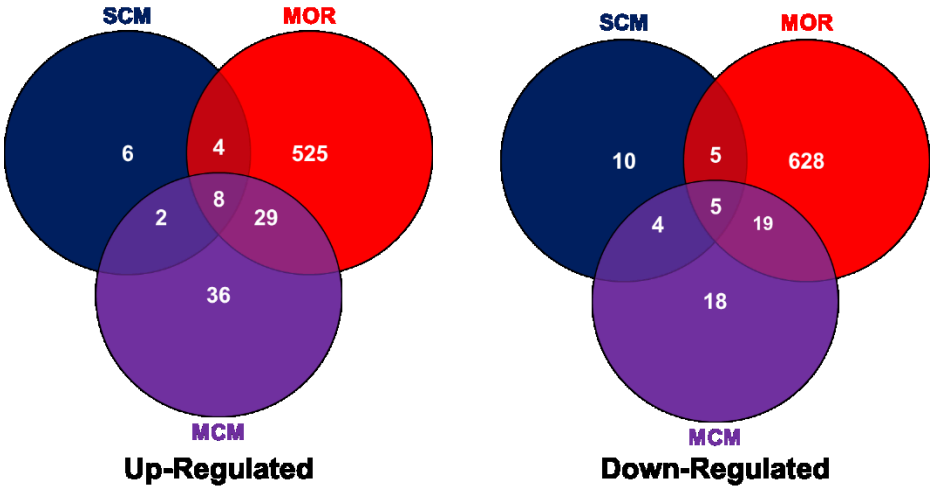

Figure 16. Venn-diagram of the number of genes that were differentially regulated in each group as compared to saline only animals. N = 10/group. SCM = Saline Cage-Mates, MCM = Morphine Cage-Mates, MOR = Morphine Only.

In order to determine the function of the gene targets that were identified using RNA-Seq, I conducted a thorough investigation using PubMed, as well as various other databases (e.g., GeneCards). Below is a table depicting the functions of the genes that were found to be altered (in the morphine-treated groups), as well which groups they were altered in (Table 4).

Table 4. A table depicting the functions of the genes that were identified in the RNA-Seq, as well as their group identification, and the direction of the gene dysregulation.

Function	Morphine Only		Morphine CM	
	Up	Down	Up	Down
Binding proteins	75	89	3	5
Cellular	21	57	3	6
Cytoskeleton	36	39	3	1
Development	46	47	4	4
Enzymes	111	131	6	12

Table 4. Continued

Function	Morphine Only		Morphine CM	
	Up	Down	Up	Down
Hormones	3	7	1	4
Ion Channels	14	6	1	3
miRNAs and other non-coding RNAs	41	46	10	13
Receptors	37	29	2	5
Signaling	35	65	2	8
Transcription	74	60	8	10
Transmembrane	12	16	0	0
Transport	31	26	0	1
Vesicle Trafficking	9	9	0	0
Unknown	21	31	3	3
Total	566	658	46	75
	1224		121	

8.3.2 qRT-PCR on candidate genes of interest

The effects of social environment on morphine-induced gene expression in the striatum were analyzed using a two-way ANOVA with housing and treatment as between-subjects variables with the fold change (ΔΔCt method) derived from the qRT-PCR as the dependent variable, followed by Bonferroni post-hoc analyses. I found that there were several gene targets with significant interactions of housing and treatment (Figure 17).

8.3.2.1 Forkhead Box J2 (Foxj2)

Data presented in Figure 17A. Two-way ANOVA revealed no significant main effects of treatment ($F(1,34) = 3.61$, $p=0.07$) or housing ($F(1,34) = 0.13$, *NS*). However, there was a significant interaction between housing and treatment ($F(1,34) = 8.46$,

$p<0.01$). Bonferroni post-hoc analysis revealed significantly higher expression levels in morphine only animals as compared to saline only animals (MOR vs. SAL, $p < 0.01$). In contrast, there was no significant difference in expression levels between the morphine cage-mates and saline-treated animals (MCM vs. SAL, *NS*). Lastly, there was a trend for higher expression levels in the morphine only than the morphine cage-mate animals (MCM vs. MOR, $p=0.1$).

8.3.2.2 Phosphatidic Acid-Preferring Phospholipase A1 (DDHD1)

Data presented in Figure 17B. Two-way ANOVA revealed that there were no significant main effects of treatment ($F(1,34) = 1.53$, *NS*) or housing ($F(1,34) = 2.48$, *NS*). However, there was a significant interaction between housing and treatment ($F(1,34) = 8.06$, $p<0.01$). Bonferroni post-hoc analysis revealed that there was significantly higher expression in the morphine only mice than the saline only animals ($p=0.03$). There was no difference in expression levels between the saline only and the morphine cage-mates. Moreover, morphine only mice exhibit higher expression levels as compared to the morphine cage-mates ($p=0.02$).

