ACCESS TO RUNNING WHEELS ATTENUATES SPONTANEOUS MORPHINE WITHDRAWAL IN MICE AS MEASURED BY THERMAL SENSITIVITY.

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ABSTRACT

REBECCA BALTER: Access to running wheels attenuates spontaneous morphine withdrawal in mice as measured by thermal sensitivity
(Under the direction of Linda A. Dykstra)

Opioid withdrawal is a critical component of opioid abuse and consists of a wide array of symptoms. For many people, the presence of, or desire to avoid, these withdrawal symptoms drives continued drug taking. There is growing evidence that aerobic exercise may be a positive intervention during the withdrawal period. The following studies seek to develop a behavioral procedure to examine one component of spontaneous opioid withdrawal in mice, hypersensitivity to a thermal stimulus, and to examine the effects of access to a running wheel during withdrawal. The experiments of Chapter 2 describe and validate the spontaneous withdrawal procedure. During the first 48 hours following the cessation of 30, 56, or 100 mg/kg morphine response latency on a hotplate is significantly decreased suggesting an increase in thermal sensitivity. The experiments described in Chapter 3 demonstrate that access to a running wheel during withdrawal reduced this increase in thermal sensitivity. Chapter 4 extended the previous results, assessing the effect of a locked wheel and group housing during withdrawal. The results provide evidence that use of the wheel not simply environmental enrichment maximized the effect on thermal sensitivity. The experiments of Chapter 5 sought to further probe the effects of wheel access.

Morphine's potency was assessed following 6 weeks of wheel access or chronic morphine injections. Under both conditions, tolerance to the antinociceptive effects of morphine developed. Immediately following behavioral testing, changes in the expression of five genes associated with the opioid system was assessed using qRT-PCR. The experimental results descried in this dissertation suggest that thermal sensitivity is a reliable and sensitive measure of spontaneous morphine withdrawal in mice and that wheel access can attenuate this sign of withdrawal.

This dissertation is dedicated to my late father, Dr. Robert Balter. You taught me that the pursuit of knowledge is a noble crusade.

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ANOVA	analysis of variance
ARRB	beta-arresting 2
C	Celsius
cAMP	cyclic adenosine monophosphate
CL	confidence limits
cm	centimeters
CREB	cAMP response element-binding protein
CRF	corticotropin releasing factor
DAMGO	[D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin
ED50	effective dose, 50%
ET10	effective time, 10 seconds
g	gram
GIRK	GPCR kinases
GPCR	G-protein coupled receptors
hr	hour
kg	kilogram
mg	milligram
MOR1	mu opioid receptor 1
MPE	maximum possible effect
PAG	periaqueductal grey
PDYN	prodynorphin
PENK	proenkephalin

POMC	proopiomelanocortin
SEM	standard error of the mean
s.c.	subcutaneous

Chapter 1

General Introduction

> [Helen] quickly dropped into the wine they were enjoying
> a drug which eased men's pains and irritations,
> making them forget their troubles.
> Odyssey IV:220-221

It is widely assumed that the pain-easing drug referred to by Homer was an opium-based preparation. Centuries later, Edgar Allen Poe references the drug by its Greek descriptor in his poem "The Raven", summoning its abilities to ease the mind: "Let me quaff this kind Nepenthe and forget this lost Lenore!" Despite all of the advances of modern medicine, morphine and its derivatives are still some of the most effective analgesics for many types of clinical pain. Unfortunately, the opioid's ability to ease the pain of the spirit ensures that it is often abused as well.

Conservatively, 2-6% of patients prescribed long-term opioids and up to 30% of illicit users develop drug dependence (Christie, 2008). This dependence is often driven by the ease and degree to which tolerance to opioids can form. Long-term illicit opioid users report consumption of doses up to a hundred fold higher than acutely effective doses (Stanford et al., 2004). Consistent consumption of such high doses often leads to physical dependence and the appearance of withdrawal symptoms when drug taking is terminated. For many

people, the presence of, or desire to avoid, these withdrawal symptoms drives continued drug taking (Le Moal and Koob, 2007). Consequently, understanding and treating opioid withdrawal is a critical component of treating opioid abuse. This dissertation presents the rationale and results from a series of studies that 1) developed a behavioral procedure to examine one component of opioid withdrawal, i.e., hypersensitivity to a thermal stimulus, 2) examined the effects of access to a running wheel on withdrawal following the development of morphine tolerance and 3) examined changes in gene expression and morphine sensitivity following chronic access to a running wheel.

The opioid receptors: expression and anatomy

To date, three opioid receptors have been identified: mu, kappa, and delta. The mu-opioid receptor, named for its stereotypical ligand morphine, shows the broadest distribution and is found throughout the brain and spinal cord. The highest concentrations can be found in the striatum, nucleus accumbens, amygdala, periaqueductal grey, and locus coeruleus (Daunais et al., 2001; McClung 2006; McDonald and Lambert 2005).

