

SOCIAL ENVIRONMENT MODULATES MORPHINE SENSITIVITY: A PARTIAL  
ROLE OF VASOPRESSIN V1B RECEPTOR

A Dissertation

by

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Submitted to the Office of Graduate Studies of  
Texas A&M University  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2012

Major Subject: Psychology

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## ABSTRACT

Social factors influence drug abuse in adolescents; this is partially attributed to peer pressure in humans. Similarly, using rodent models, some research suggests that social housing condition can influence rodents' drug taking behavior. Despite this, few studies have examined the role that intoxicated peers have on drug-naïve cage-mates. This dissertation examined how social environment affects opioid sensitivity and hormone production. This was accomplished by comparing the opioid sensitivity of mice housed in mixed cages (some animals received opioids and some were drug-naïve) to cages where all the mice were treated with the same drug (all saline or all morphine). These studies identified an adolescent-specific vulnerability to social environment-induced alteration of morphine sensitivity. Interaction with drug-intoxicated cage-mates enhanced locomotor sensitivity in previously drug-naïve males and altered their production of testosterone. Conversely, interaction of morphine experienced mice with drug-naïve cage-mates afforded protection from the rewarding properties of morphine. In other words, morphine-treated mice housed with drug-naïve cage-mates demonstrated attenuated reward compared to morphine-treated mice housed with other morphine-treated mice. In addition, part of the neurobiological basis of the social-environment effect was identified. Antagonism of V1b receptors decreased morphine reward in morphine-treated mice housed only with other morphine-treated mice. These results suggest a role of vasopressin in the peer influence on drug sensitivity observed in adolescents. This body of work further elucidates the role of peer influence on opioid

sensitivity. Future studies should further reveal the role of healthy peer relationships and should aid in combating drug abuse in this at-risk demographic.

## ACKNOWLEDGEMENTS

First, I would like to thank my committee, Drs. Shoshy Eitan, Michelle Hook, Cernel Morgan, and Paul Wellman for your patience and support during these last few months. I want to thank my “science cheerleaders”: Nick Simon, Candi LaSarge, Amanda Gabriele, Karienn Montgomery, Jeannine Tamez, Isabel Manzano, and Denise Puga. You often believed in me more than I believed in myself. To Heather Prentice and Lauren McLean: thanks for always being there. I want to thank the wonderful researchers I have had the pleasure to work with and train: Shawn Bates, Shannon Cole, Michael Emery, Katy Seloff, and Kelsey Smith in particular. I have the utmost faith that you will be nothing less than fantastic scientists. To those of you continuing to work with Shoshy, I leave the lab to you with confidence. Stephen Hodgson, thank you for setting the precedent of hard work in the lab; I think you’re amazing. To John Pekar: thank you for opening my eyes to reality outside of neuroscience. Stay free my friend.

A very big thank you goes to Shoshy. You have been extremely patient with me despite my many mistakes. You should take solace in the fact that you have taken an undergraduate psychology major with no mouse handling or molecular experience and turned her into a PhD candidate with 12 publications. Thank you for taking a chance on me; you have left me with more than just lab experience, you have instilled in me a passion for this science. Finally, thank you to my mom, dad, and Andrew. You taught me that nothing good comes for free. I am so grateful to know all of you; you make my life infinitely better. Thank you.

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## CHAPTER I

### INTRODUCTION

Drug addiction has been a problem for societies throughout the ages and will probably continue to plague people for centuries. Addiction to prescription opioids is increasing faster than addiction to other classes of drugs in certain populations (SAMHSA, 2009). This trend is especially prevalent in adolescents; they are at a higher risk of developing addiction later in life if drug use starts at this age (Grant and Dawson, 1998). While the exact cause of this age difference in vulnerability to drug addiction has not been elucidated, many theories have been proposed.

Humans and rodents are undergoing changes in brain structure during adolescence. Continued brain development at this time includes increases in myelination (Paus et al., 1999, De Bellis et al., 2001, Juraska and Markham, 2004), increases in axonal diameter (Perrin et al., 2008), and synaptic pruning (Huttenlocher and Dabholkar, 1997, Zehr et al., 2006). While total gray matter volume consistently decreases throughout the lifespan, the change during adulthood is gradual (Pfefferbaum et al., 1994). Adolescent brains are undergoing more drastic changes in both gray and white matter volume compared to adults. Brain areas undergoing the greatest changes in volume include the amygdala (Giedd et al., 1997, Koshibu et al., 2004, Zehr et al., 2006), the striatum (Teicher et al., 1995, Andersen et al., 2001, Haycock et al., 2003), and prefrontal cortex (Kalsbeek et al., 1988, Benes et al., 1996). Brain areas undergoing development are subject to toxic assault by drugs of abuse that can result in

morphological abnormalities in extreme cases (Lebel et al., 2011) and subtle behavioral changes in minor cases (Hofford et al., 2012). Interestingly, all these brain areas are involved in the development and maintenance of drug abuse and in social interaction (de Bruin, 1990, Kalivas and Duffy, 1990, Volkow et al., 1997, Hellemans et al., 2006, Harris and Aston-Jones, 2007, Volkow et al., 2007, Li et al., 2008, Arakawa et al., 2010), suggesting that addictive drug use during this time might selectively damage the brain areas involved in both drug abuse and social behavior.

Another factor likely to affect adolescents' sensitivity to drugs is levels of perceived stress. Adolescents often report more stress in their lives and they report different situations as stressful compared to adults (as reviewed in (Spear, 2000)). The links between stress and increased rates of drug abuse are well established (Sinha, 2001). This includes an increased risk of initiating drug use, continuing drug use, and an increased risk of relapse after a drug free period. Given stress's ability to potentiate the switch to compulsive drug seeking (Kreek and Koob, 1998), it is likely to contribute to the high rates of adolescent drug users that eventually become addicts.

Adolescents are also going through puberty at this time. Puberty is marked by increases in gonadal hormones and, consequently, development of secondary sexual characteristics. Increased release of gonadal hormones, testosterone in males and estrogen and progesterone in females, often initiate sex-specific behaviors (Olioff and Stewart, 1978, Meaney and Stewart, 1981) and are crucial for normal sexual behavior in adulthood (Schulz et al., 2009). These hormonal changes affect many facets of sex-

specific behavior and aid in the sexual differentiation of the brain (Perrin et al., 2008, Yates and Juraska, 2008).

Some of these sex-specific behaviors directly involve testosterone (Meaney and Stewart, 1981). Testosterone is not only crucial for the development of appropriate sexual behaviors in adulthood (Schulz et al., 2009), it also contributes to the development of healthy social behavior in male adolescents. During this time period, testosterone directly modulates levels of rough-and-tumble play in males. Castrated rats demonstrated play behavior more similar to females, but this behavior can be restored with testosterone replacement (Meaney and Stewart, 1981). In addition, testosterone affects social hierarchy status (Rose et al., 1971, Ehrenkranz et al., 1974). Disruptions of this hierarchy can affect levels of stress and potentiate drug abuse (Brady and Sinha, 2005).

Another hallmark feature of adolescence is an increased interaction with peers. Adolescence represents a time when individuals transition from spending their spare time with parents to exploring the world and spending more time with people their own age. This likely contributes to adolescents' vulnerability to substance abuse; many studies suggest that peer influence is a major factor in adolescent decision-making (Graham et al., 1991). Thus, if teenagers are surrounded by socially-deviant friends (some of whom may engage in drug use), it is more likely they will use drugs themselves (Reynolds et al., 2007, Kirillova et al., 2008).

Given the many factors that contribute to human adolescent drug abuse, rodent models of peer influence on drug sensitivity and reward could be extremely useful.

Studies in these species could more adequately control these various factors. Increased interest in peers is characteristic of human teenagers, but rodents also demonstrate increased interaction with their peers at this time. Rats and mice increase their amount of play fighting and social exploration at this time of their life (Primus and Kellogg, 1989). Adolescent rats also find social interaction more rewarding than adult rats (Douglas et al., 2004). This suggests that studying social behavior in rodents could help elucidate its role in the development of human drug abuse.

However, care should still be given when extrapolating data from rodents to humans, because even rodents differ in their response to social situations. For instance, rats are generally more social than mice, while hamsters are considered more aggressive than either rats or mice (Payne and Swanson, 1971). Even different species of voles differ in their social behavior; prairie voles are monogamous while the closely related mountain vole is not (Murie, 1971).

A few studies have been conducted on social interaction and reward in rodents. It has been shown that general social environment can influence rodents' drug reward (Gipson et al., 2011b), but most studies have focused on differences in behavior between animals housed individually compared to group-housed animals (Katz and Steinberg, 1970, Raz and Berger, 2010, Lopez et al., 2011). Rodents housed individually self-administer more drugs compared to animals housed in groups (Raz and Berger, 2010, Lopez et al., 2011). This effect could be due to increased levels of stress displayed by rodents housed in isolation (Ros-Simó and Valverde, 2012) or this could reflect some unique consequence of social housing.

Other studies that alter social environment focus on the difference in drug responsiveness after housing in an enriched environment or a standard environment. An enriched environment is one that enhances rodents' social, sensory, and/or motor experiences (Gelfo et al., 2011, Mustroph et al., 2012) and often does not distinguish the importance of one kind of stimulus over another (social compared to sensory or motor). Environmental enrichment benefits mental health in a variety of ways using both behavioral and cellular measures including increased hippocampal neurogenesis (Mustroph et al., 2012), decreased depressive like behaviors (Zhang et al., 2011), increased motor function after injury (Gelfo et al., 2011), and protection against neuropathology (Young et al., 1999, Lazarov et al., 2005, Anastasia et al., 2009). Additionally, there is evidence that environment enrichment decreases drug responding; animals housed in an enriched environment take much longer to develop consistent self-administration behavior (Gipson et al., 2011a).

Some studies have also shown that the quality of social interactions can affect abuse treatment outcomes. In humans, the presence of an emotional support system is predictive of better outcomes in substance abuse treatment (Tracy et al., 2005). Specifically, the characteristics of the emotional support were most important. If a patient was involved with a drug user or did not find a relationship fulfilling, treatment outcome was poor (Tracy et al., 2005). Studies examining relationship quality in humans often rely on self-report. For this reason, it would be advantageous to examine quality of social interaction as a determinant of drug abuse potential in an animal model.

Surprisingly, studies have demonstrated that rodents can distinguish between impoverished and quality social interaction because they have a clear preference for engaging with active, socially experienced peers. One study examined social place preference by pairing one side of an apparatus with a playful rat and the other with a scopolamine-induced paralyzed rat and found that rats preferred the chamber previously paired with a playful peer (Calcagnetti and Schechter, 1992). Another study also employed the use of a social place preference to examine age differences in social reward. This experiment examined preferences in group-housed and individually-housed rats for socially experienced or previously isolated rats. Adolescents housed in groups or individually preferred the rat-paired chamber but only if the peer had previous social experience (i.e. peer had been socially housed). Only isolated adults preferred the rat-paired chamber and to a lesser extent than adolescents (Douglas et al., 2004). According to this study, adolescents found more social situations rewarding than adults and adolescent rodents were able to distinguish between rats that had social experience and those that did not.

The efforts to elucidate the role of peers in rodent drug sensitivity have provided valuable information but the studies mentioned above mostly focus on the simple presence or absence of peers on drug sensitivity or manipulate too many factors of the rodents' environment. Only a few studies have specifically manipulated drug experience of cage-mates and observed subsequent drug preference in observers. These studies determined that alcohol-naïve adolescent rats exposed to an intoxicated peer increased their alcohol intake (Hunt et al., 2001, Fernández-Vidal and Molina, 2004). These

studies were unique because they were the first to demonstrate direct peer influence on drug taking in a rodent model.

This body of work sought to elucidate the role of different social housing environments on testosterone production and opioid response using locomotor sensitivity and conditioned place preference (CPP) in a rodent model. These studies focused on the sensitive period of adolescence. This is a time of life when both humans and rodents are directing more behavior toward their peers. The presence of peers has been shown to alter drug sensitivity in general, but the nature of those cage-mates' interactions and how previous drug treatment affects morphine sensitivity was examined specifically.



## CHAPTER II

### GENERAL METHODS

#### *1. Subjects*

Adolescent (PND 21-59) or adult (PND 60-80) C57BL/6 male or female mice (Harlan Houston) were used for the experiments. The age of the adolescent mice was based on studies done by Spear and colleagues and is the accepted time period for most studies conducted on this age group (Spear, 2000). Mice were acclimated to the colony for a minimum of 5 days before the start of any experiment. Mice received food and water *ad libitum* and were housed on a 12:00 hour light/dark cycle with the lights on at 7:00am and off at 7:00pm. All procedures were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by Texas A&M University's Institutional Animal Care and Use Committee.

#### *2. Housing*

All mice were group-housed with age- and sex-matched peers, 4 or 5 per cage, except where specifically noted. Mice were housed in one of 3 types of cages. 'Morphine only' cages contained mice that received repeated morphine injections. 'Saline only' cages contained mice that received repeated saline injections. Mixed cages contained 2 mice that received repeated saline injections, referred to as 'saline cage-

mates', and 2 mice that received repeated morphine injections, referred to as 'morphine cage-mates'.

### *3. Drugs*

Morphine sulfate and sodium pentobarbital were purchased from Sigma (St. Louis, MO, USA) and SSR149415 was purchased from Axon MedChem (Groningen, The Netherlands).

### *4. Morphine Injection Paradigm*

All mice were injected twice a day for 6 days with saline or increasing doses of morphine. Morphine-treated mice received 10 mg/kg morphine on days 1 and 2, 20 mg/kg morphine on days 3 and 4, and 40 mg/kg morphine on days 5 and 6 (s.c.). Behavioral testing or tissue collection occurred on withdrawal day 1 (WD1) or withdrawal day 9 (WD9). Different mice were used for behavioral studies and tissue collection.

## CHAPTER III

### EXPERIMENT 1: SOCIAL ENVIRONMENT EFFECT ON CIRCULATING TESTOSTERONE IN MALE MICE\*

#### *1. Background*

The injection period for all the experiments conducted in adolescents spanned from 28 days to 35 days of age. Most researchers agree this timeline is well within the adolescent period and most likely includes puberty (Becú-Villalobos et al., 1997, Spear, 2000, Shen et al., 2010). The exact onset of puberty in mice is sometimes debated depending on the measure used. Some insist that puberty is complete when animals are reproductively mature; others suggest that puberty occurs at much younger ages and corresponds to significant increases in gonadal hormone release from sex organs. These hormones include estrogen and progesterone in females (Smith and Davidson, 1968) and testosterone in males (Frasier et al., 1969).

Testosterone has been implicated in a variety of sex-specific behaviors. These include play behaviors during adolescence (Meaney and Stewart, 1981) and appropriate mating behavior (Schulz et al., 2009) during adulthood. Some studies suggest a direct role of testosterone in aggression; however the links between the two are weak at best (Ehrenkranz et al., 1974). Nevertheless, testosterone is essential to normal social development of adolescent males. Interestingly, testosterone levels can fluctuate based

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on social environment alone (Blanchard et al., 1993), with more dominant animals in a group having higher testosterone on average (Rose et al., 1971, Ehrenkranz et al., 1974). Disruptions in social hierarchies can lead to stress (Blanchard et al., 1993) which can lead to increased sensitivity to opioids (Brady and Sinha, 2005). In fact, some studies have shown that testosterone levels that are much higher than average (Reynolds et al., 2007) or much lower than average (Dawes et al., 1999) are both risk factors for substance abuse during adolescence. Testosterone seems to have an effect on morphine response in rodents as well. Castrated rats and mice did not develop physical dependence to chronic morphine (Nayebi and Rezazadeh, 2008, Sadeghi et al., 2009).

Testosterone most likely affects morphine response because it is under the control of endogenous opioid compounds and is involved in important feedback loops (Davidson et al., 1973). The normal release of gonadotropin-releasing hormone, the hormone released from the hypothalamus that controls release of follicle-stimulating hormone and luteinizing hormone (Schally, 1970), is under the control of endogenous opioids (Cicero et al., 1979). Therefore, treatment or administration of exogenous opioid compounds can cause temporary or long-lasting dysfunction within the hypothalamic-pituitary-gonadal axis. Adult humans and rodents demonstrate suppressed testosterone production after long-term treatment with opioids (Muraki et al., 1978, Yilmaz et al., 1999, Abs et al., 2000); testosterone levels usually return to normal within a week of opioid discontinuation. The effects of chronic morphine on testosterone production administered before or during puberty have not been established.

Due to its role in the maintenance of social hierarchies and morphine's ability to decrease its production, this experiment measured plasma testosterone levels in male mice housed in various social environments (i.e. 'mixed cages' compared to 'saline only cages').

## *2. Experimental Procedures*

### *Subjects*

Adolescent (PND 28-59) and adult (PND 60-80) male mice were examined for this experiment. In addition to the housing conditions listed in general methods, individually-housed adolescent males were also examined for this experiment. Briefly, group-housed mice were housed in one of two types of cages: saline only or mixed cages as listed above in general methods. Roughly 15 mice per group for a total of 231 mice were used for this experiment.