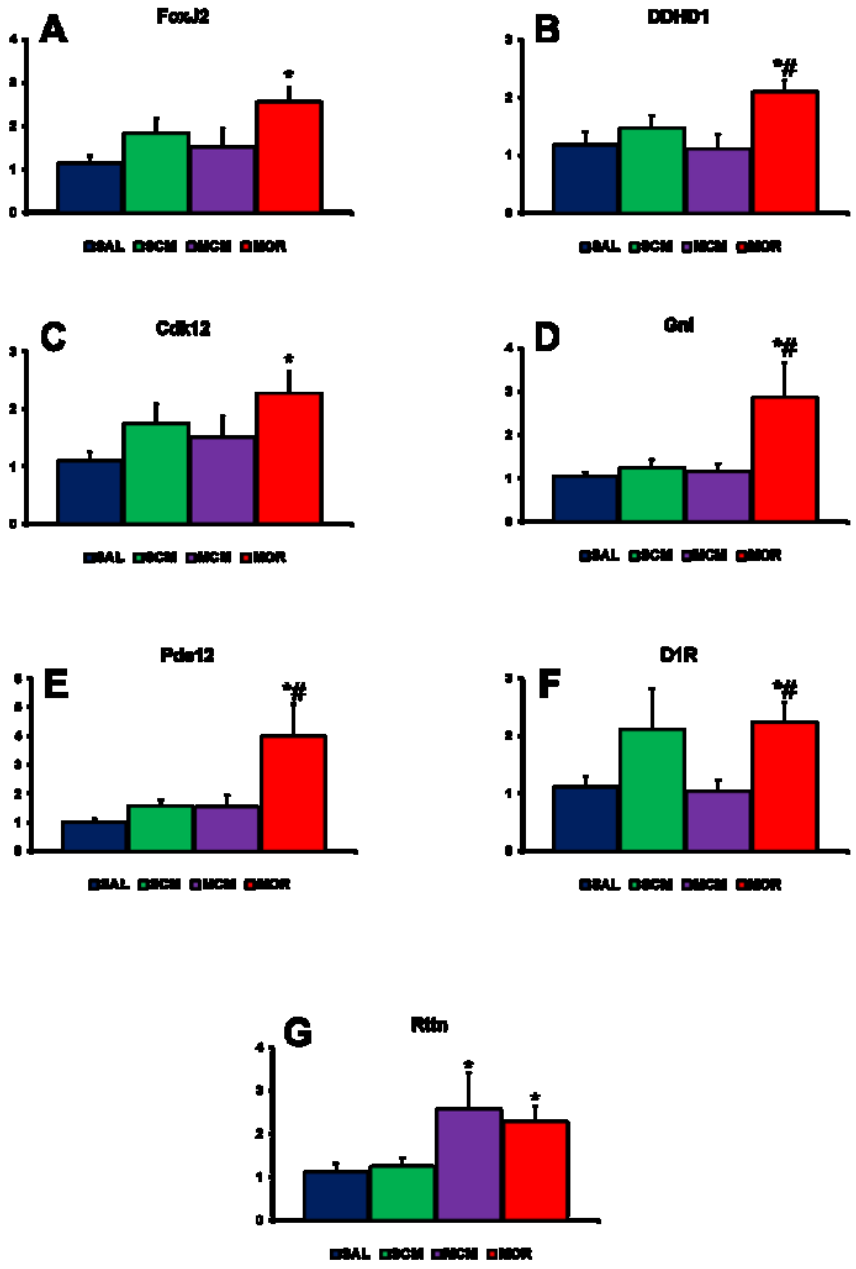

Figure 17. Genes that showed a significant treatment or housing × treatment interaction. Saline only, n = 9; saline cage-mates, n = 9; morphine cage-mates, n = 10; and morphine only, n = 10. * indicates significant differences from SAL; # indicates significant difference from MCM. Results are presented as means ± SEM.

8.3.2.3 Cyclin Dependent Kinase 12 (Cdk12)

Data presented in Figure 17C. Two way ANOVA revealed no main effects of housing ($F(1,34) = 0.02$, *NS*) or treatment ($F(1,34) = 2.19$, *NS*), but a significant housing × treatment interaction ($F(1,34) = 4.85$, $p<0.05$). Bonferroni post-hoc analysis revealed significantly higher expression in the morphine only animals than the saline only group ($p=0.04$). There was no difference between the morphine cage-mates and saline-treated animals.

8.3.2.4 G Protein Nucleolar 3 (Gnl3)

Data presented in Figure 17D. Two-way ANOVA revealed no main effect of housing ($F(1,34) = 3.36$, *NS*), but a significant main effect of treatment ($F(1,34) = 4.5$, $p<0.05$). Also, there was a significant housing × treatment interaction ($F(1,34) = 5.39$, $p<0.05$). Bonferroni post-hoc analysis revealed significantly higher expression in morphine only as compared to the saline-treated animals (MOR vs. SAL, $p = 0.02$; MOR vs. SCM, $p=0.04$). There were no differences between the morphine-cage mates and the saline-treated animals (MCM vs. SAL, *NS*; MCM vs. SCM, *NS*). Importantly, morphine only animals have significantly higher expression than morphine cage-mate mice (MCM vs. MOR, $p = 0.02$).

8.3.2.5 Phosphodiesterase 12 (Pde12)

Data presented in Figure 17E. Two-way ANOVA revealed a significant main effect of treatment ($F(1,34) = 5.74$, $p=0.02$). There was no significant main effect of

housing ($F(1,34) = 2.29$, *NS*), but there was a significant interaction between housing and treatment ($F(1,34) = 5.93$, $p=.02$). Bonferroni post-hoc analysis revealed significantly higher expression in the morphine only animals compared to the saline-treated mice (MOR vs. SAL, $p = 0.01$; MOR vs. SCM, $p=0.04$). There were no differences in expression levels between the morphine-cage mates and saline-treated animals (MCM vs. SAL, *NS;* MCM vs. SCM, *NS*). Moreover, morphine only animals exhibit significantly higher expression levels than morphine cage-mates (MCM vs. MOR, $p = 0.03$).

8.3.2.6 Dopamine 1 receptor (D1R)

Data presented in Figure 17F. Two-way ANOVA revealed no main effects of housing ($F(1,34) = 0.28$, *NS*) or treatment ($F(1,34) = 0.38$, *NS*), but a significant housing × treatment interaction ($F(1,34) = 4.69$, $p<0.05$). Bonferroni post-hoc analysis revealed significantly higher expression levels in the morphine only group compared to the saline only (MOR vs. SAL, $p=0.01$) and the morphine cage-mate groups (MOR vs. MCM, $p<0.01$).

8.3.2.7 Rotatin (Rttn)

Data presented in Figure 17G. Two-way ANOVA revealed no significant main effect of housing ($F(1,34) = 0.206$, *NS*), but there was a significant effect of treatment ($F(1,34) = 6.39$, $p=0.02$). There was no significant interaction between housing and treatment ($F(1,34) = 0.04$, *NS*). Bonferroni post-hoc analysis revealed a significant

increase in Rttn expression in both morphine-treated groups compared to both saline-treated groups ($p < 0.05$).