Extensive research suggests that activity in the nucleus accumbens is responsible for the reinforcing properties of opioids (Carlezon and Wise, 1996; Einstein et al., 2013; Pettit et al., 1984; Shippenberg et al., 1992; Spyraki et al., 1983; Stinus et al., 1989). Activity in the nucleus accumbens is likely driven by opioid induced excitation of dopaminergic neurons in the ventral tegmental area through the hyperpolarization of local inhibitory GABA-ergic interneurons

(Johnson and North, 1992). However, there is also evidence to support dopamine-independent mechanisms of reinforcement in the nucleus accumbens (Koob and Volkow, 2010).

Stimulation of the periaqueductal grey (PAG) through mu as well as kappa and delta opioid receptor activity is predominantly responsible for the analgesic effects of the opioids. Specifically, enkephalin-releasing neurons of the PAG can trigger the release of serotonin from the raphe nuclei which in turn can activate inhibitory neurons in the dorsal root ganglia reducing afferent nociceptive signaling (see Ossipov et al., 2010 for a review). The PAG as well as the locus coeruleus also play critical roles in opioid withdrawal and will be discussed in more detail later.

Kappa-opioid receptors are most highly expressed in the periaqueductal grey, locus coeruleus and amygdala as well as in the hypothalamus (McClung 2006; Mansour et al., 1995; Knoll et al., 2011). Expression in the basal lateral and central amaygdala seems to be of particular importance in mediating the anxiolytic effects of kappa-opioid receptor antagonists (Knoll et al., 2011). Finally, the highest density of delta-opioid receptors is found in the striatum, nucleus accumbens, olfactory bulb and cerebral cortex (McDonald and Lambert, 2005). Expression throughout the mesolimbic dopamine pathway may provide a neural substrate for the observed anti-depressive effects of delta receptor agonists (Jutkiewicz and Roques, 2012).

The opioid receptors: cellular activity

All three of the opioid receptors belong to a superfamily of 7-transmembrane G-protein coupled receptors (GPCR's) and are predominately found postsynaptically on dendrites and cell bodies of neurons (Ding et al., 1996).

Agonist binding of the receptors triggers the release of the alpha subunit of the coupled G_i/G_o proteins (Pennock and Hentges, 2011). The "i" of G_i references their inhibitory downstream effects. First, opioid-activated G-proteins can activate inward rectifying potassium channels, hyperpolarizing the cell and decreasing the probability of an action potential (Kelly et al., 1990; Law et al., 2000). Neuron excitation is also reduced through decreased conductance of voltage gated Ca^{2+} channels (Childers, 1991). Second, the G-proteins can inhibit adenylate cyclase activity, which leads to a decrease in cyclic AMP, PKA and phosphorylated CREB ultimately decreasing the expression of many genes including cFos, tyrosine hydroxylase, and corticotropin releasing factor (CRF) (McClung, 2006).

The opioid receptors: chronic activation and tolerance

In addition to immediate inhibitory effects on the neuron, agonist binding induces the intracellular phosphorylation of the opioid receptor by GPCR kinases (GIRK's) (Koch and Holt, 2008). The first effect of phosphorylation is a transient desensitization to further activation (Narita et al. 1995, Ueda et al 1995). Phosphorylation also increases the affinity of the receptor for beta-arrestin. Once bound, beta-arrestin accelerates the uncoupling of the receptor from its G-protein

further desensitizing the receptor, possibly through MEK/ERK pathways (Bohn et al., 2000; Connor et al., 2004; Williams et al., 2013). Finally, beta-arrestin facilitates receptor internalization through its association with clathrin (Koch and Hollt, 2008). This arrestin-mediated internalization is a critical first step in the resensitization and recycling of receptors back to the cell surface (Koch and Hollt, 2008)

It is important to note that desensitization does not always lead to internalization. In particular, morphine produces strong receptor desensitization but fails to promote efficient internalization and consequent resensitization (Bohn et al., 2004). By contrast, DAMGO (a synthetic endorphin), triggers strong internalization (Connor et al., 2004). In general, it appears that the relative ability of opioids to induce endocytosis is inversely correlated with their ability to induce opioid tolerance (Williams et al., 2013). This relative ability is sometimes referred to as an agonist's RAVE value (Relative Activation Versus Endocytosis) (Martini and Whistler, 2007). This somewhat heuristic model suggests that agonists with a high RAVE value (high activation, little endocytosis) like morphine have increased potential to produce tolerance and dependence (Whistler et al. 1999).

As tolerance develops following chronic opioid receptor activation, adenylate cyclase becomes superactivated to compensate for extended inhibition, allowing depressed cAMP levels to return to normal (Koch and Holt, 2008; Watts and Neve, 2005). It is likely that tolerance is also mediated by circuit level mechanisms as indicated by the role of the NMDA receptor in the formation of tolerance (Dykstra et al., 2011). These are a few of the most studied

mechanisms that contribute to tolerance, however, it is clear that no single mechanism can account for the massive degree of opioid tolerance that is often observed.

Opioid withdrawal: cellular mechanisms

Newton's third law of motion states that "to every action there is always an equal and opposite reaction". Though far from the realm of 18th century physics, this quite elegantly describes the theoretical framework for drug withdrawal. In the field of substance abuse, the opponent-process theory suggests that withdrawal is the product of an equal but opposite response to its foil, tolerance (Radke et al., 2011). Chronic drug exposure requires the establishment of a set of physiological parameters far outside the normal homeostatic range in order to maintain systemic stability (Sterling and Ever, 1988). This state of chronic deviation, consisting of all of the changes that allow for drug tolerance, is the allostatic state (Koob and LeMoal, 2001).