### *Injection Paradigm*

Injection paradigm was the same as listed above in general methods. Briefly, mice received twice daily injections of saline or increasing doses of morphine (10-40 mg/kg, s.c.) for 6 days.

### *Tissue Collection*

On WD1 or WD9, mice received sodium pentobarbital (100 mg/kg, i.p.). Five minutes later, blood was collected via intra-cardiac puncture and separated by centrifugation at 1,000 x g at 4° C for 15 minutes. Tissue was stored at -80° C.

### *Assessment of Hormone Levels*

Testosterone level was assessed using the Testosterone EIA kit (Cayman Chemical, Ann Arbor, MI) per assay instructions. This kit uses a competitive binding assay and requires addition of a known amount of labeled testosterone to samples with an unknown amount of unlabeled testosterone. Addition of a chemical reagent in the last step causes color change that is inversely proportional to the amount of unlabeled testosterone in the samples. Briefly, this ELISA was run as follows. Blood plasma was diluted 1:5 in EIA buffer (provided by kit) and these diluted samples were run in triplicate. Serial dilutions of a testosterone standard were run parallel to the samples. Final standards had concentrations of 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml, 7.8 pg/ml, and 3.9 pg/ml. These standards were run in duplicate and were used to generate a standard curve. Samples and standards were loaded in a 96-well plate, followed by addition of an AChE Testosterone Tracer (provided by kit) and Testosterone Antiserum (provided by kit). Plate was sealed and allowed to stir on an orbital shaker for 2 hours. Plate was rinsed 5 times with wash buffer (provided by kit) and completely dried. Ellman's reagent (provided by kit) was added to all the wells, sealed, protected from light, and allowed to shake for 1.5 hours. Color change in the

plate was measured by a spectrophotometer and a standard curve was evaluated using the standards.

### *Statistics*

For the group-housed mice, data was analyzed using a 2 (ages) x 2 (withdrawal day) x 3 (treatment groups) between-subjects ANOVA. Additionally, separate 3 (treatment groups) x 2 (withdrawal day) between-subjects ANOVAs were calculated for differences between treatment groups for both ages. For the individually-housed mice, a 2 (treatment) x 2 (withdrawal day) between-subjects ANOVA was conducted. Differences between treatment groups were calculated using Bonferroni's post-hoc comparisons. *P* values less than 0.05 were considered statistically significant.

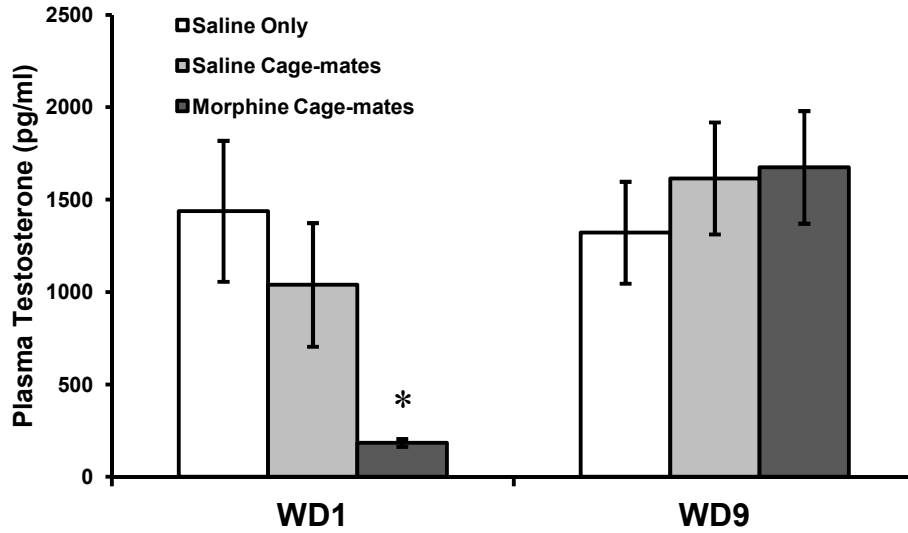
### *3. Results*

Three-way ANOVA with age (adult, adolescent), withdrawal day (WD1, WD9) and treatment group (morphine cage-mates, saline cage-mates, saline only) as factors demonstrated a significant main effect of age  $F(1, 171) = 21.36, p < 0.0001$ , a significant main effect of withdrawal day  $F(1, 171) = 15.94, p < 0.0001$ , a significant main effect of treatment group  $F(2, 171) = 4.95, p < 0.01$ , and a significant interaction between age, withdrawal day, and treatment group  $F(2, 171) = 5.31, p < 0.01$ . No significant interactions were found between age and withdrawal day, age and treatment group, or withdrawal day and treatment group ( $p > 0.05$ )

For the adult mice, a two-way ANOVA with withdrawal day (WD1, WD9) and treatment group (morphine cage-mates, saline cage-mates, saline only) as factors demonstrated a significant main effect of withdrawal day  $F(1, 77) = 7.73, p < 0.01$  and a significant interaction  $F(2, 77) = 3.96, p < 0.05$ , but no significant main effect of treatment group ( $p > 0.05$ ) on testosterone level. Bonferroni's post-hoc analyses revealed significantly lower plasma testosterone levels in the morphine cage-mates compared to the saline only mice (morphine cage-mates < saline only,  $p < 0.01$ ) on WD1 but not on WD9 (morphine cage-mates = saline only,  $p > 0.05$ ). No difference in testosterone level was found between saline cage-mates and saline only mice on either WD1 or WD9 (saline cage-mates = saline only,  $p > 0.05$ ). Results for the adult mice are presented in figure 1.

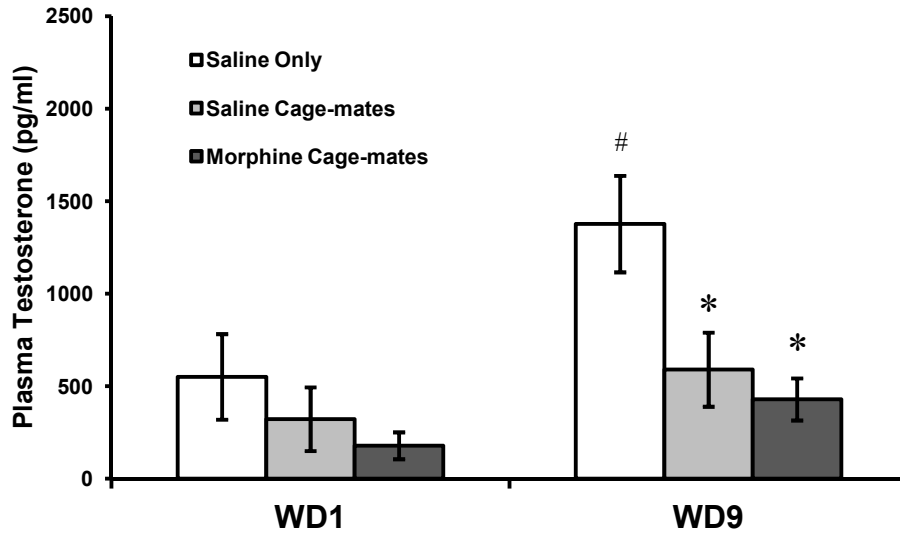
For the adolescent mice, a two-way ANOVA with withdrawal day (WD1, WD9) and treatment group (morphine cage-mates, saline cage-mates, saline only) as factors demonstrated a significant main effect of withdrawal day  $F(1, 84) = 7.90, p < 0.01$  and a significant main effect of treatment group  $F(2, 84) = 6.27, p < 0.01$ , on testosterone level, but no significant interaction ( $p > 0.05$ ). Bonferroni's post-hoc analyses revealed no significant differences in testosterone level between morphine cage-mates and saline only (morphine cage-mates = saline only,  $p > 0.05$ ) or saline cage-mates and saline only (saline cage-mates = saline only,  $p > 0.05$ ) on WD1. A trend for lower testosterone in morphine cage-mates compared to saline only mice was observed but did not reach statistical significance, perhaps due to the low levels of plasma testosterone observed at WD1 in all groups. Levels of testosterone were significantly increased by WD9 in the



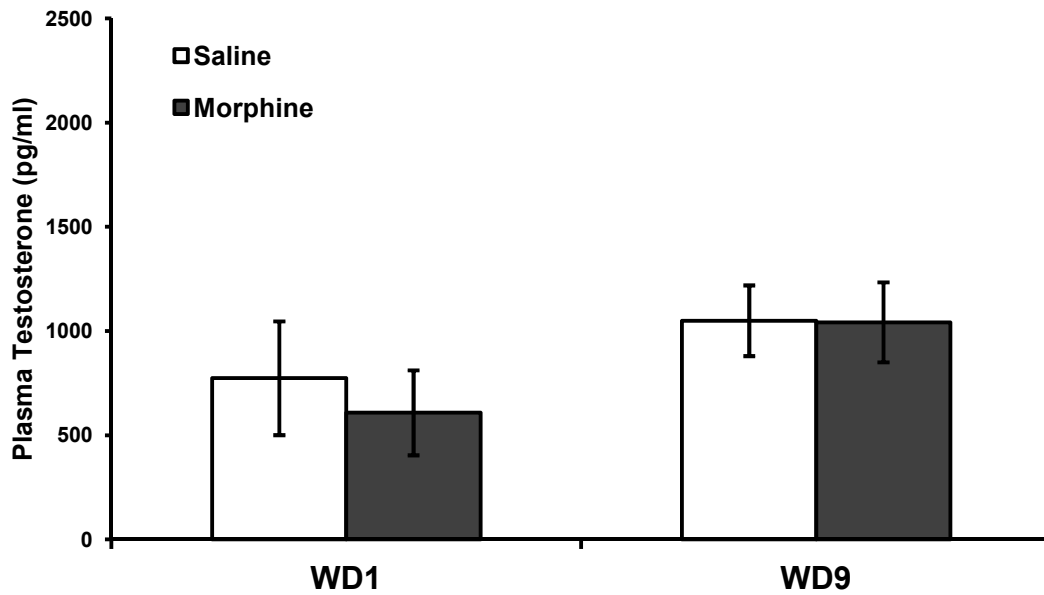


*Figure 1: Testosterone levels of group-housed adults from different social environments.*

Plasma testosterone was collected on WD9. \* Significant difference from saline only WD1 ( $p < 0.01$ ). Saline only WD1,  $n = 12$ ; saline cage-mates WD1,  $n = 14$ ; morphine cage-mates WD1,  $n = 14$ ; saline only WD9,  $n = 11$ ; saline cage-mates WD9,  $n = 16$ ; morphine cage-mates WD9,  $n = 14$ . Results are presented as means  $\pm$  SEM.



*Figure 2:* Testosterone levels of group-housed adolescents housed in different social environments. Plasma testosterone was collected on WD9. \* Significant difference from saline only WD9 ( $p < 0.01$ ); # significant difference from saline only WD1 ( $p < 0.01$ ). Saline only WD1,  $n = 18$ ; saline cage-mates WD1  $n = 12$ ; morphine cage-mates WD1,  $n = 12$ ; saline only WD9,  $n = 16$ ; saline cage-mates WD9  $n = 16$ ; morphine cage-mates  $n = 16$ . Results are presented as means  $\pm$  SEM.



*Figure 3:* Testosterone levels of individually-housed adolescents. Plasma testosterone was collected on WD9. Saline WD1,  $n = 16$ ; morphine WD1,  $n = 16$ ; saline WD9  $n = 28$ ; morphine WD9,  $n = 29$ . Results are presented as means  $\pm$  SEM.

saline only control mice (saline only at WD1 < saline only at WD9,  $p < 0.01$ ). On WD9, significantly lower plasma testosterone levels were found in the morphine cage-mates compared to the saline only mice (morphine cage-mates < saline only,  $p < 0.01$ ) and there were significantly lower plasma testosterone between saline cage-mates compared to saline only mice (saline cage-mates < saline only,  $p < 0.01$ ). Results for the adolescent mice are presented in figure 2.

For the individually-housed adolescent mice, a two-way ANOVA with treatment (morphine, saline) and withdrawal day (WD1, WD9) as factors revealed no main effect of treatment  $F(1, 85) = 2.89, p > 0.05$ , no main effect of withdrawal day  $F(1, 85) = 0.17, p > 0.05$ , and no significant interaction  $F(1, 85) = 0.14, p > 0.05$  on testosterone level. Results for the individually-housed mice are presented in figure 3 (Hofford et al., 2011).

#### *4. Discussion*

The data demonstrate a reduction in testosterone production after repeated injections of morphine in adult mice, but not in adolescents on WD1. A general trend for decreased testosterone production was observed in adolescents. Because the baseline level of testosterone production was so low in adolescents, a difference between treatment groups might exist but still not have reached statistical significance. The reduction in testosterone production observed in adults on WD1 is consistent with previous literature (Yilmaz et al., 1999, Abs et al., 2000). By WD9, testosterone level returned to normal in adult mice. In adolescents, however, testosterone production was significantly lower in morphine cage-mates compared to saline only mice at this time

point. This suggests that there is some developmental dysregulation of the hypothalamic-pituitary-gonadal axis when opioids are administered during adolescence. Additionally, saline cage-mates also produce less testosterone than saline only mice. The only difference between saline cage-mates and saline only mice is their social environment. Interestingly, there was no difference between morphine-treated and saline-treated mice that were housed individually. This suggests that social environment and prior drug treatment interact to affect levels of testosterone. Also, the overall levels of testosterone seem to be lower in individually-housed mice compared to saline only mice housed in groups on WD9. This is consistent with previous literature, as group-housed hamsters have lower testosterone compared to pair-housed hamsters (Lürzel et al., 2011).

The current set of data has several implications. For one, care should be taken when prescribing opioids to treat pain in adolescents. While testosterone level recovered by WD9 in the morphine-treated adults, adolescent testosterone did not. This may signify a delay of sexual maturation for male adolescents (Schulz et al., 2009) given long-term opioid treatment. Secondly, the current data suggests that interaction with drug intoxicated peers can affect hormone production. This could have many adverse consequences including delay of peer interaction. Lower testosterone levels have been linked to later development of depressive-like behaviors in rodents (Wainwright et al., 2011) and increased risk of drug abuse in humans (Dawes et al., 1999). Since testosterone level can predict drug sensitivity and is crucial for the development of healthy social behavior and testosterone production was affected by social housing, the following experiments focus on the role of social environment on drug sensitivity.

## CHAPTER IV

### EXPERIMENT 2: EFFECT OF SOCIAL ENVIRONMENT ON MORPHINE

#### LOCOMOTOR SENSITIVITY IN MALE MICE\*

##### *1. Background*

Locomotor behavior is increased in animals receiving multiple injections of morphine compared to animals receiving the same dose acutely. This is commonly referred to as behavioral sensitization. Morphine sensitization in lab animals has been correlated with increased drug-taking (Deroche-Gamonet et al., 2004). Additionally, both locomotor sensitization (Kalivas and Duffy, 1990) and self-administration of drugs (Wise et al., 1995) cause similar neurochemical changes, specifically increased synaptic dopamine in the striatum. Measuring rodents' locomotor response to repeated morphine injections is one way to study behavioral sensitization. While not a direct measure of drug reward or drug seeking, these tests are much more simple to run and can supply researchers with a quick screen for enhanced sensitivity to a drug's response.

As mentioned previously, alterations of a rodent's environment can result in differential sensitivity to opioids. Rodents that are group-housed normally demonstrate increased sensitivity to opioids compared to rodents that are individually-housed (Katz and Steinberg, 1970, Sudakov et al., 2003, Smith et al., 2005). However, rodents housed in groups acquire heroin self-administration more slowly and intake less heroin over

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time (Alexander et al., 1978, Bozarth et al., 1989, Raz and Berger, 2010). Additionally, rats in enriched environments, which includes the presence of social interaction, acquire self-administration more slowly (Gipson et al., 2011a).

The previous experiment measured testosterone level in male mice housed in mixed cages or in saline only cages and found that adolescents had decreased testosterone production on WD9 when housed in mixed cages regardless of drug treatment (Hofford et al., 2011). Previous studies have shown that testosterone level can predict an individual's risk of drug abuse (Dawes et al., 1999, Reynolds et al., 2007) and plays an important role in social development (Auger and Olesen, 2009, Schulz et al., 2009). Given the results of experiment 1 and testosterone's role in social behavior, this experiment examined the role of different housing environments (i.e. 'mixed cages' compared to 'saline only cages') on both adolescents' and adults' response to the locomotor stimulating properties of morphine.

## *2. Experimental Procedures*

### *Subjects*

Adolescent (PND 28-59) and adult (PND 60-80) male mice were examined for this experiment. Briefly, mice were housed in one of two types of cages: saline only or mixed cages as listed in general methods. Roughly 10-15 mice per group for a total of 107 mice were used for this experiment.

### *Injection Paradigm*

Injection paradigm was the same as listed above. Briefly, mice received twice daily injections of saline or increasing doses of morphine (10-40 mg/kg, s.c.) for 6 days.