8.3.2.8 Other genes examined

No significant differences between the experimental groups were observed in the expression levels of Pdgfra, Brd3, Irak1, Zfp518a, Farsa, Egfl7, Glce, Jag2, F5, βArr2, OtR, Ubn2, Klotho, Pdlim7, and Rabggtb. The results for these qPCRs are presented in Figure 18.

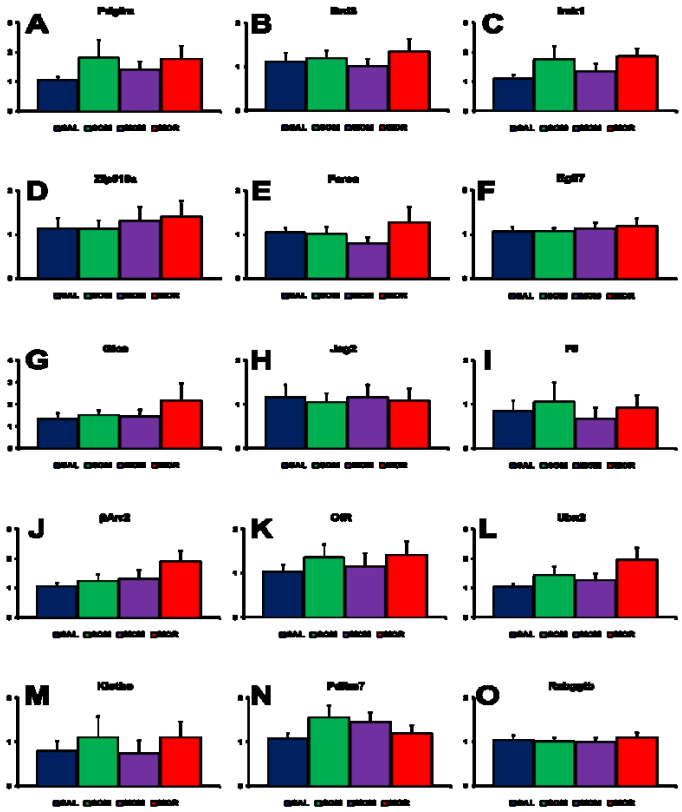

Figure 18. Genes that did not show any significant effects. Saline only, n = 9; saline cage-mates, n = 9; morphine cage-mates, n = 10; and morphine only, n = 10. Results are presented as means ± SEM.

8.4 Discussion

Opioid misuse/abuse is a debilitating problem, and there is evidence for a bidirectional relationship between its abuse liability and social environment. Opioid addiction is a severe problem given that prescription opioids are the most commonly prescribed substances for moderate-to-severe pain, and their chronic use can lead to numerous consequences. The goal of the present experiments was to elucidate potential mechanisms underlying the observed role that social environment plays in the development of morphine dependence and reward in order to identify potential novel targets for pharmaceutical treatment. Therefore, an exploratory analysis was conducted. Using RNA-Seq, I investigated the interaction between social housing conditions and morphine treatment on gene expression levels in the striatum. I observed that over 1200 genes were altered by morphine when animals were housed only with other morphine-treated animals (morphine only, MOR), as compared to saline-treated animals. However, only 121 genes were altered in morphine-treated animals housed with drug naïve animals (MCM), as compared to saline-treated animals, while only 61 of the altered genes are common between the morphine only and morphine cage-mate animals. This demonstrated that social housing conditions alter the effects of morphine on striatal gene expression.

In continuation of the RNA-Seq study, and in order to confirm its findings, I conducted qRT-PCR on genes of interest. I selected genes based on the direction of their differences and the magnitude of their fold changes, and others based on their relevance to opioid reward, dependence, and antinociception. Some of the targets revealed no

differences among groups, however, others produced some intriguing results. Among these were Dopamine D1 Receptor (D1R), DDHD1 (Phosphatidic Acid-Preferring Phospholipase A1), Cdk12 (Cyclin-dependent kinase 12), Guanine nucleotide-binding protein-like 3 (Gnl3), Forkhead Box J2 (FoxJ2), and Phosphodiesterase 12 (Pde12).

8.4.1 Dopamine D1 Receptor

Medium spiny neurons encompass most - 95% - of the neurons in the striatum (Yager et al., 2015). These neurons typically have two types, D1-receptor (D1R) expressing neurons in the direct pathway, and D2-receptor (D2R) expressing neurons in the indirect pathway (Nishi, Kuroiwa, & Shuto, 2011). Therefore, I measured expression of dopamine receptors using qPCR. Unfortunately, due to difficulties with primer design and qPCR parameters, I was unable to measure D2R gene expression. Nevertheless, I observed increased D1R expression in morphine only animals as compared to morphine cage-mates. It is known that activity at D1R contributes to the rewarding/reinforcing effects of morphine, as antagonism of D1R in the striatum decreases acquisition of morphine CPP (Shippenberg, Bals-Kubik, & Herz, 1993; Shippenberg & Herz, 1988), including single-trial CPP acquisition, like that used in experiment 5 (Fenu et al., 2006). Also, D1R-null mutant mice do not develop locomotor sensitization to morphine (A. Becker et al., 2001), and do not acquire morphine CPP (Y. P. Wang et al., 2015). Moreover, striatal D1 receptors are important for attention to drug-related cues and reward sensitivity (Agnoli & Carli, 2011; Sharp et al., 1995).

Interestingly, administration of morphine has also been shown to cause induction of ΔFosB in the accumbens (Muller & Unterwald, 2005; H. L. Wang et al., 2005), a transcription factor that accumulates during repeated drug exposure, and has been linked to drug withdrawal. Activity at D1R in NAcc is important for the acute rewarding effects of drugs of abuse, including morphine. However, in the dorsal striatum, which is important for habit formation and compulsive behavior, chronic exposure to morphine leads to induction of ΔFosB, and involves activity at D1R (Muller & Unterwald, 2005). ΔFosB is selective for D1R-expressing neurons, suggesting that D1R is critical for the development of addictive behaviors. Moreover, ΔFosB induction is more pronounced in adolescents, an age group that shows higher vulnerability to addiction, and is the focus of this dissertation. Here, I show that D1R-expression is higher in the morphine only animals than in morphine cage-mates. Increased levels of D1R-expression might correlate with the increased induction of ΔFosB that is observed following chronic exposure to morphine.