Consistent with the allostasis theory, opioid withdrawal is likely the result of hyper-excitation of brain regions and cellular processes that were chronically inhibited during extended opioid exposure. On a cellular level, cessation of opioid exposure should produce an increase in the phosphorylation of CREB via hyperactivity of sensitized adenylate cyclase (Nestler and Aghajanian, 1997; Sharma et al., 1975). Morphine withdrawal dependent increases in phosphorylated CREB have in fact been seen in both the hypothalamus and nucleus accumbens (Li et al., 2010; Martin et al. 2011).

Of the various genes whose expression is under the control of CREB, corticotropin-releasing factor or CRF may be the most important. Suppression of CRF signaling to both the amygdala and nucleus accumbens can attenuate morphine withdrawal symptoms (Almela et al., 2012; Heinrichs et al., 1995; McNally and Akil, 2002). Additionally, CRF signaling can trigger an increase in dynorphin expression in the nucleus accumbens, which contributes to the negative affective state of withdrawal (Contarino and Papaleo, 2005). Finally, aside from the pituitary gland, the locus coeruleus is probably the most important afferent structure for hypothalamic CRF signaling. Activation of the locus coeruleus causes the release of norepinephrine which drives the "fight or flight" state produced by the sympathetic branch of the central nervous system (McClung, 2006; Brodal, 2004). Not surprisingly, many of the bodily responses associated with sympathetic activation are also symptoms seen during opioid withdrawal (e.g. elevated pulse, sweating, pupil dilation).

In the drug –naïve brain, endogenous opioids play an inhibitory role and counterbalance the excitatory effects of CRF on the locus coeruleus-norepinephrine system (Curtis et al., 2001); chronic opiate administration is thought to sensitize locus coeruleus neurons to the effects of CRF (Xu, 04). The cessation of exogenous opioid administration unveils the full effects of CRF activation of the sensitized noradrenergic system (Curtis et al., 1997).

Although the locus coeruleus clearly plays an important role in opioid withdrawal, it is not necessary for opioid withdrawal. Caille et al. (1999) precipitated morphine withdrawal in rats with almost complete lesions of the

locus coeruleus. They and others conclude that opioid withdrawal also requires action in the anatomically adjacent periaqueductal grey (see review by Christie et al., 1997).

In fact, chronic morphine infusions directly into the periaqueductal grey (PAG) are sufficient to produce physical dependence in rats (Bozarth and Wise 1984). Once dependent, infusions of an opioid antagonist into the animal's PAG can precipitate withdrawal (Maldonado et al., 1992). Furthermore, morphine withdrawal triggers an increase in PAG expression of enkephalins, likely through activation of the cAMP/CREB pathway (Folkesson et al., 1989). Interestingly, infusions of enkephalin analogs into the PAG will suppress both precipitated and spontaneous withdrawal, suggesting that the PAG may also be a site of modulation of withdrawal (Fukunaga and Kishioka, 2000). Finally, opioid withdrawal is associated with rebounds in GABA-ergic signaling in the PAG (Hack et al., 2003).

Opioid withdrawal: experimental evaluation

Humans

Opioid withdrawal has been measured in many ways, most commonly by examining a range of physical symptoms (Wesson and Ling, 2003). The first withdrawal scale was published by Lawrence Kolb and C.K. Himmelsback in 1938 in the Journal of Clinical Psychiatry. A revised version from 1941 is often cited as the Himmelsback scale. More recently, Handelsman et al. (1987) developed a pair of scales to assess the subjective and objective symptoms; the

Subjective Opiate Withdrawal Scale (SOWS) and Objective Opiate Withdrawal Scale (OOWS). In 1990, a new SOWS, the short opioid withdrawal scale, was developed by Gossop. It includes 10 measures of both objective and subjective symptoms: feeling sick, stomach cramps, muscle spasms/twitching, feeling of coldness, heart pounding, muscular tension, aches and pains, yawning, runny eyes, and insomnia. The format was slightly modified by Wesson and Ling (2003) to produce the current clinical opiate withdrawal scale (COWS) which includes eleven measures of objective and subjective symptoms. These scales have been used by both researchers and health care professionals (e.g. Chu et al., 2009; Tompkins et al., 2009; Umbricht et al., 2003). In addition to multi-symptom scale, many studies have used changes in body temperature, heart rate and pain sensitivity (hyperalgeisa) to assess withdrawal (Himmelsbach, 1942; Martin and Jasinski, 1969). Hyperalgeis in particular has been reported during spontaneous withdrawal in pain patients in experimental settings (Lipman and Blumenkopf, 1989) as well as in case studies (Devulder et al., 1996). Additionally, healthy human subjects show hyperalgesia during both spontaneous (Angst et al., 2003) and antagonist precipitated withdrawal (Compton et al., 2003; Sun, 1998).