### *Behavioral Analysis: Locomotion*

All mice were examined for their locomotor response to a challenge dose of morphine. Nine days after termination of injections (WD9), mice were placed in the testing room for 30 minutes prior to the beginning of the test. Locomotion was measured in cylindrical upright containers (261 mm in diameter and 355 mm high) with white walls. Each mouse was tested separately and four chambers were recorded at a time. After habituation to the room, mice were placed in the locomotor testing apparatus for 60 minutes to measure baseline locomotor behavior. All mice received an injection of 10 mg/kg morphine (s.c.) and were video recorded from above for 60 minutes. Locomotor behavior was assessed using EthoVision 3.1 (Noldus Information Technology, Leesburg, VA). Activity chambers were washed with water and completely dried between mice.

### *Statistics*

Total distance traveled was calculated and analyzed for both baseline locomotion and locomotion post-morphine injection. Additionally, total distance traveled was summed for each 5 minute time interval post-morphine. Data was analyzed using a 2 (treatment) x 3 (treatment groups) between-subjects ANOVA for both ages. A 2 (age) x 3 (treatment groups) between-subjects ANOVA was conducted to determine if an age



difference existed. Separate 3 (treatment groups) x 24 (time) mixed-design ANOVAs were computed for baseline locomotion and locomotion post-morphine for each age. Differences between treatment groups were calculated using Bonferroni's post-hoc comparisons. *P* values less than 0.05 were considered statistically significant.

### 3. Results

For the adult mice, a two-way ANOVA with treatment (baseline, post-morphine injection) and treatment group (morphine cage-mates, saline cage-mates, saline only) as factors demonstrated a significant main effect of treatment  $F(1, 64) = 271.50, p < 0.0001$ , a significant main effect of treatment group  $F(2, 64) = 7.86, p < 0.001$ , and a significant interaction  $F(2, 64) = 8.95, p < 0.001$  on distance traveled. A two-way mixed ANOVA with treatment group (morphine cage-mates, saline cage-mates, saline only) as a between-subjects factor and time (5 minute time bins) as a within-subjects factor demonstrated a significant main effect of treatment group  $F(2, 768) = 2.79, p < 0.001$ , a significant main effect of time  $F(2, 768) = 75.42, p < 0.001$ , and a significant interaction  $F(46, 768) = 4.41, p < 0.001$ . Bonferroni's post-hoc analyses revealed no significant differences between treatment groups during baseline (morphine cage-mates = saline cage-mates = saline only,  $p > 0.05$ ) and all treatment groups had greater locomotion post-morphine compared to baseline ( $p < 0.001$  for all groups). Analyses revealed a significant difference between saline only mice and morphine cage-mates (saline only < morphine cage-mates,  $p < 0.001$ ) post-morphine but no significant difference was found

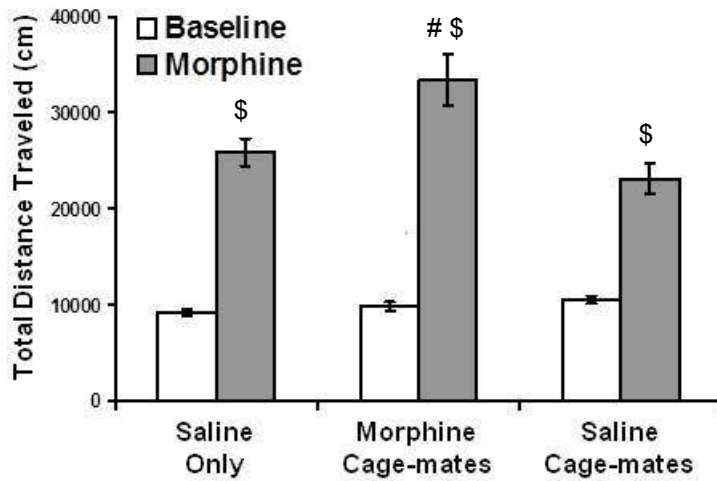


Figure 4: Adult male baseline and post-morphine (10 mg/kg) locomotion totals.

Baseline locomotion is represented by the white columns and is the total locomotion for 60 minutes pre-morphine injection. Morphine locomotion is represented by the gray columns and is the total locomotion for 60 minutes post-morphine injection. <sup>\$</sup> Significant difference from baseline ( $p < 0.001$ ); <sup>#</sup> significant difference from saline only mice ( $p < 0.001$ ). Saline only,  $n = 14$ ; saline cage-mates,  $n = 12$ ; morphine cage-mates,  $n = 8$ .

Results are presented as means  $\pm$  SEM.

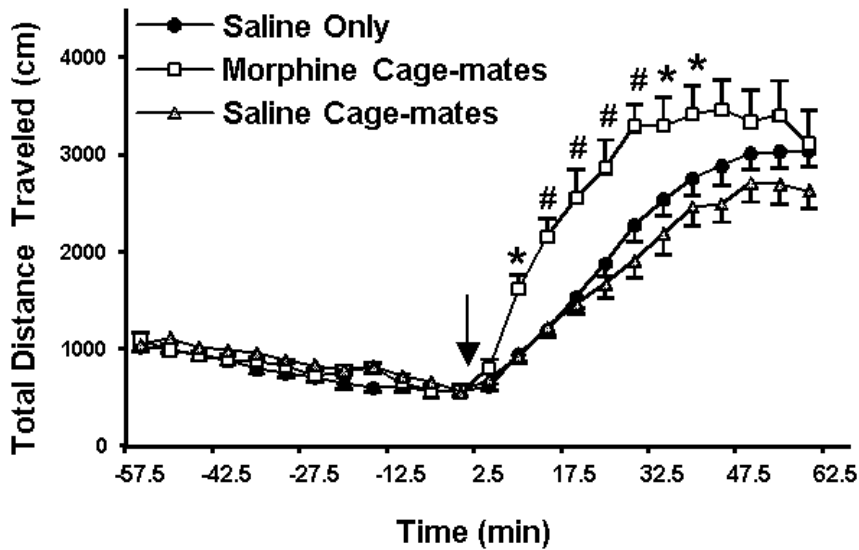
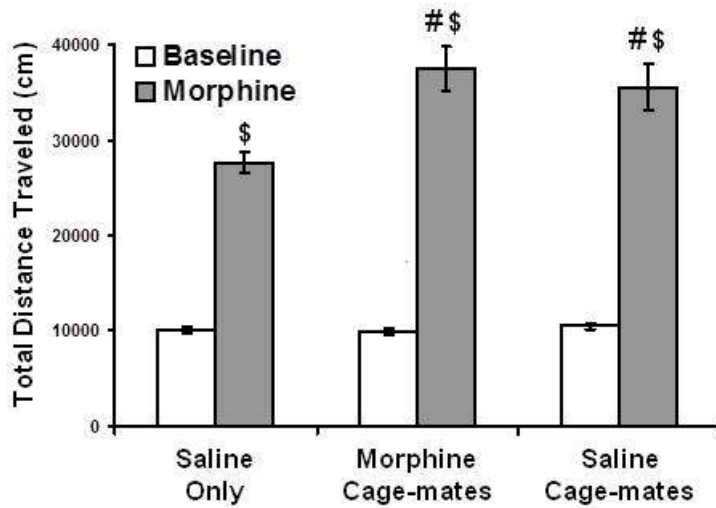


Figure 5. Timeline of adult locomotion before and after 10 mg/kg morphine challenge. Total locomotion was calculated and broken down into 5 minute time bins for each of the three groups. Negative numbers indicate times before morphine injection and the arrow indicates time of injection. \* Significant difference from saline only mice ( $p < 0.05$ ); # significant difference from saline only mice ( $p < 0.001$ ). Saline only,  $n = 14$ ; saline cage-mates,  $n = 12$ ; morphine cage-mates,  $n = 8$ . Results are presented as means  $\pm$  SEM.

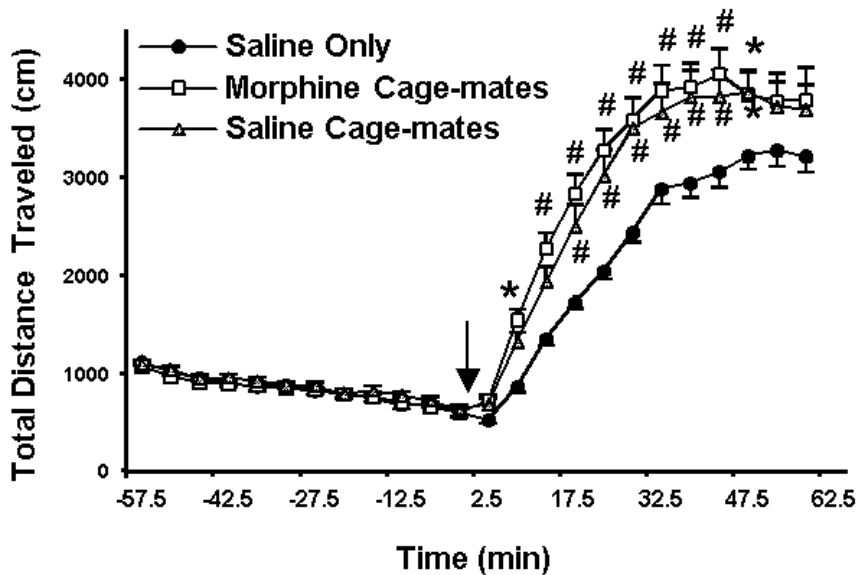
between saline cage-mates and saline only ( $p > 0.05$ ). Results for the adult mice are presented in figures 4 and 5.

For the adolescent mice, a two-way ANOVA with treatment (baseline, post-morphine injection) and treatment group (morphine cage-mates, saline cage-mates, saline only) as factors demonstrated a significant main effect of treatment  $F(1, 138) = 415.00, p < 0.001$ , a significant main effect of treatment group  $F(2, 138) = 7.11, p < 0.001$ , and a significant interaction  $F(2, 138) = 7.03, p < 0.001$ . A two-way mixed ANOVA with treatment group (morphine cage-mates, saline cage-mates, saline only) as a between-subjects factor and time (5 minute time bins) as a within-subjects factor demonstrated a significant main effect of treatment group  $F(2, 1656) = 1.65, p < 0.001$ , a significant main effect of time  $F(23, 1656) = 73.79, p < 0.001$ , and a significant interaction  $F(46, 1656) = 2.09, p < 0.0001$  on distance traveled. Bonferroni's post-hoc analyses revealed no significant differences between treatment groups during baseline (morphine cage-mates = saline cage-mates = saline only,  $p > 0.05$ ) and all treatment groups had greater locomotion post-morphine compared to baseline ( $p < 0.001$  for all groups). Analyses revealed a significant difference between saline only mice and morphine cage-mates (saline only < morphine cage-mates,  $p < 0.001$ ) post-morphine. Unlike the adult mice, a significant difference was found between saline only and saline cage-mates (saline only < saline cage-mates,  $p < 0.001$ ) after morphine injection. In fact, no significant difference in locomotion was found between saline cage-mates and morphine cage-mates (saline cage-mates = morphine cage-mates,  $p > 0.05$ ). Results for the adolescent mice are presented in figures 6 and 7.



*Figure 6:* Adolescent male baseline and post-morphine (10 mg/kg) locomotion totals. Baseline locomotion is represented by the white columns and is the total locomotion for 60 minutes pre-morphine injection. Morphine locomotion is represented by the gray columns and is the total locomotion for 60 minutes post-morphine injection.

<sup>\$</sup> Significant difference from baseline ( $p < 0.001$ ); <sup>#</sup> significant difference from saline only mice ( $p < 0.001$ ). Saline only,  $n = 26$ ; saline cage-mates,  $n = 23$ ; and morphine cage-mates,  $n = 9$ . Results are presented as means  $\pm$  SEM.



*Figure 7:* Timeline of adolescent locomotion before and after 10 mg/kg morphine challenge. Total locomotion was calculated and broken down into 5 minute time bins for each of the three groups. Negative numbers indicate times before morphine injection and the arrow indicates time of injection. \* Significant difference from saline only mice ( $p < 0.05$ ); # significant difference from saline only mice ( $p < 0.001$ ). Saline only,  $n = 26$ ; saline cage-mates,  $n = 23$ ; and morphine cage-mates,  $n = 9$ . Results are presented as means  $\pm$  SEM.

Two-way ANOVA with age (adolescent, adult) and treatment group (morphine cage-mates, saline cage-mates, saline only) as factors demonstrated a significant main effect of age  $F(1, 101) = 7.78, p < 0.001$ , a significant main effect of treatment group  $F(2, 101) = 12.72, p < 0.001$ , and a significant interaction  $F(2, 101) = 5.63, p < 0.05$ . Bonferroni's post-hoc analyses revealed a significant difference between adult saline cage-mates and adolescent saline cage-mates (adult saline cage-mates < adolescent saline cage-mates,  $p < 0.001$ ). No other significant differences were found between any groups ( $p > 0.05$ ) (Hodgson et al., 2010).

#### *4. Discussion*

Adults demonstrated classic morphine locomotor sensitization (i.e., morphine cage-mates hyperlocomoted to a challenge injection of morphine compared to both saline-treated groups). There was no significant difference in distance traveled between the saline cage-mates and saline only adults. Adolescents displayed a different pattern of locomotor activation. Adolescent morphine cage-mates underwent locomotor sensitization, as morphine cage-mates locomoted significantly more than saline only adolescents. However, saline cage-mates also demonstrated enhanced hyperlocomotion to a single morphine injection. The only difference between saline cage-mates and saline only mice was the type of social environment in which they were housed. In fact, there was no significant difference in locomotion between adolescent saline cage-mates and adolescent morphine cage-mates. This suggests that social environment affects behavioral responses to morphine in adolescents but not adults.

This result was very surprising, thus, the subject size for the morphine cage-mate adolescents and the saline cage-mate adolescents were much larger than originally planned. This was done to ensure the reliability of the effect. This experiment was conducted multiple times and the same results were obtained with each replication.

After all the data was collected, an additional group of morphine only adolescent males were run using the exact same protocol as that described above. This was done to observe any possible locomotor differences between morphine only and morphine cage-mate mice. The data was left out of this body of work for a few reasons. First, they were not run at the same time as the other groups. Second, adult male morphine only mice were not examined. Finally, their sample size was much smaller than the other groups of adolescent mice (saline only, saline cage-mates, and morphine cage-mates). The data gathered from the morphine only adolescent mice suggested that there was no difference in locomotor behavior between the morphine-treated groups (morphine only = morphine cage-mates).

A few studies have shown that periadolescent rats partake in ethanol consumption more readily when exposed to an intoxicated peer (Hunt et al., 2001, Fernández-Vidal and Molina, 2004, Eade and Youngentob, 2009). Those studies suggest enhanced sensitivity to one of ethanol's effects. The studies were important as they were the first to directly examine social mediation of a drug's effect. However, it is not always advantageous to use alcohol in studies of peer influence because ethanol can be detected on the breath (Morey et al., 2011). Rodents engage in socially-mediated food preference (Melchor-Hipólito et al., 2010). Smelling a substance on the breath of another rodent



makes an observer animal more likely to eat that food. Hence, rats in these studies may simply be engaging in socially-mediated food preference for ethanol. In contrast, this study is relatively groundbreaking, as it is the first to examine the role of peer influence on opioid sensitivity.

## CHAPTER V

### EXPERIMENT 3: EFFECT OF SOCIAL ENVIRONMENT ON MORPHINE LOCOMOTOR SENSITIVITY IN ADOLESCENT FEMALE MICE\*

#### *1. Background*

Experiment 1 demonstrated that testosterone level differed in male mice as an effect of both morphine experience and social environment (Hofford et al., 2011). This potentially suggests that the social housing-induced enhancement of morphine sensitivity observed in drug naïve mice as examined in experiment 2 might be sex-dependent (Hodgson et al., 2010).

Multiple studies have shown sex differences in cocaine (Jackson et al., 2005, Griffin et al., 2007) and nicotine (Lynch, 2009) self-administration in mice and rats. Different stages of the estrous cycle cause different intake patterns (Jackson et al., 2005). High levels of progesterone tend to decrease nicotine (Lynch, 2009) and cocaine (Feltenstein et al., 2009) self-administration in females, while high levels of estrogens increase cocaine reward (Zhao and Becker, 2010). Specifically, females have higher break points (they are willing to work harder to obtain drug) when self-administering heroin and morphine in a progressive ratio task compared to males (Cicero et al., 2003). Likewise, females are much less sensitive to the antinociceptive properties of opioids (Cicero et al., 1996).

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While both male and female rodents increase their social exploration during adolescence (Auger and Olesen, 2009), the nature of these differences are important to note. Specifically, males demonstrate a significant increase in rough and tumble play during early adolescence (Olioff and Stewart, 1978, Meaney and Stewart, 1981), while females do not show changes in this type of behavior. This behavior is dependent on pubertal hormone release; increased testosterone metabolites restore rough and tumble play in castrated males (Meaney and Stewart, 1981).