There is a discrepancy between my findings and other findings in the literature. Georges et al. (1999) observed decreased expression of D1R in the rat striatum. However, their experimental design is quite different from mine, as they implanted morphine pellets for continuous exposure to morphine, and I intermittently administered morphine to the animals once a day. Also, they did not observe any differences in locomotor activity between control animals and animals implanted with morphine pellets, while in previous experiments, there are drastic increases in locomotor activity. Thus, the differences in gene expression might represent differences in behavioral

response to the different morphine regimen used. This is given that a more continuous chronic regimen was shown to cause tolerance to the activating effects of opioids, while an intermittent injection procedure produces locomotor sensitization (Eitan et al., 2003). It could also represent an age-specific effect, given that Georges et al. (1999) only examined adult animals, while I focus on adolescents. It has been shown that D1R density is increased in adolescent animals, as compared to adults (Creese, Sibley, & Xu, 1992; Dwyer & Leslie, 2016). Moreover, adolescents are more sensitive to D1 agonists, and display greater reward sensitivity than adults (Dwyer & Leslie, 2016). Therefore, a difference between adolescents and adults in levels of D1R following morphine exposure is expected.

8.4.2 Phosphatidic Acid-Preferring Phospholipase A1

DDHD1, or phospholipase A1, is often implicated in regulating mitochondrial dynamics (Baba et al., 2014). Recently, DDHD1 was also shown to be a synthesizing enzyme of 2-arachidonoyl-LPI (LPI), an agonist for the orphan G-coupled receptor, GPR55 (Yamashita et al., 2010), which was previously identified as a cannabinoid receptor (Nevalainen & Irving, 2010; Yamashita et al., 2013). Interestingly, GPR55 antagonists show therapeutic potential in the treatment of neuropathic pain (Staton et al., 2008). GPR55-null, male mice show a reduction in hyperalgesia that developed using two models of neuropathic pain, Freund's complete adjuvant and the nerve ligation model. Therefore, if there is less GPR55 activity, there may be less neuropathic pain. Because DDHD1 is a synthesizing enzyme of the GPR55 agonist, LPI, lower levels of

DDHD1 may result in less agonism of GPR55, and thus a reduction of hyperalgesia. In the present results, morphine cage-mate mice did not exhibit the increased levels of DDHD1 observed in morphine only mice. Previously, I have shown that morphine cage-mate mice do not develop thermal hyperalgesia that was present in morphine only animals (refer to Chapter V). Therefore, there may be a link between DDHD1 levels and the development of hyperalgesia. Future studies need to be done to understand if this link between DDHD1 and hyperalgesia does indeed exist. Moreover, because there is a correlation between the antinociceptive and addictive properties of opioids (Bates et al., 2016), I propose that there is a common mediator for the two – DDHD1 may be a component of this common mediator. Nevertheless, additional work needs to be done to understand the role that DDHD1 may play in opioid reward and dependence.

Although no work has been done to directly connect DDHD1 and opioids, a recent study provides some support for a link between GPR55 and the μ-opioid receptor (oprm1), where morphine binds with high-affinity (Wolozin & Pasternak, 1981). Viudez-Martinez et al. (2017) examined the effects of cannabidiol, an anxiolytic compound found in the *Cannabis sativa* plant on ethanol reinforcement, as well as on the expression of genes related to reinforcement in the nucleus accumbens (i.e., ventral striatum). It was found that cannabidiol treatment significantly reduced expression of GPR55 and oprm1 almost identically (Viudez-Martinez et al., 2017). While this is certainly not a causal link, these data suggest a relationship between GPR55 and oprm1. Future experiments should examine whether treatments that upregulate oprm1 also

induce an upregulation of GPR55, and if the upregulation of GPR55 also increases DDHD1 levels.

8.4.3 Cyclin-dependent kinase 12

Another target of interest is Cyclin-dependent kinase 12 (Cdk12). Cdk12 was upregulated in morphine only animals as compared to morphine cage-mates. Cdk12, like many cyclin dependent kinases, is important for regulation of the cell cycle, and is involved in transcription and posttranscriptional processes. Cdk12 is a regulator of transcription elongation (Bartkowiak et al., 2010; K. Liang et al., 2015). Also, Cdk12 is important for genomic stability, as cells depleted of Cdk12 are sensitive to DNA damage (Blazek et al., 2011; Juan et al., 2016). Cdk12 was also shown to be involved in the regulation of axonal elongation in cortical neurons (H. R. Chen, Lin, et al., 2014). Chronic morphine has been shown to alter presynaptic proteins (Abul-Husn et al., 2011; Berrios, Castro, & Kuffler, 2008). Interestingly, animals with established morphine self-administration behavior exhibited an upregulation of genes involved in axonal development and outgrowth (Tapocik et al., 2013). More work should be done to understand the precise patterns of axonal alterations produced by morphine dependence, and how neuroplasticity-associated genes are involved. Here, I observed an increased expression of Cdk12 in morphine only animals compared to morphine-cage mates. It has also been observed that morphine only animals display increased levels of morphine dependence as compared to morphine cage-mates. This provides a potential mechanistic

link between the increased levels of Cdk12 reported here, and increases in morphine dependence.