Non-human Primates

Concurrent with the initial development of opioid withdrawal scales for humans was the development of the first withdrawal scale tailored to non-human primates (Seevers, 1936). Seevers' scale included a wide range of symptoms

quite similar to those seen in humans indicative of mild (yawning, shivering, hiccups, etc), moderate (tremor, anorexia, cramps, etc) and severe withdrawal (vomiting, diarrhea, insomnia, crying, etc). Variations of this scale have been used to assess withdrawal in the decades since (e.g. Deneau and Seevers, 1963; Holtzman and Villarreal, 1969; Sell et al., 2005). In addition to somatic symptoms, a number of research groups have used changes in operant responding to assess opioid withdrawal. One approach, measures disruption in food reinforced responding to quantify withdrawal (e.g. Thompson and Schuster,1964; Holtzman and Villarreal, 1973). A second approach uses drug discrimination to identify interoceptive withdrawal states in which naltrexone is used as the discriminative stimulus (Brandt and France, 1998; Becker et al., 2008; France and Woods, 1989; McMahon et al., 2009).

Rodents

The majority of rodent studies assess opioid withdrawal by measuring the presence of behavioral signs such as jumping, wet dog shakes, piloerection, diarrhea, writhing, and ptosis (e.g. Kest et al., 2002; Papaleo and Contarino 2006). An alternative approach assesses changes in body temperature, heart rate, and blood pressure (Froger-Colleaux et al., 2011) Though some studies consider a single sign of withdrawal, most use a weighted scale adapted from the first global rodent scale described by Gellert and Holtzman (1978). Such an approach mimics the withdrawal scales used in humans and primates. Although many symptoms are consistent across species such as tremors, diarrhea and

piloerection (goose bumps), one of the most striking behaviors, jumping, is unique to rodents. Jumping during opioid withdrawal was first described in 1969 in a paper by Way et al. Although the original procedure measured the number of rats that jumped off a platform, recent experiments measure the number of times a mouse or rat jumps when contained inside a beaker or activity chamber.

Beyond somatic signs of withdrawal, conditioned place aversion is often used to evaluate the aversive state produced during withdrawal (e.g. Gómez-Milanés et al., 2012; Wang et al., 2012). The elevated plus maze, open field test, and Morris water maze can also be used to assess the cognitive and anxiogenic effects of withdrawal (Miladi-Gorji et al., 2011, 2012). Although these approaches are excellent for answering many questions, they are limited in their ability to measure subtle changes during withdrawal produced by behavioral interventions. Many of the somatic signs appear in a binary present/absent dichotomy. Conditioned place aversion and cognitive measures may provide more subtle data but are limited to precipitated withdrawal and are sensitive to repeated testing, respectively.

Hyperalgesia, another measure of withdrawal, was first presented by Tilson et al. in 1973. They reported that sensitivity to electric foot shock increases following the cessation of chronic morphine in rats. Like many of the somatic symptoms, hyperalgesia has translational validity considering that “an increase in pain or sensitivity to pain” is one of the symptoms that make up the Clinical Opiate Withdrawal Scale, used to assess withdrawal in humans.

Since Tilson's 1973 study, a modest number of papers have described hyperalgesia in animal models of opioid withdrawal. In rats, hyperalgesia occurs during both precipitated and spontaneous morphine withdrawal (Devillers et al., 1995; Dunbar and Pulai, 1998; Grilly and Gowans, 1986; Jin et al., 2012; Li et al., 2001). Hyperalgesia in rats also occurs during withdrawal from other opioids such as fentanyl (Laulin et al., 2002) and heroin (Devillers et al., 1995; Laulin et al., 1998).

To the best of our knowledge only two prior studies employ a hyperalgesia model for examining opioid withdrawal in mice. Rubovich et al. (2009) examine only a single time point during spontaneous withdrawal and Crain and Shen (2007) employ a precipitated withdrawal procedure.

Opioid withdrawal: existing treatments

As stated previously, for many people, the presence or desire to avoid withdrawal symptoms will drive continued drug taking (Le Moal and Koob, 2007). As such, having effective treatments for withdrawal is a critical component of addiction treatment. In the 1960's, the introduction of methadone replacement therapies revolutionized the treatment of opioid addiction by providing an effective pharmacological intervention. Though methadone is still the primary long-term treatment for opioid dependence, there is increasing support for buprenorphine, a low efficacy mu agonist, as an alternative agonist replacement therapy (e.g. Connock et al., 2007; Kraus et al., 2011). The primary advantages of buprenorphine are its greatly decreased risk of respiratory depression and its

ability to suppress spontaneous opioid withdrawal symptoms during the induction phase of treatment (Strain et al., 2011). Additionally the anxiolytic, clonidine, and the opiate antagonist naloxone are approved as detoxification treatments (Nicholls et al., 2010).

Though these treatments are highly effective, they all have unwanted effects including constipation, nausea and respiratory depression (Fiellin et al., 2002). Additionally some methadone maintained patients still report cue-induced cravings that increase the risk of relapse (Fareed et al., 2011). Finally, there are always questions about potential abuse and/or diversion of these compounds.