Evidence suggests that females respond to drugs of abuse differently than males. Females also exhibit different social behaviors than males that are dependent on gonadal hormones (Meaney and Stewart, 1981). Given the sex differences observed in opioid sensitivity and drug self-administration, as well as general differences in androgen-dependent social behavior, it was possible that females would respond differently to the manipulation of social housing environment (i.e. ‘mixed cages’, ‘saline only cages’, and ‘morphine only cages’) compared to males. This study examined the role of social housing environment on female locomotor sensitivity in adolescent females. Adult females were not examined in this study because the initial social housing effect was only found in adolescent males (Hodgson et al., 2010).

## *2. Experimental Procedures*

### *Subjects*

Adolescent (PND 28-59) female mice were examined for this experiment. Briefly, group-housed mice were housed in one of three types of cages: saline only,

morphine only, or mixed cages as listed in general methods. Roughly 10 mice per group for a total of 44 mice were used for this experiment.

### *Injection Paradigm*

Injection paradigm was the same as listed above. Briefly, mice received twice daily injections of saline or increasing doses of morphine (10-40 mg/kg, s.c.) for 6 days.

### *Behavioral Analysis: Locomotion*

All mice were examined for their locomotor response to a challenge dose of morphine. On WD9, mice were placed in the testing room for 30 minutes prior to the beginning of the test. Locomotion was measured in cylindrical upright containers (261 mm in diameter and 355 mm high) with white walls. Each mouse was tested separately and four chambers were recorded at a time. After habituation to the room, mice were placed in the locomotor testing apparatus for 60 minutes to measure baseline locomotor behavior. Activity was video recorded from above for 60 minutes. Locomotor behavior was assessed using EthoVision 3.1 (Noldus Information Technology, Leesburg, VA). Activity chambers were washed with water and completely dried between mice.

### *Statistics*

Total distance traveled was calculated and analyzed for both baseline locomotion and locomotion post-morphine injection. Additionally, total distance traveled was summed for each 5 minute time interval post-morphine. Data was analyzed using a 2

(treatment) x 4 (treatment groups) between-subjects ANOVA. Separate 4 (treatment groups) x 24 (time) mixed-design ANOVAs were computed for baseline locomotion and locomotion post-morphine. Differences between treatment groups were calculated using Bonferroni's post-hoc comparisons. *P* values less than 0.05 were considered statistically significant.

### 3. Results

A two-way ANOVA with treatment (baseline, post-morphine injection) and treatment group (morphine only, morphine cage-mates, saline cage-mates, saline only) as factors demonstrated a significant main effect of treatment  $F(3, 80) = 13.58, p < 0.0001$ , a main effect of treatment group  $F(1, 80) = 1059.00, p < 0.0001$ , and a significant interaction  $F(3, 80) = 12.95, p < 0.001$  on distance traveled. A two-way mixed ANOVA with treatment group (morphine only, morphine cage-mates, saline cage-mates, saline only) as a between-subjects factor and time (5 minute time bins) as a within-subjects factor demonstrated a significant main effect of treatment group  $F(3, 960) = 103.30, p < 0.0001$ , a significant main effect of time  $F(23, 960) = 469.50, p < 0.0001$ , and a significant interaction  $F(69, 960) = 6.04, p < 0.0001$ . Bonferroni's post-hoc analyses revealed no significant differences between the treatment groups at baseline (morphine only = morphine cage-mates = saline cage-mates = saline only,  $p > 0.05$ ) and all treatment groups had greater locomotion post-morphine compared to baseline ( $p < 0.001$  for all groups). Analyses revealed no significant differences between saline-treated groups post-morphine (saline cage-mates = saline only,  $p > 0.05$ ). Results also

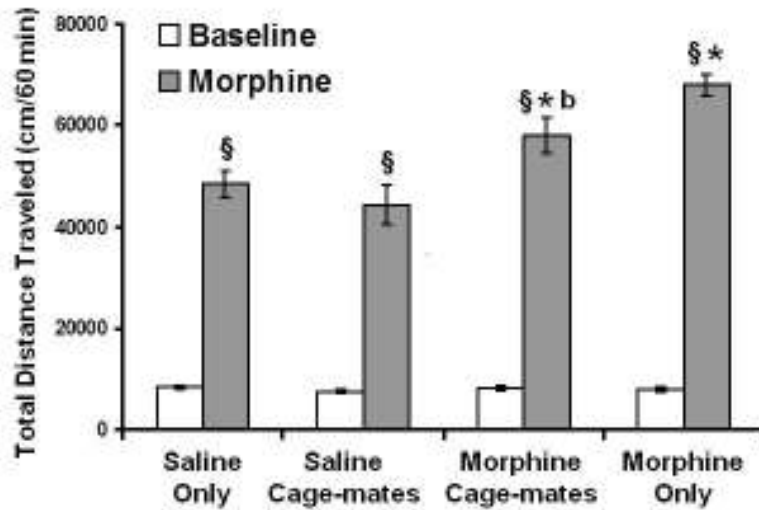


Figure 8: Adolescent female baseline and post-morphine (20 mg/kg) locomotion totals. Baseline locomotion is represented by the white columns and is the total locomotion for 60 minutes pre-morphine injection. Morphine locomotion is represented by the gray columns and is the total locomotion for 60 minutes post-morphine injection.

<sup>§</sup> Significant difference from baseline ( $p < 0.001$ ); \* significant difference from saline only mice ( $p < 0.05$ ); <sup>b</sup> significant difference between morphine cage-mates and morphine only ( $p < 0.01$ ). Saline only,  $n = 12$ ; saline cage-mates,  $n = 10$ ; morphine cage-mates,  $n = 10$ ; and morphine only,  $n = 12$ . Results are presented as means  $\pm$  SEM.

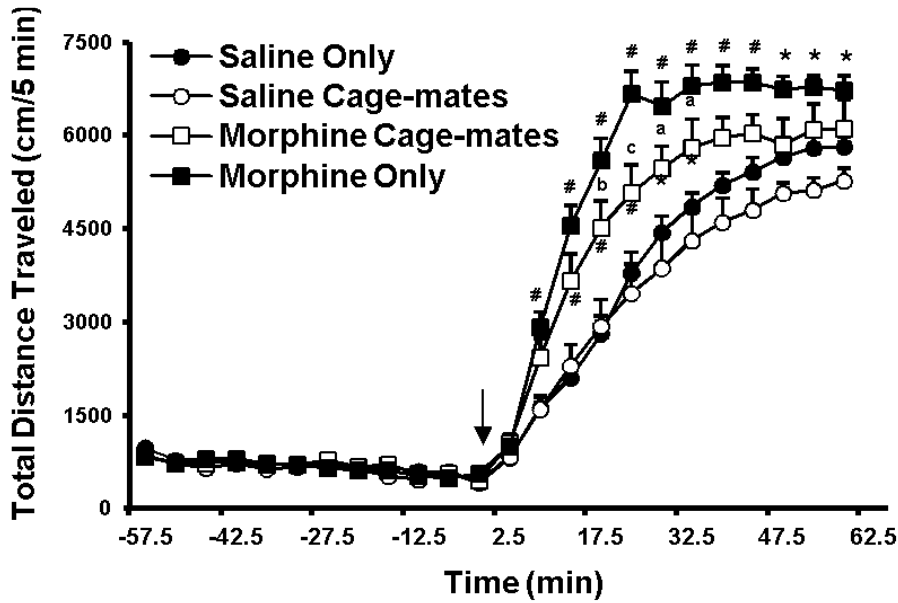


Figure 9: Timeline of female locomotion before and after 20 mg/kg morphine challenge. Total locomotion was calculated and broken down into 5 minute time bins for each of the four groups. Negative numbers indicate times before morphine injection and the arrow indicates time of injection. \* Significant difference from saline only mice ( $p < 0.05$ ); # significant difference from saline only mice ( $p < 0.001$ ); <sup>a</sup> significant difference from morphine only mice ( $p < 0.05$ ); <sup>b</sup> significant difference from morphine only mice ( $p < 0.01$ ); <sup>c</sup> significant difference from morphine only mice ( $p < 0.001$ ). Saline only,  $n = 12$ ; saline cage-mates,  $n = 10$ ; morphine cage-mates,  $n = 10$ ; and morphine only,  $n = 12$ . Results are presented as means  $\pm$  SEM.

show that both morphine-treated groups demonstrated morphine locomotor sensitization (saline only < morphine only,  $p < 0.001$ , and saline only < morphine cage-mates,  $p < 0.01$ ). However, there was a significant difference between groups receiving morphine, with morphine only mice demonstrating greater locomotor sensitization compared to morphine cage-mates (morphine cage-mates < morphine only,  $p < 0.01$ ) (Hofford et al., 2010). Results are presented in figures 8 and 9.

#### *4. Discussion*

Similar to the effect observed in adolescent male mice, manipulation of social housing produced differences in locomotor behavior in response to morphine challenge in adolescent females. Unlike the adolescent males, the difference was between the morphine-treated groups. Morphine only females were significantly more sensitized to morphine than morphine cage-mate females. There was no significant difference between the saline-treated groups.

Preliminary data suggested that the morphine challenge dose of 10 mg/kg that was administered to males was not high enough to induce locomotor sensitization in the females. No sensitization in either morphine-treated group was observed at that dose. By increasing the morphine dose to 20 mg/kg, locomotor sensitization was observed in both morphine-treated groups. This general lack of morphine sensitivity is not surprising; females are less sensitive to certain properties of opioids compared to males (Cicero et al., 1996).



This experiment added a morphine only group that was not present originally in either experiment 1 or experiment 2. However, a morphine only group was examined in experiment 2 after the original data was collected and revealed no difference in locomotor sensitization between morphine-treated adolescent males. The experimenters included a morphine only group in this experiment to gain a more complete interpretation of the social environment effect on morphine sensitivity. This study indicates that the social environment effect is not only specific to adolescents, the enhancement in locomotor behavior between the treatment groups are sex-dependent as well.

## CHAPTER VI

### EXPERIMENT 4: SOCIAL ENVIRONMENT EFFECT ON MORPHINE REWARD IN ADOLESCENT MICE AS MEASURED BY CONDITIONED PLACE PREFERENCE\*

#### *1. Background*

Experiments 2 and 3 focused on measuring morphine locomotor sensitization in mice after being housed in various social environments. This paradigm, however, only measures behavioral sensitization to one of morphine's effects. While increased locomotor sensitization is correlated with enhanced drug seeking (Wise and Bozarth, 1987, Deroche-Gamonet et al., 2004), it does not adequately assess drug reward. A more valid way of measuring a drug's reinforcing properties involves testing mice using either self-administration or CPP. Self-administration in adolescent mice is not possible given their small size. CPP is considered an adequate substitute for the measure of reward in mice (Bardo and Bevins, 2000). CPP requires an animal to associate a context with a drug's effects. If an animal finds a drug rewarding, it will spend more time in the chamber previously paired with drug.

This experiment employed a short CPP paradigm compared to most other studies examining morphine reward (Gadd et al., 2003, Tenayuca and Nazarian, 2012). This was done to avoid a ceiling effect on morphine CPP. Multiple morphine-chamber pairings

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cause morphine CPP in previously drug-naïve rodents (Gadd et al., 2003, Tenayuca and Nazarian, 2012) (similar to the ‘saline only’ mice). Conversely, previous studies have shown that rats given repeated injections of morphine before CPP training acquired morphine CPP faster than rats that were morphine-naïve (Simpson and Riley, 2005). This phenomenon is an enhancement in the rewarding properties of an addictive drug after previous experience with that drug and can be referred to as “reward sensitization”. The same idea was applied to this study. The purpose of this experiment was to maximize reward in morphine only mice (obtain reward sensitization in this group), while keeping the level of morphine CPP lower in saline only mice. In other words, the experiment aimed to observe differences between ‘saline only’ and ‘morphine only’ mice and to examine the place preference in the other treatment groups (‘saline cage-mates’ and ‘morphine cage-mates’).

This experiment measured drug reward in adolescent mice using CPP after animals were housed in different social housing environments (i.e. ‘mixed cages’ compared to ‘saline only cages’ and ‘morphine only cages’). Since social housing only affected adolescents’ sensitivity to morphine, only adolescents were examined for morphine reward after social housing manipulation.

## *2. Experimental Procedures*

### *Subjects*

Adolescent (PND 28-59) male mice were examined for this experiment. Briefly, group-housed mice were housed in one of three types of cages: saline only, morphine

only, or mixed cages as listed in general methods. Roughly 10 mice per group for a total of 220 mice were used for this experiment.

### *Injection Paradigm*

Injection paradigm was the same as listed above in general methods. Briefly, mice received twice daily injections of saline or increasing doses of morphine (10-40 mg/kg, s.c.) for 6 days.

### *Behavioral Analysis: Conditioned Place Preference*

On WD7, mice were placed in the testing room for 30 minutes. Each mouse was then placed in a CPP apparatus and was allowed to freely explore for 30 minutes. The CPP apparatus contained 3 distinct chambers; one chamber was neutral with white walls and no scents. From this chamber, a mouse could enter one of two conditioning chambers: one chamber accented with cow spotted wallpaper and lemon scent (Adams Extract and Spices, LLC., Gonzales, TX) and the other with checkered wallpaper and almond scent (Adams Extract and Spices, LLC., Gonzales, TX). Each CPP apparatus was placed inside an automated optical beam activity monitor (Model RXYZCM-16; Accuscan Instruments, Columbus, OH, USA) which recorded each mouse's position. Any mouse spending more than 70% of its time in one chamber was excluded from the final analysis. On either WD8 or WD9, mice were given saline, 10 mg/kg, 20 mg/kg, or 40 mg/kg morphine and confined to their least preferred conditioning chamber for 60 minutes. On the other day, mice received saline and were confined to their most

preferred conditioning chamber for 60 minutes. On WD10, mice were allowed to freely explore the entire apparatus. Time spent in each chamber was recorded. All chambers were cleaned with 70% ethanol and water between mice.

### *Statistics*

Percent time spent in each chamber was calculated on habituation day and on test day using the formula: [(time spent in the chamber in seconds/total time in apparatus recorded in seconds) X 100]. Individual chamber preference was calculated as: [percent time spent in the chamber on test day - percent time spent in the chamber on habituation day]. Some animals did not receive morphine on their conditioning day. These mice were included as a control and were injected and housed as all other animals (i.e. they were either 'saline cage-mates', 'morphine cage-mates', 'morphine only', or 'saline only'). Their individual chamber preferences were calculated as stated above. Each treatment group's percent  $\Delta$  average chamber preference was normalized to their saline conditioned group using the formula: [(percent  $\Delta$  time spent in the chamber/average percent  $\Delta$  time spent in the chamber of the control saline conditioned animals for this treatment group X 100)-100]. Data was analyzed using a 2 (pretreatment) x 2 (cage condition) x 4 (conditioning dose of morphine) between-subjects ANOVA. Differences between treatment groups were calculated using Bonferroni's post-hoc comparisons. *P* values less than 0.05 were considered statistically significant.

### 3. Results

Mean chamber preference on habituation day was calculated for each group of mice; these were calculated to assure that the bias against the least preferred chamber was similar in all treatment groups. A three-way ANOVA with pretreatment (saline, morphine), cage condition (mixed cage, 'only' cage), and conditioning dose (0 mg/kg, 10 mg/kg, 20 mg/kg, 40 mg/kg) as factors demonstrated that there was a significant main effect of pretreatment  $F(1, 217) = 4.59, p < 0.05$ ; saline pretreated mice preferred the chamber they would be conditioned in  $27.72 \pm 0.36\%$  of the time, while the morphine pretreated mice preferred the chamber they would be conditioned in  $28.81 \pm 0.35\%$  of the time. However, there were no significant main effects of cage condition (i.e. no significant differences between saline only and saline cage-mates or between morphine only and morphine cage-mates) or conditioning dose, as well as no significant interactions between any of these factors ( $p > 0.05$ ).

The conditioned chamber preferences of the control mice, those receiving only saline on their conditioning day, were calculated and analyzed using a two-way ANOVA with pretreatment (saline, morphine) and cage condition (mixed cage, 'only' cage) as factors. Mean chamber preferences of these mice were calculated in order to normalize the results of mice conditioned with 10 mg/kg, 20 mg/kg, or 40 mg/kg morphine. This analysis demonstrated a significant main effect of cage condition  $F(1, 56) = 4.25, p < 0.05$ , but no significant main effect of pretreatment and no significant interaction ( $p > 0.05$ ). Results for the control mice are presented in figure 10.

Morphine CPP was calculated as the [(percent  $\Delta$  time spent in the chamber/average percent  $\Delta$  time spent in the chamber of the control saline conditioned animals for this treatment group X 100)-100]. Results for the mice conditioned with 20 mg/kg morphine before normalization are presented in figure 11. Three-way ANOVA of the normalized conditioned chamber preference with pretreatment (saline, morphine), cage condition (mixed cage, 'only' cage), and conditioning dose (0 mg/kg, 10 mg/kg, 20 mg/kg, 40 mg/kg), demonstrated a significant main effect of pretreatment  $F(1, 217) = 49.82, p < 0.0001$ , a significant main effect of cage condition  $F(1, 217) = 50.50, p < 0.0001$ , and a significant main effect of conditioning dose  $F(3, 217) = 7.97, p < 0.0001$ . Analysis also revealed a significant interaction between pretreatment and cage condition  $F(1, 217) = 41.62, p < 0.001$ , a significant interaction between pretreatment and conditioning dose  $F(3, 217) = 7.68, p < 0.001$ , a significant interaction between cage condition and conditioning dose  $F(3, 217) = 7.67, p < 0.001$ , and a significant interaction between pretreatment, cage condition, and conditioning dose  $F(3, 217) = 7.76, p < 0.001$ .