Cyclin-dependent kinases, like Cdk12, typically require an association with a specific cyclin subunit. Recently, it has been shown that Cdk12 most closely associates with cyclin K (Kohoutek & Blazek, 2012). The cyclinK/Cdk12 complex is reported to be important for maintaining genomic stability for cell survival (Blazek, 2012; Blazek et al., 2011). Cyclin K is also a transcriptional target of p53, a tumor suppressor protein (Mori et al., 2002). Mori et al. (2002) showed that cyclin K may begin to act on cell survival after being targeted for transcription, and activated, by p53. Interestingly, p53 is involved in the development of antinociceptive tolerance to morphine, as chronic exposure to morphine induces over-expression of p53 (Shoae-Hassani et al., 2011; Tsujikawa et al., 2009) and antinociceptive tolerance to morphine was blunted following administration of a p53 inhibitor (Tsujikawa et al., 2009). Therefore, p53 may be overexpressed in morphine only animals as compared to morphine cage-mates, which would explain their increased antinociceptive tolerance to morphine, and their increased levels of Cdk12. Future studies would need to examine think link further, as well as levels of p53 tumor suppressor protein in these groups.

8.4.4Gnl3/Nucleostemin

Guanine nucleotide-binding protein-like 3, also known as nucleostemin, is a recently identified nucleolar protein that regulates cell cycle progression and has been implicated in modulation of p53 (Tsai & McKay, 2002). Overexpression of Gnl3 acts

on murine double minute (MDM2) to prevent degradation of p53 (Dai, Sun, & Lu, 2008; H. Ma & Pederson). While Gnl3 has yet to be linked to morphine-induced apoptosis, my results suggest that it may play a role in the increased dependence and reward that has been observed in morphine only animals. I found that mRNA expression of Gnl3 was significantly higher than all other groups, which provides further evidence for a role of regulators of the cell cycle in the differences produced by social environment. Because like Cdk12 it is involved in p53 signaling, this finding also suggests that there are differences in p53-induced apoptosis in the striatum among groups. Future experiments should examine this, as well as study levels of p53 activity in the striatum.

8.4.5 Forkhead Box J2

FoxJ2 is a member of the fork head family of transcriptional activators; the expression of which starts very in embryonic development and is distributed widely in the adult (Granadino et al., 2000). I showed that FoxJ2 was upregulated in morphine only animals compared to morphine cage-mates. Recently, it was shown that overexpression of FoxJ2 in utero is lethal in some mice, and leads to hypertrophy of the heart in the few surviving adults (Martin-de-Lara et al., 2008). Martin-de-Lara et al. (2008) also showed that FoxJ2 may be involved in the transcription of Cadherin 1, as FoxJ2 overexpression produced a phenotype similar to overexpression of this gene. Moreover, FoxJ2 binds to promoter regions encoding for Cadherin1 and activates their transcription.

Interestingly, cadherins have been shown to be differentially expressed based on substance history. Higher levels of cadherin mRNA were observed in the hippocampus of mice with established oxycodone self-administration compared to those of yoked-saline controls (Zhang et al., 2015). Similarly, levels of cadherins and protocadherins in the nucleus accumbens were increased following cocaine exposure and during cocaine withdrawal (Eipper-Mains et al., 2013). Therefore, cadherins may be involved in the acquisition and maintenance of drug self-administration behavior and dependence. While these findings do not directly implicate FoxJ2, the fact that FoxJ2 is involved in their signaling pathways suggests that it may be involved in drug-related behaviors.

8.4.6 Phosphodiesterase 12

Phosphodiesterase 12 (Pde12) breaks phosphodiester bonds, as is typical of the phosphodiesterase family. Also, like DDHD1, Pde12 is associated with the mitochondria, where it acts to regulate translation of RNA to protein (Rorbach, Nicholls, & Minczuk, 2011). Recently, Pde12 was identified as a negative regulator of 2'5' oligoadenylate (OA) (Wood et al., 2015), which is activated by interferons, induces RNAse L, and binds to and degrades viruses upon infection (Silverman, 2007; Sim et al., 2016). Interferons are a group of proteins that are released upon sensing a pathogen (De Andrea et al., 2002). Relative to the present work, interferons are decreased in opioid addicts and individuals who use them chronically. Chronic use of opioids often leads to immunosuppression (for review, see (Plein & Rittner, 2017)). Also, I observed a trend for a significant difference between morphine only and saline only animals in expression

of Interleukin-1 receptor-associated kinase-1, which is involved in immune function (Deng et al., 2003). Moreover, morphine decreases RNAse L enzyme activity, an effect that was blocked with agonism of 2'5' OA (Homan et al., 2002).

In a series of experiments, Dafny and colleagues showed that interferons affect morphine dependence and withdrawal. Animals that were chronically exposed to morphine and given interferon treatment exhibited a dampened withdrawal syndrome compared to those without interferon treatment (Dafny, 1983; Dafny, Zielinksi, & Reyes-Vazquez, 1983; Dougherty et al., 1987), an effect that occurred with multiple classes of interferons (Dafny & Reyes-Vazquez, 1985). Moreover, physical dependence to morphine produces an inhibition in interferon response (Lorenzo et al., 1987).

Phosphodiesterases have also been implicated in morphine reward. Phosphodiesterase 10A (Pde10A) is highly expressed in medium spiny neurons of the striatum. It was found that administration of a Pde10A inhibitor decreased acquisition, and accelerated extinction, of morphine CPP (Mu et al., 2014). While Mu and colleagues did not directly examine Pde12, these findings suggest that the phosphodiesterase family can influence morphine CPP. In the present experiment, I found that the morphine cage-mates do not exhibit the increased levels of Pde12 observed in morphine only animals. These animals also show decreased acquisition of morphine CPP, and accelerated extinction. Therefore, it is possible that decreasing Pde12 would decrease acquisition of morphine CPP.

Because Pde12 negatively regulates OA, it is possible that increased levels of Pde12 correlate with decreased levels of OA and interferon activity. Therefore, the

increased levels of Pde12 gene expression and decreased immune activity in the morphine only animals would correlate with the more severe withdrawal symptoms that I observe in this group. Conversely, because morphine cage-mates do not exhibit the morphine-induced increase in Pde12 expression observed in morphine only animals, then interferon activity would be normal and they would display fewer withdrawal symptoms than the morphine only animals, which they do. It would be interesting to examine immune function among the different housing conditions to see if they differ.