Improving existing treatments

The American Psychological Association, as well as most treatment programs, emphasizes the fact that treatment effectiveness is optimized when pharmacological interventions are combined with psychosocial approaches. At the present time, there is growing interest and evidence for exercise as a positive behavioral intervention for optimizing the treatment of drug addiction.

Specifically, it has been reported that short periods of aerobic exercise can decrease the desire for alcohol (Ussher et al., 2004), tobacco (Taylor and Katomeri, 2007) and cannabis (Buchowski et al., 2011) in humans. Exercise has also been shown to reduce symptoms of nicotine withdrawal and aids in smoking cessation (Taylor and Ussher, 2005; Taylor and Katomeri, 2007).

Rodents with access to running wheels reduce their self-administration of amphetamine (Kanarek et al., 1995), heroin (Smith and Pitts, 2012) and alcohol

(Hammer et al., 2010) and show a decrease in morphine conditioned place preference (Lett et al., 2002). Beyond altering acute drug effects, limited evidence from the animal literature suggests that voluntary wheel running is beneficial during drug withdrawal. For example, wheel running attenuates seizures induced by ethanol withdrawal (Devaud et al., 2012) and reduces cognitive deficits and anxiety associated with spontaneous morphine withdrawal in rats (Miladi-Gorji et al., 2011, 2012).

It is possible that these behavioral effects are due to increases in levels of endogenous opiates following aerobic exercise. In humans, beta- endorphin levels increase three-fold following treadmill exercise (Mahler et al., 2009) and pain sensitivity decreases following rowing exercise (Cohen et al., 2010).

In animals, many studies have shown that wheel running can produce rightward shifts in a morphine dose-effect curve (Kanarek et al., 1998; Mathes and Kanarek, 2001; Smith and Yancey 2003; Smith and Lyle, 2006). Opioid-like withdrawal has even been precipitated after chronic exercise in rats (Kanarek et al., 2009). Taken together, these studies provide evidence that wheel running can alter the functioning of the opiate system.

Goals of this dissertation

The primary hypothesis of this dissertation is the following: The severity of spontaneous morphine withdrawal, as measured by hypersensitivity to a thermal stimulus, is reduced in mice that are given access to running wheels in their home cages. Aim I (described in Chapter II) addresses the first step in testing

this hypothesis by developing and validating a sensitive measure of spontaneous morphine withdrawal in mice. Aim II (described in Chapters III and IV) directly tests the primary hypothesis that wheel access reduces withdrawal severity and examines potential mechanisms for the effect. Aim III (described in Chapter V) further addresses the mechanism by which wheel access alters brain and behavior by comparing the effects of chronic wheel access with those of chronic morphine.

Aim I: Thermal sensitivity, measured by response latency on a hot plate, is a sensitive measure of spontaneous morphine withdrawal in mice.

Aim I validated the use of thermal sensitivity as a measure of spontaneous morphine withdrawal. To test this hypothesis, physical dependence was induced by 5.5 days of twice daily injections of 56 mg/kg morphine. At multiple time points following the final injection, withdrawal was assessed in two ways. First, thermal sensitivity was evaluated by latency to respond on the hot plate at a range of temperatures (50, 52, 54 and 56^{o}C). Second, within and between subject changes in thermal sensitivity were compared to changes in jumping behavior. The ability of a dose of buprenorphine to attenuate withdrawal-induced changes in thermal sensitivity was also tested.

Aim II: Access to a running wheel in the home cage attenuates increases in thermal sensitivity observed during spontaneous morphine withdrawal in mice.

To test this hypothesis, mice were given morphine injections for 5.5 days to establish physical dependence. Following termination of this chronic regimen, mice were given access to running wheels throughout the subsequent withdrawal period. Withdrawal was measured by determining thermal sensitivity on the hot plate at baseline and at 8, 24, 32, 48 hrs and 1 week following termination of the chronic regimen of morphine administration.

Aim II examined these effects further by determining the effects of wheel access on morphine withdrawal under conditions in which running wheels were present in the mice cages, but were in a “locked” position. Since mice were housed singly in the experiments involving access to a running wheel, an additional set of experiments examined the effects of group housing on morphine withdrawal.

Aim III: Chronic wheel access reduces morphine’s antinociceptive potency and produces changes in gene expression that are similar to changes seen following chronic morphine administration.

The behavioral portion of this aim used the tail-flick procedure to assess morphine’s antinociceptive effects. Research in our laboratory, as well as in many others, has shown that morphine’s antinociceptive effects in the tail-flick procedure are dose-dependent and reliable (Fisher et al., 2005; 2008). Moreover, the development of tolerance following chronic administration of morphine can be readily observed with the tail-flick procedure (e.g. Huidobro, 1971; Kamei et al., 1973; Fernandes et al., 1977; Bhargava, 1978). Therefore, the effects of chronic

morphine in the tail-flick procedure were compared to the effects of access to running wheels.