Bonferroni's post-hoc analyses revealed that saline only mice did not develop morphine CPP at any dose tested (saline only 0 mg/kg = saline only 10, 20, 40 mg/kg,  $p > 0.05$ ). Saline cage-mates developed morphine CPP to the highest conditioning dose of morphine (saline cage-mates 0 mg/kg < saline cage-mates 40 mg/kg,  $p < 0.01$ ) but not at any other dose examined (saline cage-mates 0 mg/kg = saline cage-mates 10, 20 mg/kg,  $p > 0.05$ ). Morphine only mice demonstrated morphine CPP to 10 mg/kg morphine

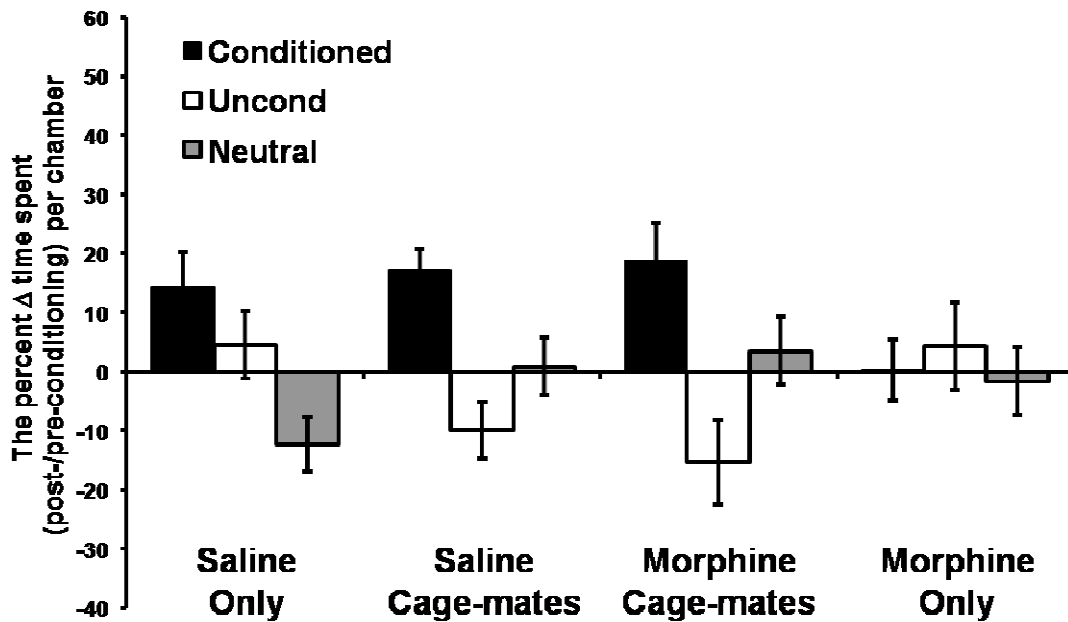


Figure 10: Time spent in each of the three chambers in saline conditioned controls.

Place preference time is calculated as: [percent time spent in the chamber on test day - percent time spent in the chamber on habituation day]. These mice received saline on both conditioning days. Saline only,  $n = 12$ ; saline cage-mates,  $n = 16$ ; morphine cage-mates,  $n = 16$ ; and morphine only,  $n = 16$ . Results are presented as means  $\pm$  SEM.



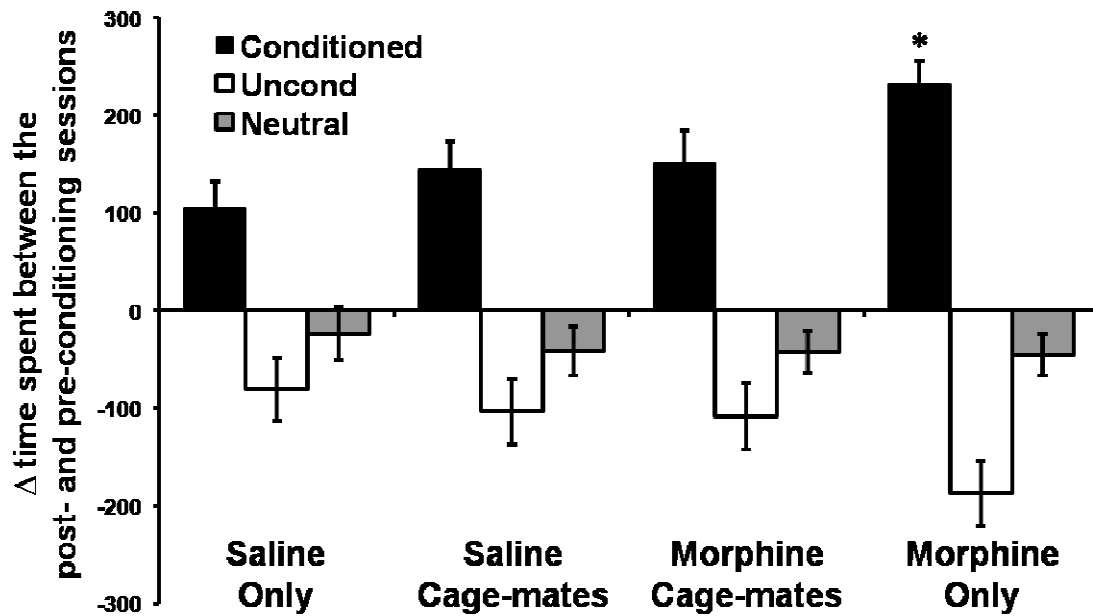


Figure 11: Non-normalized time spent in each of the three chambers in all groups conditioned with 20 mg/kg morphine. Place preference time is calculated as: [percent time spent in the chamber on test day - percent time spent in the chamber on habituation day]. \* Significant difference from saline only mice ( $p < 0.01$ ). Saline only,  $n = 16$ ; saline cage-mates,  $n = 16$ ; morphine cage-mates,  $n = 16$ ; and morphine only,  $n = 16$ . Results are presented as means  $\pm$  SEM.

(morphine only 0 mg/kg < morphine only 10 mg/kg,  $p < 0.01$ ) and to 20 mg/kg morphine (morphine only 0 mg/kg < morphine only 20 mg/kg,  $p < 0.01$ ), but not to 40 mg/kg morphine (morphine only 0 mg/kg = morphine only 40 mg/kg,  $p > 0.05$ ). In contrast, morphine cage-mates did not develop morphine CPP at any conditioning dose (morphine cage-mates 0 = morphine cage-mates 10, 20, and 40 mg/kg,  $p > 0.05$ ). Additionally, normalized morphine CPP was higher in morphine only mice compared to saline only mice (saline only < morphine only,  $p < 0.01$ ) and morphine cage-mates (morphine cage-mates < morphine only,  $p < 0.01$ ) at all doses (Cole et al., 2012). Normalized morphine CPP results are presented in figure 12.

#### *4. Discussion*

Morphine only mice demonstrated enhanced morphine reward compared to all other groups as measured by CPP when conditioned with 10 mg/kg or 20 mg/kg morphine. In other words, morphine only mice, but not morphine cage-mates, demonstrated enhanced sensitivity to the rewarding effects of morphine. Most interestingly, there was a difference in morphine reward between morphine cage-mates and morphine only mice despite the same morphine treatment regimen. The only difference between the morphine-treated groups was their social environment. Additionally, saline cage-mates demonstrated a small, albeit significant, place preference at 40 mg/kg. This was not observed in the saline only group.

Another noticeable difference between morphine only mice and all other groups is seen in figure 10. Morphine only saline conditioned controls did not shift their

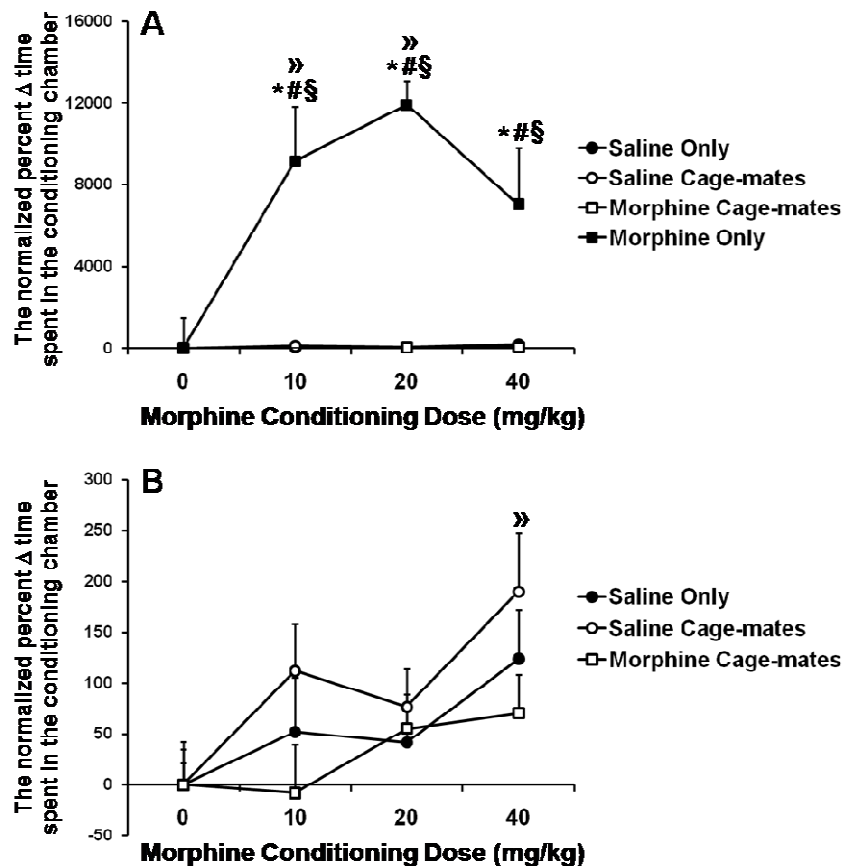


Figure 12: Normalized morphine conditioned place preference. A) Normalized place preference including the morphine only group. B) Normalized place preference without the morphine only group. » Significant difference from the equivalent treatment controls, (i.e. those conditioned with saline) ( $p < 0.01$ ); \* significant difference from saline only mice ( $p < 0.01$ ); # significant difference from saline cage-mates ( $p < 0.01$ ); § significant difference from morphine cage-mates ( $p < 0.01$ ). Results are presented as means  $\pm$  SEM.

preference to the conditioned chamber (which, in this case, was the chamber they least preferred on habituation day). All other groups did. This suggests that morphine only mice, but not morphine cage-mates or either of the saline-treated groups, perseverate more than the other groups. This could be caused by a switch to a caudate/putamen-based memory strategy (Packard and McGaugh, 1996), possibly due of increased levels of stress in these mice (Schwabe et al., 2008).

Morphine only mice demonstrated significant CPP at 10 mg/kg and 20 mg/kg, but their reward was reduced at the highest dose used, 40 mg/kg. This high dose of morphine might be aversive to the morphine only group. Morphine only mice demonstrated sensitivity to reward and were likely sensitized to other behavioral effects of morphine (i.e. morphine's ability to inhibit memory formation); this might explain why a decrease in morphine CPP at 40 mg/kg only occurred in this group. The attenuated place preference observed at this dose might also be caused by morphine's ability to inhibit the formation of contextual memory (McNally and Westbrook, 2003). Again, the morphine only mice might be more affected by this high dose due to general sensitization.

The largest difference in morphine sensitivity was found between the morphine-treated mice (morphine cage-mates < morphine only), while experiment 2 found a difference in morphine sensitivity between the saline-treated groups (saline only < saline cage-mates). Together, these two experiments suggest that there might be a difference in morphine sensitivity between morphine-treated groups at certain doses and in certain behavioral paradigms. In support of this, saline cage-mates did demonstrate enhanced

reward compared to saline only mice, but this was at a higher dose. Experiment 2 demonstrated a difference in morphine sensitivity between saline-treated groups administered 10 mg/kg while the current experiment observed significant differences at 40 mg/kg.

Taken together, the testosterone data with the results of the behavioral experiments suggest that morphine only mice are experiencing more stress than morphine cage-mates or either of the saline groups. It is well known that morphine withdrawal is stressful and causes increased release of corticosterone (Nunez et al., 2007). Usually, increased levels of corticosterone are observed at shorter time points after withdrawal in adults, but the length of this effect is rarely examined in adolescents (Hofford et al., 2011). Previous psychosocial stress can sensitize rodents to an addictive drug's effects (Sinha et al., 1999). Increased stress due to morphine withdrawal explains the difference in reward between morphine only and saline-treated groups (saline only and saline cage-mates), but does not adequately explain the difference between the morphine-treated groups (morphine only compared to morphine cage-mates).

It is possible that morphine only mice are undergoing more stress than morphine cage-mates. This could be due to increased fighting or disruption of the social hierarchy in a cage where four mice receive morphine compared to a cage where just half of the mice receive morphine. Because of this possibility, we quantified corticosterone level on WD9 in mice from the different social housing environments.

## CHAPTER VII

### EXPERIMENT 5: SOCIAL ENVIRONMENT EFFECT ON CIRCULATING CORTICOSTERONE IN ADOLESCENT MALE MICE\*

#### *1. Background*

Stress affects a variety of behaviors and is often implicated as a risk factor for initiating drug use, sensitivity to drugs of abuse, and is correlated with poor treatment retention during prolonged abstinence (Erb et al., 1996, Ahmed and Koob, 1997, Sinha et al., 1999, Tracy et al., 2005). Many environmental stimuli will cause rodents to demonstrate stress-related behaviors and these stressful stimuli can cause corticosterone release, the main glucocorticoid in rodents (Hyde and Skelton, 1961). For this reason, levels of circulating corticosterone are often measured to indicate if an animal is experiencing stress.

Corticosterone is released from the adrenal glands in response to circulating adrenocorticotrophic hormone (ACTH), which is released from the pituitary (Bergner and Deane, 1948). Corticosterone and ACTH, as well as other hormones in this pathway, act as part of a feedback loop, with regulation during many stages of the release cycle preventing overstimulation by corticosterone (Kendall et al., 1964, Sato et al., 1975, Di and Tasker, 2008). This entire pathway is commonly referred to as the hypothalamic-pituitary-adrenal axis, named after the major structures involved. Functionally,

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corticosterone inhibits its own release by decreasing release of ACTH from pituitary (Kendall et al., 1964) and by decreasing corticotropin releasing factor (CRF) from hypothalamus (Sato et al., 1975).

As stated above, many things can cause elevated corticosterone levels in rodents. Isolated housing is generally considered stressful to rats and mice. While the effect of isolation on corticosterone release is debated (Benton et al., 1978, Frances et al., 2000, Hunt and Hambly, 2006), isolation has been shown to cause exaggerated response to later stressors (Serra et al., 2005). Additionally, alterations of social hierarchies via subordination stress result in elevated corticosterone levels (Blanchard et al., 1993).

One factor that affects both social behavior and corticosterone is the gonadal hormone testosterone. Testosterone is also inversely related to corticosterone, with low levels of testosterone correlated with high levels of corticosterone (Blanchard et al., 1993). Experiment 1 demonstrated that adolescent mice living in mixed cages had lower levels of testosterone compared to mice in saline only cages, regardless of drug treatment (Hofford et al., 2011).

Considering the results of experiment 1 together with the role stress plays in drug sensitivity and relapse, it is possible that an alteration in social housing environment differentially affects levels of stress in adolescent male mice. To quantify stress, this study measured levels of corticosterone in adolescent male mice housed in the different housing environments (i.e. 'saline only', 'mixed cages', and 'morphine only').

## *2. Experimental Procedures*

### *Subjects*

Adolescent (PND 28-59) male mice were examined for this experiment. Briefly, group-housed mice were housed in one of three types of cages: saline only, morphine only, or mixed cages as listed in general methods. Roughly 15 mice per group for a total of 61 mice were used for this experiment.

### *Injection Paradigm*

Injection paradigm was the same as listed above in general methods. Briefly, mice received twice daily injections of saline or increasing doses of morphine (10-40 mg/kg, s.c.) for 6 days.

### *Tissue Collection*

On WD9, mice received sodium pentobarbital (100 mg/kg, i.p.). Blood was collected via intra-cardiac puncture and separated by centrifugation at 1,000 x g at 4° C for 15 minutes. Tissue was stored at -80° C.