I only investigated alterations in mRNA in this study, more work should be done to determine if the mRNAs explored here translate into proteins. Also, I analyzed changes in expression using homogenization of the whole striatum, thus future work should seek to reveal the precise locations of the alteration in these mRNAs, and their associated proteins, in order to develop a clearer picture of the present findings. Morphine has been shown to alter gene expression in the striatum. Here, I show that social housing conditions can affect these alterations, in that, for a variety of genes morphine cage mates show differential regulation as compared to morphine only animals. This study suggests that the gene × environment interactions in altering striatal gene expression likely explain the effects of social environment on the abuse potential of opioids in adolescent mice.

CHAPTER IX

GENERAL DISCUSSION AND CONCLUSIONS

The studies in the present dissertation provide evidence for a role of social environment on morphine sensitivity and morphine-induced behaviors in adolescent mice. The role of social environment in human drug abuse is well-known. Therefore, I sought to examine this in a rodent model. Because social environment is influential during adolescence, the experiments described here focus on this age group. Animals were housed in the 'only' condition, or in the 'mixed' condition. In the only condition, all animals in a cage were given repeated injections of the same treatment. However, in the mixed condition, half of the animals in a cage were given repeated injections of saline, and half of them were given repeated injections of morphine.

The goal of the first three experiments was to further investigate the role of social housing on morphine-induced behaviors. Previously, it was observed that social environment affected sensitization to morphine-induced locomotor activation, as well as sensitization to morphine reward (measured by morphine CPP) (Cole et al., 2013; Hodgson et al., 2010). More specifically, there was a protective effect of being housed with drug-naïve animals during morphine exposure in adolescent mice. In order to extend these observations, I examined whether or not social environment would affect the extinction of morphine CPP, spontaneous withdrawal symptoms following morphine cessation, and morphine antinociception.

Given that social environment sensitized morphine only, but not morphine cage-mate, animals to more readily acquire morphine CPP, I sought to examine, using a classical CPP procedure, whether social housing conditions would alter the acquisition and extinction of morphine CPP. In this experiment, mice housed in the morphine only condition acquired CPP faster as well as took significantly longer to extinguish morphine CPP than those housed as morphine cage-mates. While it took cage-mates longer to acquire CPP, they did eventually acquire it. However, they extinguish CPP readily using an extinction protocol of 30-minute extinction sessions. In striking contrast, the morphine only animals failed to extinguish morphine CPP using this extinction protocol. Therefore, the memory of morphine reward appears to be less robust in morphine cage-mates than in morphine only animals. Because length of time to extinguish morphine CPP relates to increased drug reward (X. Ma et al., 2012), these data suggest that morphine only mice find morphine more rewarding than the morphine cage-mates. Also, these findings suggest that neural alterations involved in morphine memory may be more drastic in morphine only animals. Thus, the findings in this experiment show that being housed with drug-naïve animals attenuates the development of morphine reward in adolescent mice.

The observation that social environment modulates morphine reward is exciting and quite relevant to human addiction. In addition, many addicts report that their continued use of opioids is not due to its rewarding properties, which have decreased, but rather to stave off withdrawal symptoms (Volkow et al., 2010). Therefore, in the next experiment, I examined the expression of somatic withdrawal symptoms in

adolescent mice. Animals that received morphine while housed with drug-naïve animals displayed significantly fewer withdrawal symptoms than those that were housed in the morphine only condition. Because withdrawal symptoms correlate with the degree of morphine dependence, this finding indicates that morphine cage-mates develop less physical dependence on morphine than morphine only animals (Schulz & Herz, 1977). These results corroborate previous findings that morphine's effects are more robust in morphine only animals than in morphine cage-mates. Because morphine reward and dependence are very relevant to human addiction, the results of these experiments suggest that, in adolescent mice, social environment may mitigate the development of addiction.

Opioids are the most commonly used substances for pain. Reward and dependence are the focus of this dissertation, but it was also important to examine if social housing conditions in the present paradigm affected morphine antinociceptive processes. Moreover, the development of paradoxical pain symptoms, like tolerance and hyperalgesia, is related to morphine dependence (Ueda & Ueda, 2009). There is evidence that implies that morphine analgesic tolerance and physical dependence may share some similar mechanisms (D. Y. Liang et al., 2007). Additionally, animals that show tolerance to morphine analgesia also show increased jumping behavior, which is indicative of dependence and relevant to this dissertation (D. Y. Liang et al., 2006). Therefore, I studied if social environment affects morphine-induced analgesic tolerance and hyperalgesia.

In experiment 3, I used four different measures of morphine analgesia: tail withdrawal, sensitivity to mechanical and cold allodynia, and thermal nociception, as measured by the hot plate test. I found that social environment did mediate the alteration of nociceptive processes following repeated morphine exposure. Morphine only mice, the mice that were more vulnerable to morphine reward and physical dependence, developed antinociceptive tolerance that was more robust than the tolerance that was developed by morphine cage-mates. Similarly, these animals were the only group to develop opioid-induced hyperalgesia. Therefore, the results of this study indicate that social environment not only affects the neurobiological processes underlying morphine reward and dependence, but morphine antinociception as well. This provides more support for previous findings, and also suggests that the effects of social environment are diverse.

My findings are in line with previous studies that demonstrated the impact of social factors on nociceptive processes (for review see (Morales-Rivera et al., 2014)). Social play deprivation and peer-rejection during adolescence were demonstrated to modulate pain reactivity in rodents (E. H. Schneider, Neumann, & Seifert, 2014). Moreover, opioid antinociception is affected by social stress (Huhman et al., 1991; Miczek et al., 1982; Rodgers & Hendrie, 1983). Lastly, the observation of a cage-mate in pain or the observation of a cage-mate who does not experience pain increases or reduces, respectively, the responses to pain in their pain-inflicted cage-mates (Langford et al., 2006). This suggests that social environment can have profound effects on neurobiological processes underlying pain mechanisms.