The second section of Aim III used quantitative polymerase chain reaction (qPCR) to compare gene expression following chronic wheel access and chronic morphine administration. The expression of five genes was assessed: POMC, PENK, PDYN, MOR1, and ARRB2. Proopiomelanocortin (POMC), proenkephalin (PENK) and prodynorphin (PDYN) were selected because they code for the precursor proteins that are post-translationally modified into the three major endogenous opioids: beta-endorphin, enkephalin, and dynorphin, (Aghajanian and Sanders-Bush 2002). MOR1 gene codes for the mu-opioid receptor (Ammon-Treiber et al 2005). Beta-arrestin 2 (BARR2) encodes the protein beta-arrestin which regulates mu-opioid receptor desensitization and internalization (Bohn et al., 2004). Gene expression was assessed in four brain regions integral to the formation and expression of morphine tolerance: the striatum, nucleus accumbens, hypothalamus and periaqueductal grey.

Together, these experiments will support the use of a new method to evaluate spontaneous morphine withdrawal and extend our knowledge of the effects of wheel running during withdrawal. Ultimately, these studies carry great translational potential to support the use of aerobic exercise in the treatment of opioid addiction.

Chapter 2

Thermal sensitivity as a measure of spontaneous morphine withdrawal in mice

INTRODUCTION

The opioid withdrawal syndrome consists of a constellation of symptoms that appear following the termination of a prolonged period of opioid administration. The presence or desire to avoid these symptoms may even contribute to continued drug taking (Le Moal and Koob, 2007). As such, withdrawal is a critical component of opioid abuse. One of the many symptoms that make up the Clinical Opiate Withdrawal Scale or COWS (Tompkins et al., 2009) is an increase in pain or sensitivity to pain. An increase in pain sensitivity or hyperalgesia during spontaneous withdrawal occurs in pain patients in experimental settings (Lipman and Blumenkopf, 1989) and is reported in case studies, as well (Devulder et al., 1996). Healthy human subjects show hyperalgesia during both spontaneous (Angst et al 2003) and antagonist precipitated withdrawal (Compton et al., 2003; Sun, 1998).

The development of pharmacological and environmental interventions to mitigate hyperalgesia during opioid withdrawal requires reliable preclinical models of this symptom of withdrawal. In 1973, Tilson et al. reported that

sensitivity to electric foot shock increases following the cessation of morphine in rats. Since then a modest number of papers have described hyperalgesia in animal models of opioid withdrawal. In rats, hyperalgesia occurs during both precipitated as well as spontaneous morphine withdrawal and is observed with multiple pain assays: hot plate, tail-flick, and shock discrimination (Devillers et al., 1995; Dunbar and Pulai 1998; Grilly and Gowans 1986; Jin et al., 2012; Li et al., 2001; Tilson et al., 1973). Hyperalgesia in rats also occurs during withdrawal from fentanyl (Laulin et al., 2002) and heroin (Devillers et al., 1995; Laulin et al., 1998). Beyond rodents, withdrawal hypersensitivity is seen in both dogs (Martin et al., 1987) and cats (Johnson and Duggan, 1981).

Traditionally, opioid withdrawal in mice is measured by the presence of behavioral signs such as jumping, wet dog shakes, piloerection, diarrhea, and ptosis (e.g. Kest et al., 2002; Papaleo and Contarino 2006). To the best of our knowledge only two studies from laboratories other than our own employ a hyperalgesia model for examining opioid withdrawal in mice. These studies examine only a single time point during spontaneous withdrawal (Rubovich et al., 2009) or employ a precipitated, rather than a spontaneous, withdrawal procedure (Crain and Shen 2007).

The current study describes a new method for assessing hyperalgesia in a mouse model of spontaneous morphine withdrawal. We hypothesize that thermal sensitivity on a hot plate will increase during spontaneous withdrawal from a range of morphine doses. Further, we hypothesize that buprenorphine treatment during the withdrawal period will attenuate the increase in sensitivity.

Buprenorphine, a low efficacy mu agonist, was selected because it is commonly used in agonist replacement therapy for opioid dependence (e.g. Connock et al. 2007 and Kraus et al., 2011), and used to suppress spontaneous opioid withdrawal symptoms during the induction phase of treatment (Strain et al. 2011).

METHODS

Animals

All experiments were conducted in male C57BL/6J mice (Jackson Labs, Raleigh, NC), 10 weeks of age upon delivery. Male C57BL/6J mice were selected to allow comparison with other data collected in our laboratory regarding morphine's pharmacological effects as well as the extensive literature on the behavioral effects of opioids in C57BL/6 mice. Additionally, in comparison to other inbred strains, C57BL/6J mice are known to be highly sensitive across many behavioral assays. Specifically, they exhibit high sensitivity in measures of acute nociception (Mogil et al., 2000), naloxone precipitated morphine withdrawal (Kest et al. 2002) and morphine self-administration (Elmer et al. 2009).

Mice were individually housed in polycarbonate cages (floor area=335cm^2) with continuous access to food and water throughout the study. The colony room was maintained on a 12-hr, reverse, light/dark cycle (lights off at 7:00 am) and all behavioral testing was conducted during the dark cycle, between 9:00 am and 7:00 pm. Mice were habituated to handling and the colony room environment for two weeks prior to any experimental manipulation. Mice were also exposed to the testing environment for at least two days prior to initiation of an experiment and

for 1 hr prior to all behavioral testing. Although a criterion was set such that mice <20 g or those that lost >20% of initial body weight would be removed from the study, it was not necessary to remove any mice from the study. Animal protocols were approved by the Institutional Animal Care and Use Committee, and the methods were in accord with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council, 2011).