### *Assessment of Hormone Levels*

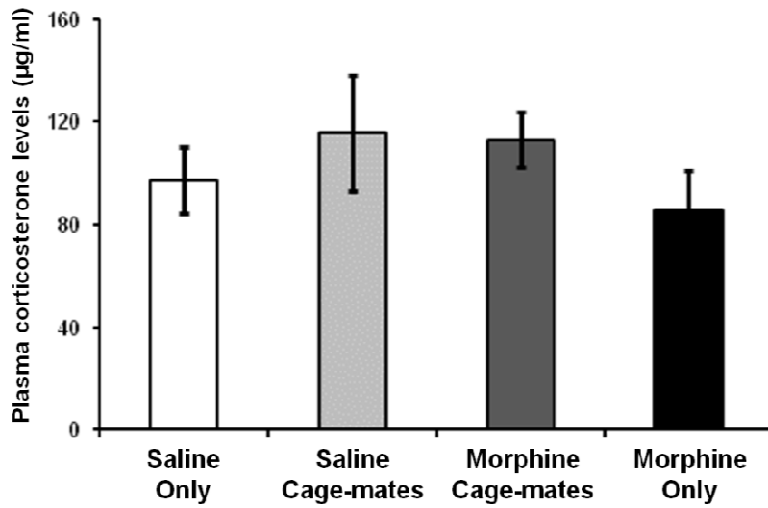
Corticosterone level was assessed using the Corticosterone EIA kit (Cayman Chemical, Ann Arbor, MI) per assay instructions. This kit uses a competitive binding assay and requires addition of a known amount of labeled corticosterone to samples with an unknown amount of unlabeled corticosterone. Addition of a chemical reagent in



the last step causes color change that is inversely proportional to the amount of unlabeled testosterone in the samples. Briefly, this ELISA was run as follows. Blood plasma was diluted 1:1000 in EIA buffer (provided by kit) and these diluted samples were run in triplicate. Serial dilutions of a corticosterone standard were run parallel to the samples. Final standards had concentrations of 10,000  $\mu\text{g/ml}$ , 4,000  $\mu\text{g/ml}$ , 1,600  $\mu\text{g/ml}$ , 640  $\mu\text{g/ml}$ , 256  $\mu\text{g/ml}$ , 102.4  $\mu\text{g/ml}$ , 41  $\mu\text{g/ml}$ , and 16.4  $\mu\text{g/ml}$ . These standards were run in duplicate and were used to generate a standard curve. Samples and standards were loaded in a 96-well plate, followed by addition of an AChE Corticosterone Tracer (provided by kit) and Corticosterone Antiserum (provided by kit). Plate was sealed and allowed to stir on an orbital shaker for 2 hours. Plate was rinsed 5 times with wash buffer (provided by kit) and completely dried. Ellman's reagent (provided by kit) was added to all the wells, sealed, protected from light, and allowed to shake for 1.5 hours. Color change in the plate was measured by a spectrophotometer and a standard curve was evaluated using the standards.

### *Statistics*

Data was analyzed using a 2 (pretreatment) x 2 (cage condition) between-subjects ANOVA. Differences between treatment groups were calculated using Bonferroni's post-hoc comparisons. *P* values less than 0.05 were considered statistically significant.



*Figure 13:* Corticosterone levels in adolescent male mice in the different social environments. Plasma corticosterone was collected on WD9. Saline only,  $n = 10$ ; saline cage-mates,  $n = 8$ ; morphine cage-mates,  $n = 8$ ; and morphine only,  $n = 16$ . Results are presented as means  $\pm$  SEM.

### *3. Results*

Two-way ANOVA with pretreatment (saline, morphine) and cage condition (mixed cage, 'only' cage) as between-subjects variables revealed no significant main effect of pretreatment,  $F(1, 60) = 0.35, p > 0.05$ , cage condition  $F(1, 60) = 3.40, p > 0.05$ , or interaction  $F(1, 60) = 0.14, p > 0.05$  on corticosterone level (Cole et al., 2012). Results are shown in figure 13.

### *4. Discussion*

This study found that there were no differences in corticosterone production on WD9 between any of the treatment groups. Blood plasma was only collected on WD9 to represent the circulating corticosterone at the time equivalent to all relevant behavior (i.e. locomotor sensitivity and morphine CPP were all conducted at this time). This data suggests that stress at the time of behavioral testing is not the only factor involved in the expression of altered morphine sensitivity in adolescent males in different social environments.

It is still possible, and quite likely, that stress plays some role in the development of this behavior. Corticosterone levels were only measured on WD9 during the second half of the light cycle. Higher stress levels could have been occurring during the time of drug treatment or during early withdrawal. Additionally, corticosterone levels fluctuate throughout the day under normal circumstances (Ungar, 1964). It is possible that corticosterone levels would differ at other times of day, for instance, at night or early morning.

Measuring basal corticosterone is sometimes not the best way to quantify the physiological effects of a stressor. This method is easy and provides some clues to what an animal finds acutely stressing. However, it might not take into account the sensitizing effect of stress. While the mice in this experiment did not have elevated (or reduced) basal corticosterone levels at the equivalent time of behavioral testing, it is possible that a later stressor would result in differential corticosterone release between mice housed in the different social environments.

Regardless of the utility of the method, the data demonstrate no social environment effect on basal corticosterone levels on WD9. This suggests that corticosterone and stress have very little to no effect on the expression of the social housing effect on morphine sensitivity.

## CHAPTER VIII

### EXPERIMENT 6: ROLE OF VASOPRESSIN V1B RECEPTOR IN THE EXPRESSION OF A SOCIAL ENVIRONMENT EFFECT ON MORPHINE REWARD

#### *1. Background*

Arginine vasopressin is a peptide synthesized in the supraoptic nucleus of the hypothalamus (Sachs et al., 1971) and functions as a peripheral hormone (Dicker and Greenbaum, 1954). Vasopressin was originally identified as a stress hormone; release of vasopressin in hypothalamus induces release of ACTH from the pituitary (Rivier and Vale, 1983, Zelena et al., 2009). Centrally, it also acts to modulate anxiety (Murgatroyd et al., 2004) and helps regulate social behavior (Bielsky et al., 2005, Arakawa et al., 2010) in limbic areas, such as lateral septum, bed nucleus of stria terminalis, (Veenema et al., 2010) and amygdala (Arakawa et al., 2010). While classically viewed as a stress hormone, vasopressin, along with oxytocin, is considered a social hormone. Limbic vasopressin is essential for the recognition of familiar peers (Everts and Koolhaas, 1997, Bielsky et al., 2005) and, in the monogamous prairie vole, regulates pair bonding (Winslow et al., 1993). Also, in the hamster, vasopressin regulates aggression (Albers et al., 2006).

Interestingly, the vasopressin system is highly sexually dimorphic. There is a much richer expression of vasopressin peptide in male limbic system compared to females (De Vries and Panzica, 2006). This is not true of the hypothalamic and peripheral vasopressin system. This sex difference is due to higher circulating androgens

in males, as castrated male rats exhibit limbic vasopressin expression patterns more similar to females (De Vries et al., 1985, Auger et al., 2011). Specifically, higher testosterone levels result in decreased methylation of the vasopressin promoter (Auger et al., 2011). Vasopressin also modulates behaviors sex-dependently; limbic vasopressin regulates anxiety and aggression in males (Murgatroyd et al., 2004, Veenema et al., 2010).

Finally, vasopressin expression can affect drug behavior. Vasopressin action at the V1b receptor has been shown to modulate alcohol (Zhou et al., 2011) and heroin seeking after a period of abstinence in rats. Specifically, antagonism of the V1b receptor decreased drug- and stress-primed heroin reinstatement (Zhou et al., 2007).

Vasopressin's role in both social behavior and opioid seeking as well as its regulation by testosterone, suggest that it plays some part in the expression of social environment-induced morphine reward. The V1b receptor was chosen as a target over V1a due to its role in heroin seeking during relapse (Zhou et al., 2007).

This study evaluated the role of V1b receptors in the expression of altered morphine reward after exposure to different social housing conditions. Since the largest difference in morphine reward was between the morphine only and the morphine cage-mate mice, this study examined the role of V1b receptor on the environmental effect on opioid sensitivity in the morphine-dependent mice only (i.e. morphine CPP between adolescent morphine cage-mates and adolescent morphine only mice).

## *2. Experimental Procedures*

### *Subjects*

Adolescent (PND 28-59) morphine cage-mates and morphine only males were examined for this experiment. Briefly, mice were housed in one of two cages, morphine only or mixed cages as listed in general methods. Data was not collected for saline cage-mates. Roughly 15 mice per group for a total of 57 mice were used for this experiment.

### *Injection Paradigm*

Injection paradigm was the same as listed above in general methods. Briefly, mice received twice daily injections of saline or increasing doses of morphine (10-40 mg/kg, s.c.) for 6 days.

### *Behavioral Analysis: Conditioned Place Preference*

On WD7, mice were placed in the testing room for 30 minutes. Each mouse was then placed in a CPP apparatus and was allowed to freely explore for 30 minutes. The CPP apparatus contained 3 distinct chambers; one chamber was neutral with white walls and no scents. From this chamber, a mouse could enter one of two conditioning chambers: one chamber accented with cow spotted wallpaper and lemon scent (Adams Extract and Spices, LLC., Gonzales, TX) and the other with checkered wallpaper and almond scent (Adams Extract and Spices, LLC., Gonzales, TX). Each CPP apparatus was placed inside an automated optical beam activity monitor (Model RXYZCM-16; Accuscan Instruments, Columbus, OH, USA) which recorded each mouse's position.

Any mouse spending more than 70% of its time in one chamber was excluded from the final analysis.

On either WD8 or WD9, mice were given vehicle or SSR149415, a V1b antagonist (suspended in 0.1% Tween 80/0.6% methyl cellulose in saline, 10 mg/kg, i.p.) and were returned to their cages for 30 minutes (Urani et al., 2011). This was followed by s.c. saline or 20 mg/kg morphine and confinement to their least preferred conditioning chamber for 60 minutes. On the other day, mice did not receive any i.p. injection. On this day, they received s.c. saline and they were confined to their most preferred conditioning chamber for 60 minutes. On WD10, mice were allowed to freely explore the entire apparatus. Time spent in each chamber was recorded. All chambers were cleaned with 70% ethanol and water between mice.

### *Statistics*

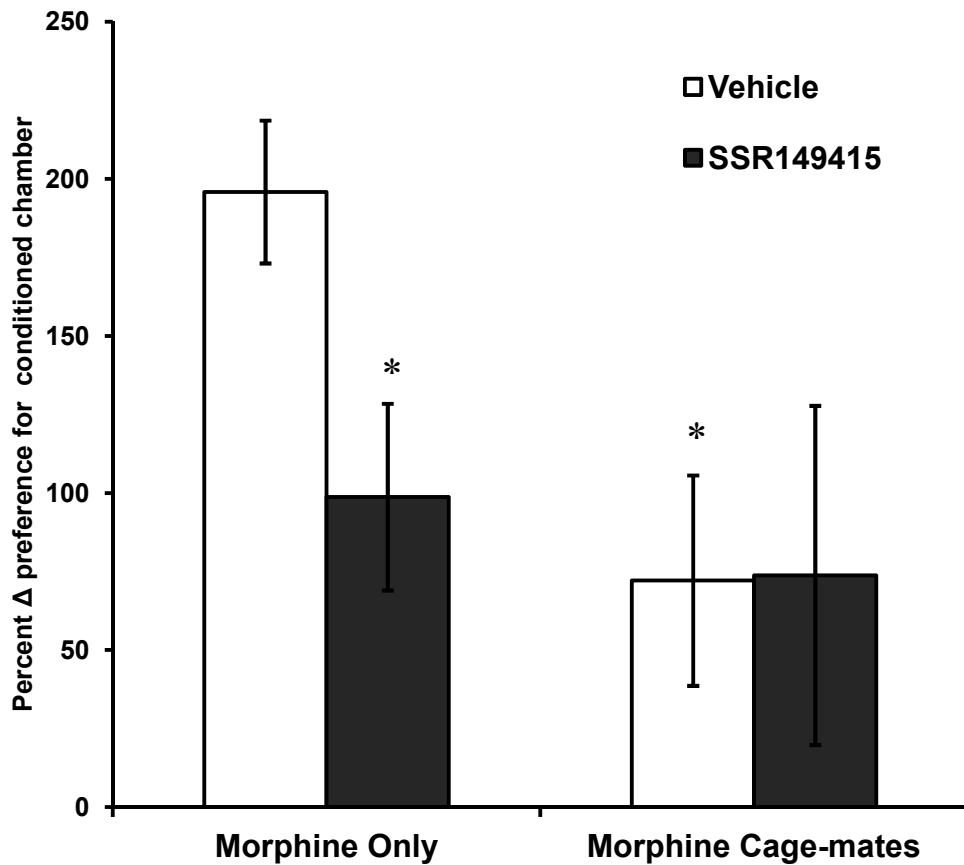
Percent time spent in each chamber was calculated on habituation day and on test day using the formula:  $[(\text{time spent in the chamber in seconds} / \text{total time in apparatus recorded in seconds}) \times 100]$ . Individual chamber preference was calculated as:  $[\text{percent time spent in the chamber on test day} - \text{percent time spent in the chamber on habituation day}]$ . Some animals did not receive morphine on their conditioning day. These mice were included as a control and were injected and housed as all other animals (i.e. they were either ‘morphine cage-mates’ or ‘morphine only’). Their individual chamber preferences were calculated as stated above. Each treatment group’s percent  $\Delta$  average chamber preference was normalized to their saline conditioned group using the formula:



[(percent  $\Delta$  time spent in the chamber - average percent  $\Delta$  time spent in the chamber of the control saline conditioned animals for this treatment group X 100)-100]. Data was analyzed using a 2 (cage condition) x 2 (antagonist treatment) between-subjects ANOVA. Differences between treatment groups were calculated using Bonferroni's post-hoc comparisons. *P* values less than 0.05 were considered statistically significant.

### 3. Results

Morphine conditioned place preference (CPP) was calculated as the [(percent  $\Delta$  time spent in the chamber-average percent  $\Delta$  time spent in the chamber of the control saline conditioned animals for this treatment group X 100)-100]. Two-way ANOVA of the normalized conditioned chamber preference with cage condition (mixed cage, 'only' cage), and antagonist treatment (vehicle, 10 mg/kg SSR149415) demonstrated a significant main effect of cage condition  $F(1, 55) = 4.78, p < 0.05$ , but no significant main effect of antagonist treatment ( $p > 0.05$ ) and no significant interaction ( $p > 0.05$ ). Bonferroni's post-hoc analyses revealed significantly less acquisition of morphine CPP by morphine cage-mates receiving vehicle compared to morphine only mice receiving vehicle (morphine cage-mates < morphine only,  $p < 0.05$ ). Additionally, administration of SSR149415 significantly reduced the acquisition of morphine CPP in the morphine only mice (morphine only SSR149415 < morphine only vehicle,  $p < 0.05$ ). Results for this study are presented in figure 14.



*Figure 14:* Effect of V1b antagonism on morphine reward in morphine cage-mates and morphine only adolescents. Morphine place preference was calculated as: [(percent  $\Delta$  time spent in the chamber-average percent  $\Delta$  time spent in the chamber of the control saline conditioned animals for this treatment group X 100)-100]. \* Significant difference from morphine only vehicle. Morphine only vehicle,  $n = 26$ ; morphine only SSR,  $n = 24$ ; morphine cage-mates vehicle,  $n = 14$ ; morphine cage-mates SSR,  $n = 14$ . Results are presented as means  $\pm$  SEM.

#### *4. Discussion*

V1b antagonism resulted in a decrease in morphine reward in morphine only mice but not morphine cage-mates. The data from this experiment suggests a role of the vasopressin system in the social environment effect on morphine CPP in morphine only mice. V1b antagonism is unlikely to be affecting morphine reward in morphine-treated mice in general, as SSR419145 administration had no effect on morphine cage-mates' CPP. However, any decrease in morphine reward would be harder to detect in morphine cage-mates given the low level of morphine CPP observed in this treatment group. Additionally, it is unlikely that SSR419145 causes general aversion in morphine only mice because morphine cage-mates' place preference did not change after pretreatment with the compound.

The data suggests that the vasopressin system is altered in morphine only mice. This could be due to higher expression levels of vasopressin compared to morphine cage-mates or due to other changes in signaling throughout the system. Higher levels of vasopressin could reflect increased levels of stress in these animals (Zelena et al., 2009). As mentioned above, morphine withdrawal causes stress and increases corticosterone release (Nunez et al., 2007, Hofford et al., 2011) and vasopressin has been correlated with increased corticosterone and stress (Knepel et al., 1985, Nunez et al., 2007). However, no significant differences in corticosterone production were observed between any of the treatment groups on WD9 as observed in experiment 5 (Cole et al., 2012). Hence, it is unlikely that stress alone can explain why V1b antagonism decreases reward in morphine only mice specifically.