The previous studies offer evidence that supports the notion that social environment affects morphine reward, dependence, and antinociception. Specifically, they showed that being housed with drug-naïve animals during morphine exposure provides protection from the effects of morphine. However, all of the previous experiments focus on behavior, with little exploration of underlying mechanisms. The only differential experimental manipulation between morphine only animals and morphine cage-mates is the conditions in which they were housed. Thus, the findings suggest that the differences arise due to an interaction between social environment and opioid treatment. It is possible that mechanisms that mediate social interaction are involved in the results seen here. Therefore, the next set of studies sought to determine those neurobiological processes. In experiment 4, I focus on an affiliative behavior in rodents, social grooming, and its underlying mechanisms, while in experiment 5, I focus on one of the most commonly implicated peptides in social interaction, vasopressin. Lastly, in experiment 6, I conduct a general exploration of possible genetic mechanisms using high-throughput, next-generation genomic sequencing and qPCR.

I was interested in deciphering possible behavioral mechanisms by which the protective effect that I observed might be conferred to morphine cage-mates. Observational data in the laboratory suggests that differential patterns of social interaction between animals housed in the morphine only condition, and those housed as morphine cage-mates might be responsible. One form of affiliative social behavior in the rodent is social grooming. Recently, a novel neuronal subtype involved in social grooming, MRGPRB4+ neurons, was discovered (Vrontou et al., 2013). In this study,

Vrontou et al. (2013) developed a DREADD model to explore the behavioral effects of manipulating MRGPRB+ neuronal activity. They were able to show that activation of these neurons produced reward in juvenile mice. Therefore, using DREADD technology, I manipulated these neurons and the sensation of social grooming in morphine-treated animals in the different housing conditions. More specifically, I activated the sensation of grooming in morphine only animals and inhibited it in morphine cage-mates.

I found that inhibition of the sensation of social grooming in morphine cage-mates increased morphine dependence. These results suggest that reducing the sensation of social grooming by decreasing activity at MRGPRB4+ neurons has the ability to affect the previously observed social housing effect. Moreover, there was a trend that did not reach significance in these animals for increased time to extinguish morphine CPP. Conversely, activating MRGPRB4+ neurons did not affect morphine dependence, but it did decrease acquisition of reward in morphine only animals. However, it did not affect extinction of CPP. This may be due to over activation of these neurons, which could have induced excessive stimulation to these animals that was similar to social crowding, a form of social stress (Beery & Kaufer, 2015).

There are other possible behavioral mechanisms that may confer the observed protective effect to the morphine cage-mates. One such mechanism may be social learning. While social learning has not been studied in opioids, there are extensive data that show that it is relevant to the acquisition of drug-taking behaviors. The effect of peer drug use on the social transmission of ethanol intake has been explored using the

"observer-demonstrator procedure" (Hunt & Hallmark, 2001; Hunt, Lant, & Carroll, 2000). In these studies, a demonstrator rat is administered ethanol, and observed by a cage-mate, and then this cage-mate is examined for ethanol consumption 30 minutes later. Both preweanling (Postnatal day 8-12) (Hunt et al., 2000) and periadolescent (Hunt et al., 2001) observer rats that were exposed to an intoxicated peer voluntarily consumed more ethanol than did rats that were exposed to sober peers. Moreover, the observer rats only increased their ingestion of ethanol, not a control solution, which suggests that only ethanol preference was transferred, and not a preference for general consumption. Therefore, the effects of morphine may have been compounded in morphine only animals because of their interaction with only other morphine-dependent animals. However, the morphine cage-mates would be spared from this because of their interaction with only one other morphine-dependent animal, as well as their interactions with drug-naïve animals. One way to examine this would be by preventing the ability of the animals to interact, or to prevent them from being able to transmit information by inducing anosmia (i.e., blocking their sense of smell). Indeed, preference for nicotine has been shown to be socially transmitted through carbon disulfide, a compound present in the exhaled breath of rodents (T. Wang & Chen, 2014). Therefore, examining the social transmission of opioid preference may be a worthwhile venture in the future.

The reasoning behind considering social grooming was very sound because adolescence is the period in development when social interaction is its most salient. Moreover, in rodents, social play behaviors occur more frequently than any other developmental stage (Vanderschuren, Spruijt, et al., 1995). Also, in adolescent rats,

opioids seem to decrease social play (Panksepp et al., 1985). Morphine decreases social investigation in mice (Kennedy et al., 2011; Landauer & Balster, 1982), and opioid withdrawal increases social aggression (Kantak & Miczek, 1986, 1988). Therefore, it may be that there are decreased play behaviors in morphine only cages than in mixed cages. In continuation of the above reasoning, in this next experiment I sought to understand the role of vasopressin in the social housing effect because of its importance in social recognition and social play behavior (Veenema, Bredewold, & De Vries, 2013). In agreement with previous literature, I found that blockade of AVP activity at the V1b receptor decreased morphine CPP acquisition in morphine only animals, while having no effects in morphine cage-mates. Consistent with these results, AVP gene expression in the striatum was significantly higher in morphine only animals as compared to all other groups, which suggests a role for AVP in the effect that social environment has on drug reward and dependence.

In the final experiment, I examined gene expression in the striatum among all of the experimental groups and observed that morphine has markedly larger effects on alteration in striatal gene expression in morphine only animals compared to all other experimental groups, including morphine cage-mates. This confirmed my hypothesis that social environment interacts with the ability of morphine to regulate genetic mechanisms. The genes that were identified had various functions, including activity as signaling mechanisms, transcription factors, and enzymatic activity.

The goal of these experiments was to identify mechanisms that may have been involved in the observed interaction between social environment and morphine treatment

as related to the addictive properties and pain-relieving effects of opioids. In experiment 6 I identified, and confirmed with qPCR, multiple gene targets that were significantly increased in the morphine only animals. Two of these genes, Cdk12 and Gnl3, interact with the tumor suppressor gene, p53. There is emerging evidence that suggest that p53 might mediate some of the effects of morphine in morphine-dependent animals.