Experimental Procedures

Thermal Sensitivity: Thermal sensitivity was assessed using a hot plate analgesia meter (25.3 × 25.3 cm), Columbus Instruments, Columbus, OH. During each 1-hr hot plate testing period, a temperature-effect curve was determined for each mouse. Sensitivity was evaluated by recording the latency to lick or flutter the hind paw(s), or to jump from the hot plate surface at each of four temperatures presented in the following order: 50, 54, 52, 56^{o}C with 15-min intervals between temperatures. Response latency was measured to the nearest 0.1 sec. To prevent tissue damage, a predetermined cutoff time of 20 sec was defined as the maximal trial duration. Immediately following the termination of a trial, whether due to a mouse's response or elapsed cutoff time, mice were removed from the hot plate surface. Parameters were selected based on prior work in our laboratory regarding responses on the hot plate (e.g. Fischer et al. 2008; Balter and Dykstra, 2012).

Jumping: To measure jumping, mice were removed from their home cages and placed in a 4L beaker in the center of a Med Associates Inc. activity chamber. Vertical beam breaks, monitored by a computer, were used to count the number of jumps that occurred in a 30-min period.

Pharmacological Procedure: During the saline/morphine administration period, doses of saline, 30 mg/kg, 56 mg/kg or 100 mg/kg of morphine were administered daily for 5.5 days, with injections occurring at 10:00 am and 8:00 pm daily (11 injections total). Morphine sulfate and buprenorphine hydrochloride, provided by the National Institute on Drug Abuse (Bethesda, MD, USA), were both dissolved in 0.9% saline to yield all concentrations. Doses were injected subcutaneously at a volume of 0.1 ml /10 g.

Experimental Design

Experiment 1: Thermal sensitivity following saline, 30, 56, or 100 mg/kg of morphine

On day one, thermal sensitivity was assessed in all four groups of mice (n=8) at 10:00 am (baseline 1) and at 6:00 pm (baseline 2). A 2-way repeated measures ANOVA revealed no difference between baseline 1 and baseline 2; therefore, baselines were averaged for all analyses and figures. At 10:00 am on day two 30, 56, 100 mg/kg morphine or saline administration began as described above and continued for 5.5 days. Following the last dose of morphine on day seven, thermal sensitivity was assessed six more times: immediately after the

final injection (10:00 am on day 7), at 8 hrs (6:00 pm on day 7), at 24 hrs (10:00 am on day 8), at 32 hrs (6:00 pm on day 8), at 48 hrs (10:00 am on day 9) and at 1 week (10:00 am on day 14). This period (days 7-14) was designated as the withdrawal period.

Experiment 2: Buprenorphine and thermal sensitivity

In order to select a dose of buprenorphine that did not produce antinociception on its own, a cumulative dose-effect curve (0.01 to 0.32 mg/kg) was obtained for buprenorphine at each of the four temperatures tested during the thermal sensitivity assessment (50, 52, 54 and 56 ±0.1^{o}C). Baseline response latencies on the hot plate were determined twice prior to the beginning of the buprenorphine dose-effect curve and spaced 30 min apart. Data from these baselines were averaged to yield one baseline value. Following baseline determination, responding on the hot plate was examined over multiple cycles, and doses of buprenorphine were spaced 30 min apart. Drugs were administered at the start of each cycle and latency on the hot place was determined during the last minute of the cycle. Drug doses were increased cumulatively, with the dose increasing in one-half log unit increments prior to each cycle (0.01, 0.03, 0.1, 0.32 mg/kg). Buprenorphine effects were expressed as a percentage of the maximal possible effect (% MPE) using the following formula:

$$\%MPE = \frac{[\text{Postdrug latency} - \text{baseline latency}]}{[\text{cutoff time (20sec)} - \text{baseline latency}]}$$

During the withdrawal experiment, on day one thermal sensitivity was assessed in two groups of mice (n=8) at 10:00 am (baseline 1) and 6:00 pm (baseline 2). A 2-way repeated measures ANOVA revealed no difference between baseline 1 and baseline 2; therefore, baselines were averaged for all analyses and figures. At 10:00 am on day two 56 mg/kg morphine administration began for all mice as described above and continued for 5.5 days. Following the last dose of morphine on day seven, thermal sensitivity was assessed five more times: immediately after the final injection (10:00 am on day 7), at 8 hrs (6:00 pm on day 7), at 24 hrs (10:00 am on day 8), at 32 hrs (6:00 pm on day 8), and at 48 hrs (10:00 am on day 9). A dose of 0.01 mg/kg buprenorphine or saline was administered subcutaneously 30 minutes prior to each testing session on days 7-9. This period (days 7-9) was designated as the withdrawal period.