SSR419415 was administered systemically via i.p. injection. While systemic injections are useful for researchers, this route of administration offers very few clues to a compound's site of action. Receptors for vasopressin exist in the periphery (Dicker and Greenbaum, 1954, Gózdź et al., 2002) as well as within the CNS (De Vries et al., 1985, Allaman-Exertier et al., 2007, Arakawa et al., 2010, Auger et al., 2011). Interactions between the periphery and the central nervous system can sometimes be complex, but evidence suggests that vasopressin is acting in the brain to affect social housing-induced morphine reward. First, morphine reward (Olmstead and Franklin, 1997) and social behavior (Carballo-Márquez et al., 2009, Arakawa et al., 2010) are both mediated by the brain. Second, vasopressin affects male anxiety by acting on limbic brain areas (Bielsky et al., 2005). Thus, vasopressin is most likely working centrally to modulate social housing-induced morphine sensitivity. Given the results of this experiment, the following study measured levels of vasopressin and vasopressin receptors in specific brain regions known to affect social behavior.

## CHAPTER IX

### EXPERIMENT 7: SOCIAL ENVIRONMENT EFFECT ON EXPRESSION OF VASOPRESSIN AND VASOPRESSIN RECEPTOR MESSENGER RNA

#### *1. Background*

Experiment 6 found differences in behavior between morphine cage-mates and morphine only mice receiving a V1b antagonist. Morphine only mice, but not morphine cage-mates, demonstrated decreased reward after pretreatment with a V1b antagonist, suggesting that the vasopressin/V1b system is partially responsible for the social environment effect on morphine reward in adolescent males.

As suggested above, data from experiment 6 support the notion that levels of vasopressin are higher in morphine only mice compared to morphine cage-mates. Vasopressin synthesis increases during morphine withdrawal (Nunez et al., 2007), but vasopressin levels have only been assessed immediately after naloxone injection in adults. There are no studies that have examined vasopressin expression during protracted spontaneous opioid withdrawal in adolescents. This study examined the hypothesis that morphine only mice have higher expression levels of central vasopressin in limbic target areas.

## *2. Experimental Procedures*

### *Subjects*

Adolescent (PND 28-59) males were examined for this experiment. Briefly, group-housed mice were housed in one of three types of cages: saline only, morphine only, or mixed cages as listed in general methods. Roughly eight mice per group for a total of 31 were used for this experiment. Dissections were unable to be done on 2 brains; 29 brains total were examined in this experiment.

### *Injection Paradigm*

Injection paradigm was the same as listed above in general methods. Briefly, mice received twice daily injections of saline or increasing doses of morphine (10-40 mg/kg, s.c.) for 6 days.

### *Tissue Collection*

On WD9, mice received sodium pentobarbital (100 mg/kg, i.p.). Whole brains were removed and frozen immediately with methylbutane. Brain sections were extracted from amygdala that included central amygdala, basolateral amygdala, lateral amygdala, cortical medial nucleus of amygdala, medial amygdala, and parts of piriform cortex and extended from -1.58 to -1.94 mm from bregma, 4 to 6 mm deep, +/- 1.5 to 4 mm lateral; septum that included medial and lateral subregions and extended from 0.50 to 0.26 mm from bregma, 2 to 4 mm deep, -1 to 1 mm lateral; and hypothalamus that included paraventricular nucleus, supra-chiasmatic nucleus, lateral hypothalamus, arcuate nucleus,

ventromedial hypothalamus and extended from -0.36 to -0.94 mm from bregma, 4.5 to 6 mm deep, -1 to 1 mm lateral. Brain sections were stored at -80°C.

### *Quantitative PCR*

RNA was extracted from brain sections using RNeasy Lipid Tissue Mini Kit according to assay instructions (Qiagen, Valencia, CA, USA) and total RNA was quantified using a Nanodrop 1000 spectrophotometer (ThermoScientific, Waltham, MA, USA). Extracted RNA was made into cDNA using the High Capacity cDNA Reverse Transcription Kit per assay instructions (Applied Biosystems, Carlsbad, CA, USA). Quantitative reverse transcription PCR using SensiMix SYRB Hi-ROX kit (Bioline, Taunton, MA, USA) was conducted on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). All primers were purchased from Eurofins mwg Operon (Huntsville, AL, USA). Primer sequences used in this experiment included: control sequence 18S rRNA forward: 5'-TCCTGCCAGGTAGCATATGCTTGT-3', 18S rRNA reverse: 5'-CGACTCACCACGTCACACACCATC-3'; V1a forward: 5'-GCTTCTGGTCACGCCTTGTGTCAG-3', V1a reverse: 5'-GTGATCGTGGTGGGAAGGGTTTTTCGG-3'; and vasopressin forward: 5'-CTTCTCCTCCGCCTGCTACTTCCA-3', vasopressin reverse: 5'-GGTGCCACGAAGCAGCCCAG-3'. V1b receptor mRNA was measured using 4 different primers, but amplification was too low using all of them to adequately assess

any differences between treatment groups. Differences between groups were calculated using the  $\Delta\Delta\text{Ct}$  method.

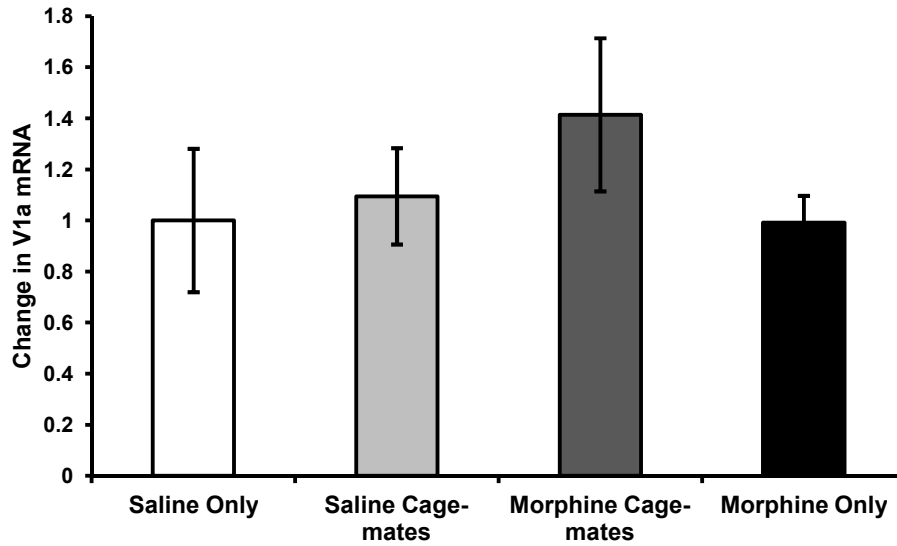
### *Statistics*

Data was analyzed using a 2 (pretreatment) x 2 (cage condition) between-subjects ANOVA for each brain region and mRNA target. Differences between treatment groups were calculated using Bonferroni's post-hoc comparisons. *P* values less than 0.05 were considered statistically significant.

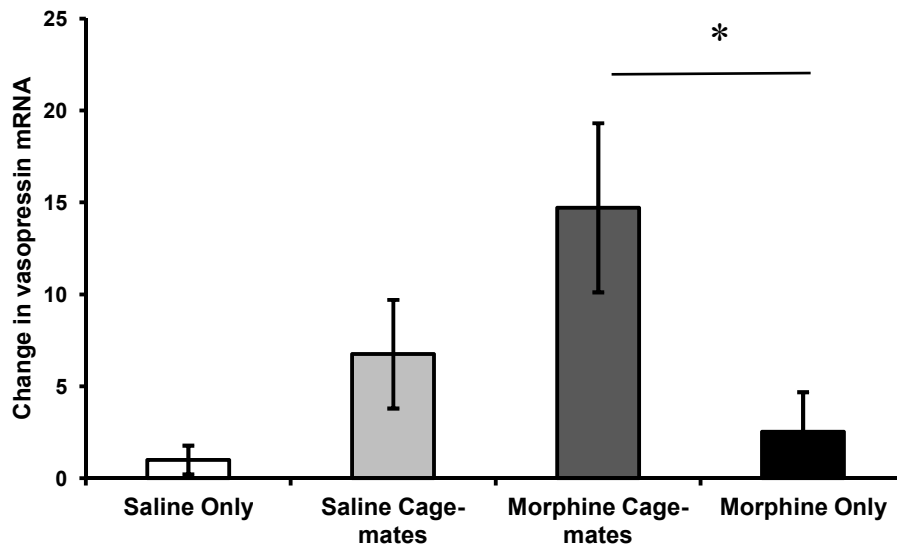
### *3. Results*

For V1a in the amygdala, two-way ANOVA with pretreatment (saline, morphine) and cage condition (mixed cage, 'only' cage) as between-subjects variables revealed no significant main effect of pretreatment ( $p > 0.05$ ), no significant main effect of cage condition ( $p > 0.05$ ), and no significant interaction ( $p > 0.05$ ). Results for V1a in the amygdala are presented in figure 15. For vasopressin in the amygdala, two-way ANOVA revealed a significant main effect of cage condition  $F(1, 24) = 8.20, p < 0.01$  but no main effect of pretreatment ( $p > 0.05$ ), and no significant interaction ( $p > 0.05$ ). Bonferroni's post-hoc analyses revealed significant differences in vasopressin mRNA between morphine cage-mates and morphine only mice (morphine only < morphine cage-mates,  $p < 0.05$ ). No other significant differences were observed ( $p > 0.05$  for all). Results for vasopressin in the amygdala are presented in figure 16.

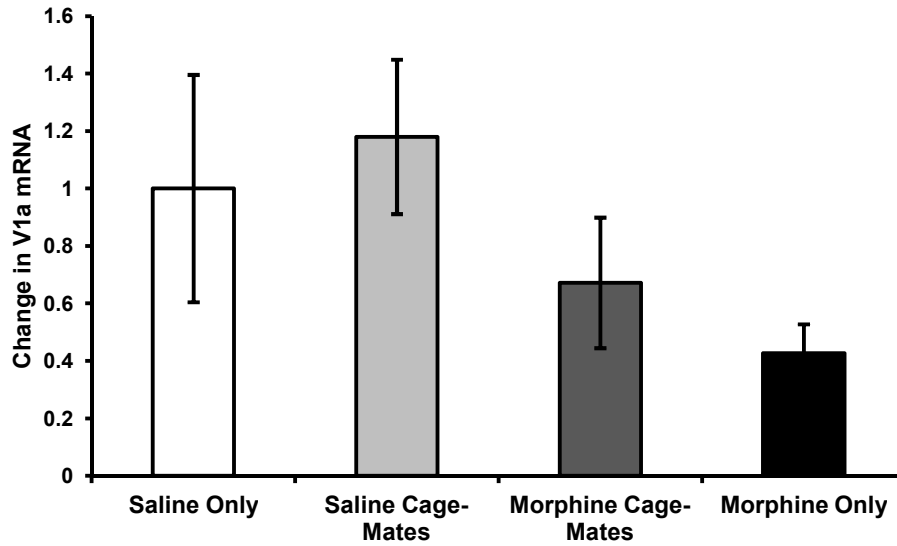




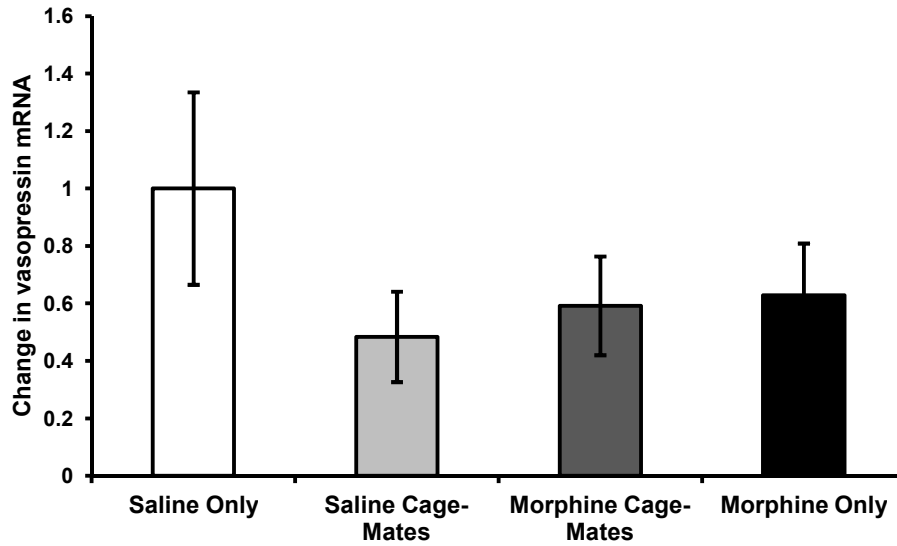
*Figure 15:* V1a receptor mRNA levels in amygdala. Saline only,  $n = 6$ ; saline cage-mates,  $n = 7$ ; morphine cage-mates,  $n = 8$ ; and morphine only,  $n = 8$ . Results are presented as means  $\pm$  SEM.



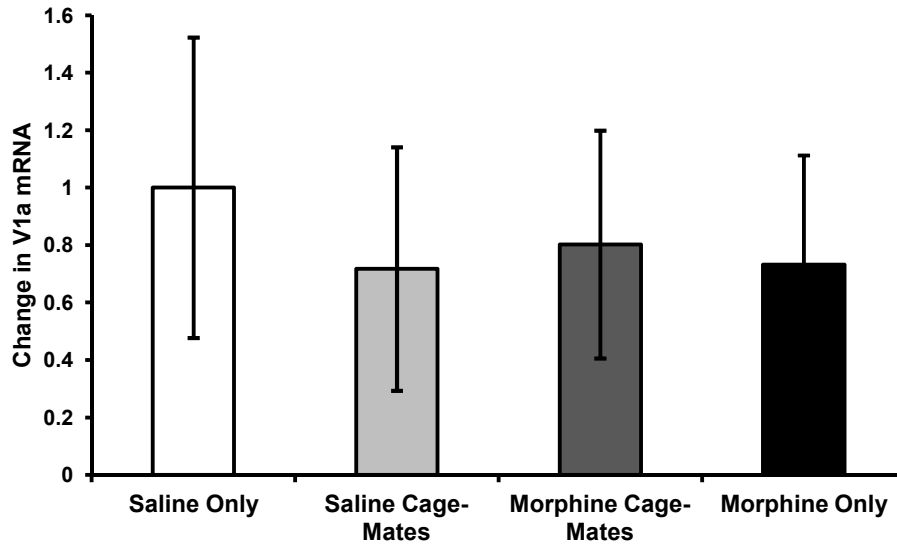
*Figure 16:* Vasopressin mRNA levels in amygdala. Saline only,  $n = 6$ ; saline cage-mates,  $n = 7$ ; morphine cage-mates,  $n = 8$ ; and morphine only,  $n = 8$ . \* Significant difference in vasopressin mRNA between morphine cage-mates and morphine only mice. Results are presented as means  $\pm$  SEM.



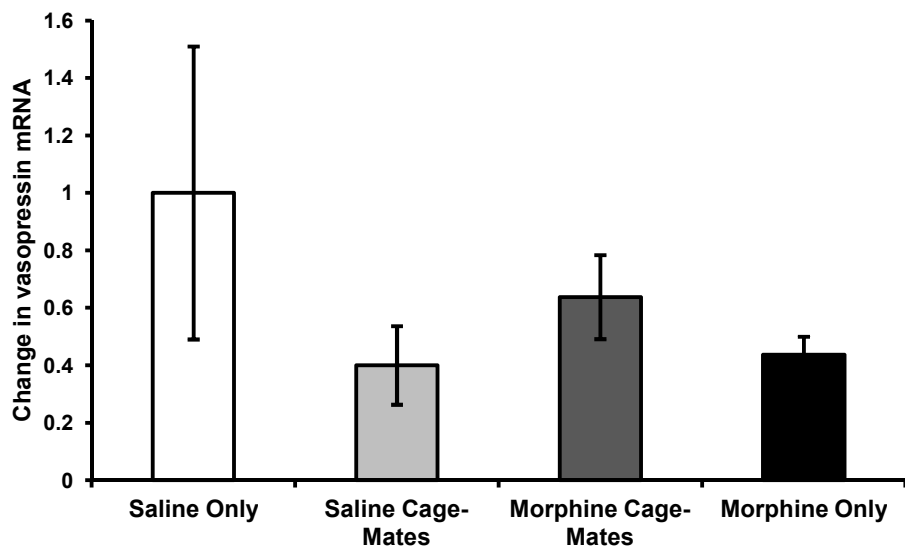
*Figure 17:* V1a receptor mRNA levels in septum. Saline only,  $n = 6$ ; saline cage-mates,  $n = 7$ ; morphine cage-mates,  $n = 8$ ; and morphine only,  $n = 8$ . Results are presented as means  $\pm$  SEM.



*Figure 18:* Vasopressin mRNA levels in septum. Saline only,  $n = 6$ ; saline cage-mates,  $n = 7$ ; morphine cage-mates,  $n = 8$ ; and morphine only,  $n = 8$ . Results are presented as means  $\pm$  SEM.



*Figure 19:* V1a receptor mRNA levels in hypothalamus. Saline only,  $n = 6$ ; saline cage-mates,  $n = 7$ ; morphine cage-mates,  $n = 8$ ; and morphine only,  $n = 8$ . Results are presented as means  $\pm$  SEM.