Morphine dependence has been shown to produce apoptosis in many brain regions, including the striatum and other parts of the reward circuitry (Katebi et al., 2013). Previous data suggest that p53 is elevated during apoptotic states, and its activity may signal apoptosis, as levels of p53 were increased during NMDA-mediated apoptosis in striatal medium spiny neurons (Qin et al., 1999). Therefore, p53 might produce apoptosis in morphine-dependent animals. CyclinK/Cdk12 complex and Gnl3 are involved in p53 cell signaling to produce apoptosis. Because levels of these genes are lower in morphine cage-mates than morphine only animals, this may correlate with my observation that morphine dependence and reward are decreased in morphine cage-mates. Nevertheless, more work needs to be done in order to determine whether or not levels of p53 activity, and apoptosis in striatal neurons, differ between these groups.

Interestingly, p53 expression has been shown to be altered by social enrichment. Typically, prenatal alcohol exposure leads to an increase in p53 expression in the striatum of adolescent rats. However, adolescent animals exposed to social enrichment displayed decreases in levels of striatal p53 (Ignacio, Mooney, & Middleton, 2014). While morphine and alcohol are different compounds that act at different receptors, they share the ability to alter function of dopaminergic neurons in the striatum to produce

reward. Moreover, both morphine and ethanol induce p53 activity. Therefore, Ignacio et al. (2014)'s findings with ethanol may relate to the present results with morphine, and suggest that social environment can attenuate the expression of signaling molecules that mediate the effects of drug exposure.

In experiment 6, I also measured levels of dopamine D1 receptor (D1R). D1R is often implicated in drug reward, and the association of drug cues with a drug, including the acquisition of CPP. Moreover, excess dopamine activity, via D1R, was previously shown to induce apoptosis in juvenile mice (J. Chen et al., 2009; Iwatsubo et al., 2007), indicating a possible relationship between apoptotic processes and D1R levels. Similarly, methamphetamine, a potent agonist of dopaminergic activity, was shown to increase p53 activity in the striatum (Asanuma et al., 2002). Nevertheless, a relationship between morphine exposure, p53, and D1R has not been explored, but is a potential explanation for the effects seen here.

There is also a relationship between dopamine activity and AVP in the striatum. AVP has been shown to affect dopamine concentration, as antivasopressin serum, which lowers AVP levels, decreases dopamine turnover in the striatum (Tanaka et al., 1977). Moreover, similar to effects seen with morphine, intra-striatum administration of AVP increases dopamine turnover there, suggesting that AVP affects dopamine release in the striatum (Sugrue, 1974; van Heuven-Nolsen & Versteeg, 1985). It is known that chronic administration of morphine leads to increases in dopamine release in the striatum (Bosse & Kuschinsky, 1976) – AVP may be involved in this increase. Also, low levels of AVP enhanced the dopamine-induced activation of adenylate cyclase in the striatum

(Courtney & Raskind, 1983). Activity of adenylate cyclase has been shown to be increased in morphine-dependent states (Shijun et al., 2009), so it is possible that the increased AVP in the striatum is also associated with the augmentation of adenylate cyclase activity seen in morphine-dependent animals.

If the increased level of AVP in the striatum is related to increased dopamine release there, then this may be a potential mechanism by which social housing affects morphine CPP. The mesolimbic circuitry is involved in drug reward. It may be the case that there is decreased dopaminergic activity in the striatum in morphine cage-mates. Indeed, I found decreased levels of D1R in the striatum of morphine cage-mates. Future studies should explore whether or not dopamine activity differs between morphine only animals and morphine cage-mates, as well as whether or not levels of other dopamine receptors differ between these groups.

While I was thorough, there are many other candidates that were not examined here. One such candidate would be oxytocin. Oxytocin induces profound prosocial effects in both humans and non-human animals, including increases in the salience of social stimuli, as well as increases in the time spent interacting with conspecifics in a social interaction test (Domes et al., 2007; Lukas et al., 2011). Conversely, oxytocin and oxytocin-receptor (OT-R) knockout mice show deficits in social recognition (Choleris et al., 2006; Ferguson et al., 2000; Takayanagi et al., 2005). Moreover, it has been shown to be modulate opioid-induced reward and dependence (Brown et al., 2005; Kovacs, Borthaiser, et al., 1985; Kovacs et al., 1987). Also, as mentioned in the Discussion for experiment 4, oxytocin is activated during social grooming, and may be a downstream

mechanism by which grooming exerts its rewarding properties. Nevertheless, I was not able to report any differences in oxytocin activity here. There were technical difficulties that lead to low amplification of oxytocin in the qPCR. Moreover, while I was able to measure oxytocin receptor gene expression, I found no differences among the groups. This could be due to the fact that oxytocin receptor expression is not very high in the striatum (Ostrowski, 1998). Nonetheless, future studies should measure oxytocin levels in the striatum, or other parts of the brain, including hypothalamus and amygdala.

It is also interesting to note that here I identify genes in which expression levels differ between saline only and saline cage-mate animals. Previous studies show that saline cage-mates develop "sensitization" to morphine, as compared to saline only animals (Hodgson et al., 2010). Moreover, saline cage-mates acquired morphine CPP after a single conditioning session with 40 mg/kg dose, but not saline only animals (Cole et al., 2013). Moreover, saline cage-mates demonstrated altered testosterone plasma levels similar to morphine-treated animals that were not observed in saline only animals (Hofford et al., 2011). This data indicates that the association with morphine-treated animals increases the abuse liability of opioids. Thus, the identification of genes, in the present dissertation, that are altered in saline cage-mates animals might explain the increased abuse liability in this group. However, these effects were not further studied here and should be explored in-depth in future studies.

The present experiments added to a body of work that characterized morphine-induced behavior in adolescent rodents housed in different social conditions. Therefore, these studies provided further support for the effect of peer influences on morphine-

related responses. Moreover, the experiments explored potential mechanisms for these peer influences, and identified possible avenues for further study on the effects of social environment on the development of drug abuse in adolescents. Unfortunately, treatments for adolescent drug addiction are lacking. Opioid use remains a major issue, and the present data may present avenues for potential development of new treatments for adolescent opioid addicts.

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