*Figure 20:* Vasopressin mRNA levels in hypothalamus. Saline only,  $n = 6$ ; saline cage-mates,  $n = 7$ ; morphine cage-mates,  $n = 8$ ; and morphine only,  $n = 8$ . Results are presented as means  $\pm$  SEM.

For V1a in the septum, two-way ANOVA revealed a significant main effect of pretreatment  $F(1, 25) = 5.49, p < 0.05$ , but no significant main effect of cage condition ( $p > 0.05$ ), and no significant interaction ( $p > 0.05$ ). Bonferroni's post-hoc analyses revealed no significant differences in V1a mRNA between any of the groups ( $p > 0.05$ ). Results for V1a in the septum are presented in figure 17. For vasopressin in the septum, two-way ANOVA revealed no significant main effects ( $p > 0.05$  for pretreatment and cage condition) and no significant interaction ( $p > 0.05$ ). Results for vasopressin in the septum are presented in figure 18.

For both V1a and vasopressin in the hypothalamus, two-way ANOVA revealed no significant main effects ( $p > 0.05$  for pretreatment and cage condition) and no significant interactions ( $p > 0.05$ ). Results for V1a in the hypothalamus are presented in figure 19 and results for vasopressin in the hypothalamus are presented in figure 20.

#### *4. Discussion*

The current experiment found a social environment effect on synthesis of vasopressin and V1a receptor. Unexpectedly, expression of V1a receptor was lower in morphine-treated mice (morphine only and morphine cage-mates) compared to saline-treated mice (saline only and saline cage-mates) in septum. In amygdala, vasopressin levels were significantly lower in morphine only mice compared to morphine cage-mates, which was contrary to the hypothesis. No significant differences were found in either V1a or vasopressin expression in hypothalamus.

It was not surprising that no differences were found in vasopressin or V1a expression in hypothalamus; vasopressin in this brain area does not mediate social behavior (Arakawa et al., 2010, Veenema et al., 2010). The surprising part of this study was the difference in vasopressin expression in the amygdala. Experiment 6 found that antagonism of V1b receptor decreased morphine CPP in morphine only mice but not morphine cage-mates and this suggested that vasopressin levels would be higher in morphine only mice compared to the morphine cage-mates. This was not confirmed, as vasopressin mRNA in amygdala had higher levels of expression in morphine cage-mates compared to morphine only mice.

However, only levels of mRNA were assessed in this experiment. It is possible that a difference in vasopressin signaling is due to altered release of vasopressin or altered synthesis of vasopressin. Lower rates of release and/or synthesis might be causing a compensatory increase in mRNA production. According to this assumption, vasopressin mRNA might be higher in morphine cage-mates compared to morphine only mice because vasopressin synthesis or release was lower in this group.

Although experiment 6 suggests that vasopressin influences the expression of the social environment-induced alteration of morphine sensitivity, the brain region responsible for this effect is still unknown. It likely involves a limbic brain region that expresses high levels of vasopressin receptors and/or cell bodies, such as amygdala or septum (De Vries and Panzica, 2006). While the amygdala was the brain region that demonstrated a difference in vasopressin mRNA levels between morphine only and morphine cage-mates, it might not be the brain area responsible for the social



environment-induced difference in morphine sensitivity. In the septum, there was significantly less V1a mRNA expression in morphine-treated groups compared to the saline-treated groups although there was no difference between morphine cage-mates and morphine only mice. This might reflect a homeostatic downregulation of this receptor after a period of increased activation by vasopressin during morphine intoxication or, more likely, during early withdrawal (Nunez et al., 2007). While levels of V1b were not assessed in this experiment due to low amplification, it is possible that levels of V1b would also be lower in morphine-treated mice due to increased vasopressin release during early spontaneous withdrawal. This idea is still compatible with experiment 6 because V1b antagonism might be causing a general decrease in reward in morphine-treated mice. Any decrease in reward would not be observable in morphine cage-mates due to their low level of morphine CPP.

## CHAPTER X

### GENERAL DISCUSSION AND CONCLUSION

This set of studies is the first to demonstrate a role of peers in sensitivity to morphine in a rodent model. These experiments manipulated social housing condition by placing adolescent or adult mice in either ‘saline only’ cages, where all 4 mice in a cage received repeated saline injections over 6 days, ‘morphine only’ cages, where all 4 mice received repeated morphine injections, or in ‘mixed’ cages, which consisted of two mice that received morphine and two mice that received saline. Mice that received saline in mixed cages were referred to as ‘saline cage-mates’ and mice that received morphine in mixed cages were referred to as ‘morphine cage-mates’. This set of experiments examined how changes in social housing environment affected morphine sensitivity and hormone production during adolescence. Experiment 1 measured the testosterone level of mice in mixed cages and saline only cages and found that testosterone level is lower in adolescent mice housed in mixed cages compared to saline only cages on WD9. This was true for both the saline cage-mates and the morphine cage-mates. Adult morphine cage-mates only demonstrated decreased testosterone production on WD1; this recovered by WD9 (Hofford et al., 2011).

In addition to affecting hormone production, social environment has also been shown to affect responses to drugs of abuse (Gipson et al., 2011b), so experiment 2 examined one behavioral outcome of morphine administration, enhanced locomotion. Both adolescent morphine cage-mates and adult morphine cage-mates demonstrated

locomotor sensitization (i.e. they hyperlocomoted compared to their respective saline only groups). However, adolescent saline cage-mates hyperlocomoted to an acute morphine injection as well. The magnitude of locomotor increase after morphine injection was nearly the same in adolescent morphine cage-mates and adolescent saline cage-mates (Hodgson et al., 2010). This suggests that proximity to morphine-treated mice somehow increases sensitivity to morphine in previously drug-naïve adolescent mice, but this effect was not observed in adults.

Due to the difference in testosterone production found in experiment 1 (Hofford et al., 2011) and its role in sex-dependent play behavior during adolescence (Meaney and Stewart, 1981), experiment 3 examined the sex-dependence of the social environment effect on morphine locomotor sensitivity. Social environment helped determine locomotor sensitivity in adolescent females. However, the difference was not found between the saline-treated groups, but was found between the morphine-treated groups. Both morphine cage-mates and morphine only female adolescents demonstrated locomotor sensitization, but morphine only mice locomoted significantly more than morphine cage-mates (Hofford et al., 2010). Taken together with the results of experiment 2, this suggests that social environment alters adolescent responses to morphine, but in a sex-dependent manner.

While locomotor sensitization is often used as an initial screen of a drug's abuse potential, more direct measures of drug reward are often more appropriate in assessing addiction-like behavior in rodents. Experiment 4 measured drug reward in adolescent male mice using the CPP paradigm. This experiment revealed another social

environment-induced behavioral effect, with adolescent morphine only mice preferring the morphine-paired chamber significantly more than all other groups, including morphine cage-mates. This was considered reward sensitization because mice with previous morphine experience required less pairings of morphine to achieve morphine CPP. This was not observed in the morphine cage-mate mice. Additionally, saline cage-mates developed morphine place preference at 40 mg/kg, but this was not observed in saline only mice (Cole et al., 2012).

Experiments 2, 3, and 4 determined the behavioral consequences of social environment on tests of drug responsiveness. However, the underlying neurobiological cause of this age- and sex-dependent phenomenon was still unknown. While evidence from this dissertation suggested that testosterone level fluctuated based on an interaction between morphine treatment and social environment (Hofford et al., 2011), it was unlikely to be the causative factor because there is no known role of testosterone in morphine reward. The most plausible explanation was that stress simultaneously decreased testosterone production (Blanchard et al., 1993) and caused cross-sensitization (Sinha et al., 1999) in both saline cage-mates and morphine only to the locomotor stimulating properties of morphine and the rewarding properties of morphine, respectively. This stress was hypothesized to come from increased fighting or disruptions in social status by morphine administration and withdrawal (Blanchard et al., 1993, Covington and Miczek, 2001). Thus, experiment 5 measured levels of the rodent stress hormone, corticosterone, to assess the possible role of stress in the expression of the social environment-induced effect on morphine sensitivity in adolescents. No

differences in corticosterone levels were observed on WD9, the same time behavior was conducted in the previous experiments. From this it was concluded that stress is not solely responsible for the social environment effect.

While it is unlikely that either testosterone or corticosterone is responsible for the social environment-induced enhancement of morphine sensitivity, it is important to note that these two hormone systems work in two separate feedback loops that can influence each other. Interestingly, both testosterone and corticosterone synthesis are regulated, in part, by vasopressin. Increases in vasopressin cause increases in testosterone (Auger et al., 2011) and corticosterone (Rivier and Vale, 1983). Most importantly, this hormone acts centrally to regulate social behavior in males (Everts and Koolhaas, 1997). Thus, experiment 6 tested the idea that vasopressin modulated the social housing-induced enhancement of reward seen in morphine only adolescents during CPP. This was done by administering a vasopressin V1b antagonist to adolescent morphine cage-mates and morphine only mice before their morphine-chamber pairing. Pretreatment with V1b antagonists resulted in decreased reward in morphine only animals with no change in reward in morphine cage-mates. Based on these studies, vasopressin helped mediate the increase in reward that was previously observed in morphine only mice.

Because morphine reward was decreased after pretreatment with a V1b antagonist in the morphine only adolescents, it was hypothesized that vasopressin levels were higher in morphine only mice compared to morphine cage-mates. Experiment 7 measured levels of vasopressin mRNA in hypothalamus, amygdala, and septum. The latter two areas are crucial for vasopressin's roles in social behavior (De Vries and

Panzica, 2006). Surprisingly, amygdalar vasopressin mRNA levels were significantly higher in morphine cage-mates compared to morphine only mice. There was a significant pretreatment difference in V1a expression in septum, but no interaction with social environment.

These experiments modeled adolescents' propensity to develop drug abuse as determined by drug treatment of their peers. The focus was placed on adolescents' responses because previous literature suggests that adolescents are heavily influenced by their peers (Graham et al., 1991). Combined with increases in perceived stress (Spear, 2000) and vulnerability of brain areas still under development (Giedd et al., 1996), it is not surprising that the social environment-induced differences in drug sensitivity observed in these experiments were unique to adolescents.

The fact that peers have so much influence on measures of drug sensitivity and hormone regulation are not surprising, even in this rodent model. Housing environment affects a variety of rodent behavior (Mustroph et al., 2012) including reward processing (Gipson et al., 2011a). More specifically, the aspect of environment that is most important in affecting this behavioral change seems to be the presence of cage-mates. Animals housed in groups demonstrate diminished drug taking compared to animals housed in isolation (Raz and Berger, 2010, Lopez et al., 2011). Moreover, some studies suggest that the quality of social interaction between cage-mates matters as well. Active, playful partners with previous social experience are preferred over peers without social experience (Calcagnetti and Schechter, 1992, Douglas et al., 2004). Not surprisingly, the importance of social experience in a peer differs by age, with the largest difference in

preference between isolated and group-housed peers observed in adolescents and juveniles (Calcagnetti and Schechter, 1992, Douglas et al., 2004).

Results from this dissertation clearly show that vasopressin influences the expression of the social environment effect. In experiment 6, a V1b selective antagonist reduced morphine CPP in morphine only mice, but did not alter reward in morphine cage-mates. This suggested that vasopressin would be higher in morphine only mice compared to morphine cage-mates. However, vasopressin was not higher in this group in any of the brain regions examined. In fact, vasopressin mRNA was significantly lower in morphine only mice compared to morphine cage-mates in the amygdala.

A few things could explain this discrepancy. For one, the technique used only measured synthesis of mRNA. It is possible that this mRNA is never made into protein. Additionally, none of the experiments in this dissertation measured vasopressin release. There certainly are many contributing factors in the release of a neuropeptide and it is possible that social environment actually modulates the release of vasopressin and not its synthesis. Second, vasopressin levels might have been high in both morphine-treated groups at some time before WD9 in a brain area other than the amygdala. In theory, this could have caused a long-lasting downregulation of vasopressin receptors in some limbic brain regions. Unfortunately, levels of V1b were not assessed due to technical difficulty. However, morphine-treated mice expressed less V1a mRNA in septum, which does support this general idea. Although not detectable using quantitative RT-PCR, V1b receptor might also be lower in septum if vasopressin levels were higher in this area sometime before WD9. The differences in morphine CPP after V1b antagonism could

have been observed simply because morphine cage-mates did not demonstrate morphine CPP at all. Any reduction in reward in this group would not be detectable. Neither of these possibilities negate the finding that vasopressin is directly mediating the expression of the social environment-induced morphine reward sensitization seen in morphine only mice. The brain area responsible for this effect still needs to be identified.

Vasopressin is clearly involved in the expression of the social environment-induced effect. However, two aspects of vasopressin's role remain unexplained. One, how does social environment affect vasopressin expression? Two, how does altered vasopressin expression or activity affect morphine reward? There is one candidate mechanism that might explain how social environment affects vasopressin expression, and that is play behavior.

As mentioned above, rodents demonstrate a preference for socially experienced partners over partners that do not interact (Calcagnetti and Schechter, 1992, Douglas et al., 2004). The preference given to socially active partners in the past literature is most likely explained by their ability to engage in play behavior. Consequently, altered play behavior of cage-mates in mixed cages might help explain differential expression of vasopressin. Given morphine's role in decreasing social interaction in mice (Kennedy et al., 2011), it would be expected that cages with the least amount of morphine-dependent animals would engage in the most play behavior. Assuming this, saline only cages would play the most, while morphine only cages would play the least. Decreased play behavior in saline cage-mates and morphine only mice most likely results in decreased vasopressin release in these groups compared to saline only and morphine cage-mates,



respectively. Previous literature supports this; vasopressin level in amygdala is positively correlated with play behavior (Taylor et al., 2012). In fact, some of our results also support this. Experiment 7 demonstrated that morphine only mice express less vasopressin in amygdala compared to morphine cage-mates. This likely reflects a difference in play behavior, with morphine only mice engaging in play behavior less than morphine cage-mates. However, there was no significant difference in vasopressin expression between saline only and saline cage-mates. For this hypothesis to be completely true, saline cage-mates should express less vasopressin than saline only mice. It is possible that saline cage-mates express different levels of vasopressin at some other time point, but this is complete conjecture.

Results from experiment 3 also fit with this hypothesis; adolescent females did not demonstrate the same pattern of locomotor sensitivity to morphine as adolescent males did. Because females do not demonstrate increased play behavior during adolescence like males (Meaney and Stewart, 1981), any decrease in social interaction from the presence of morphine-treated mice might not be affecting females as much. In addition, altered play behavior to the presence of morphine-treated mice would probably not change vasopressin levels in amygdala because play behavior is not correlated with levels of vasopressin in females (Taylor et al., 2012). This hypothesis does not, however, explain why morphine only females demonstrate greater locomotor sensitization than morphine cage-mates. Future experiments need to measure play behavior in the different types of cages throughout pretreatment and withdrawal as well as measure vasopressin synthesis or release throughout those same time periods.

Finally, the question remains as to how altered vasopressin signaling might be affecting reward. Very few studies exist that examine this question directly. Some studies demonstrate a role of V1b in alcohol and heroin seeking (Zhou et al., 2007, Zhou et al., 2011) but the underlying mechanism was not examined. The authors of these studies suggest that vasopressin affects reward by increasing stress. Vasopressin increases corticosterone and stress (Knepel et al., 1985), by increasing ACTH release in hypothalamus (Rivier and Vale, 1983). Given that increased stress can sensitize mice to drug reward and can increase locomotor sensitivity (Kreek and Koob, 1998, Sinha et al., 1999), increased stress might explain vasopressin's role in morphine reward. This hypothesis is not congruent with the results from experiment 7 that demonstrated decreased vasopressin mRNA in morphine only mice compared to morphine cage-mates. As proposed above, it is possible that vasopressin levels are higher earlier in withdrawal and are decreased by WD9. Vasopressin mRNA levels are higher during early morphine withdrawal (Nunez et al., 2007), which supports this general idea.

There are likely many factors contributing to the social environment-induced alteration of morphine sensitivity found in adolescents. Lack of play behavior, stress, and vasopressin are interacting in some way to affect morphine reward in adolescents in a sex-dependent manner. It is important to note that these behaviors are age-specific, as adults did not demonstrate any social housing effects. Regardless of its cause, more studies need to be conducted on the role of vasopressin and V1b receptors on morphine reward in adolescents after exposure to morphine-dependent and morphine-naïve cage-mates.

This set of experiments characterized the behavioral responses to morphine when adolescent rodents were housed in different social environments. These studies helped establish an animal model of peer influence on drug sensitivity as well as helped build the foundation for examining the underlying neurobiological mechanisms that contribute to social effects on the development of drug abuse in adolescence. Human addiction is a complicated disorder and is unlikely to have a simple solution. However, it may be possible to decrease the incidences of opioid abuse by understanding the neurobiological effects of adolescents' social networks. This may provide a pharmacological (targeting the V1b receptor) as well as a non-pharmacological intervention in the treatment and prevention of this disorder in young people.

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