Experiment 3: Jumping responses following saline, 30, 56, or 100 mg/kg of morphine

On day one, jumping was assessed in all four groups of mice (n=8) at 10:00 am (baseline 1, AM) and at 6:00 pm (baseline 1, PM). One week later on day 8, a second baseline measure (baseline 2, AM and PM) was taken at 10:00 am and 6:00 pm. The second set of baselines (10:00 am and 6:00 pm on day 8) was used for data analysis. At 10:00 am on day nine 30, 56, 100 mg/kg morphine or saline administration began as described above and continued for 5.5 days. Following the last dose of morphine on day 14, thermal sensitivity was assessed five more times: immediately after the final injection (10:00am on day 14), at 8

hrs (6:00 pm on day 14), at 24 hrs (10:00 am on day 15), at 32 hrs (6:00 pm on day 15), at 48 hrs (10:00 am on day 16). This period (days 14-16) was designated as the withdrawal period.

Data analysis

All data are presented as means (±SEM). In Experiments I and II, response latencies were used to derive a measure of thermal sensitivity, designated as ET10. The ET10 represents the theoretical temperature required to produce a response latency of 10 sec (half the maximal response latency of 20 sec) and was derived using log-linear interpolation. In Experiment III, jumping responses during the withdrawal period are presented and analyzed as jumps during the withdrawal period minus the average number of jumps that occurred during the corresponding baseline period (i.e., Since data for the 0, 24, and 48 hrs withdrawal period fell in the AM, baseline measures from the morning period were used. Likewise since data for the 8 and 36 hrs withdrawal period fell in the PM, baseline measures from the evening period were used.)

Analysis of the latency data used a 3-way repeated measures ANOVA with time and temperature as repeated measures factors and group as an independent factor. ET10 and jumping data were analyzed using a 2-way repeated measures ANOVA with time as the repeated measures factor and group as an independent factor. For the 2- and 3-way ANOVA, an alpha level of significance was set at $p<0.01$. Following the 3-way ANOVA, appropriate follow-up contrasts and Student's t-tests were performed using a fully saturated mixed

model of the data. The model was a straight model of the means and included random intercepts for each mouse. Following the 2-way ANOVAs, appropriate follow-up contrasts were performed using a model of jumps or ET10 as a function of time and group. The null hypothesis assumed no mean difference in the number of jumps or the ET10 values. Standard error was adjusted for multiple observations within each mouse.

Statistical analyses were conducted with an alpha level of significance set at $p<0.001$. The alpha level was determined using Bonferoni corrections to account for the large number of comparisons. The ANOVAs were performed using SPSS for Windows software, version 9.0. All post hoc analysis was performed using SAS for Windows software, version 9.2. Figures were created with GraphPad Prism 5.

RESULTS

Thermal sensitivity following spontaneous withdrawal from 30, 56, or 100 mg/kg morphine.

Fig. 2.1 shows the latency to respond on the hot plate as a function of temperature at baseline, 8, 24, 32, 48 hrs and 1 wk following termination of the 5.5 day treatment period of either 30, 56, or 100 mg/kg morphine or saline. In general, two findings were consistent across all time points. First, latency to respond on the hot plate decreased as a function of temperature. Response latencies in both saline and morphine-treated mice were at or close to the

maximal value of 20 sec when the hot plate was set at 50^{o}C; at 52, 54 and 56^{o}C, latencies averaged 12.8, 9.4 and 5.7 sec, respectively. Second, response latencies at the 0 (data not shown), 8, 24, 32 and 48-hr and 1 wk time points for saline-treated mice were never significantly different from baseline, calculated as the average of baseline 1 and 2, indicating that repetition of testing did not produce measurable effects on response latency. In addition, immediately following the final morphine injection (0 hr), response latencies were at the cut off value of 20 sec at all temperatures for morphine-treated mice; consequently these data are not shown. The failure to respond within in the 20 sec maximal trial duration indicates a full antinociceptive response to acute morphine exposure.

A 3-way repeated measures ANOVA revealed a time x temperature x group interaction $F(45, 405) = 1.974$, $p<0.001$. Follow up Student's t-tests were then used to compare individual groups, time points, and temperatures.

In general, the curves obtained in the morphine-treated mice were displaced downward from those obtained at baseline and from those of saline-treated mice. Significant differences in response latencies were apparent between morphine-treated and saline- treated mice throughout the withdrawal period. Significant differences between the 30 mg/kg morphine- and saline-treated mice were apparent at 32 and 48 hrs (52^{o}C) $t_{621}= 3.87, 4.43$, $p<0.001$, respectively. Significant differences between the 56 mg/kg morphine- and saline-treated mice were apparent at 8 hrs (52^{o}C) $t_{621}= 3.41$, $p<0.001$; 24 hrs (52 and 54^{o}C) $t_{621}= 6.13, 5.25$, $p<0.001$; 32 hrs (50, 52, 54^{o}C) $t_{621}=6.12, 4.96, 5.13$,

$p<0.001$; 48 hrs (50, 52, 54^{o}C) $t_{621}=3.65, 6.787, 3.82$, $p<0.001$; and at 1 wk (50 and 54^{o}C) $t_{621}=3.45, 3.55$, $p<0.001$. Significant differences between the 100 mg/kg morphine- and saline-treated mice were apparent at 48 hrs (50 and 52^{o}C) $t_{621}= 4.30, 5.21$, $p<0.001$ and at 1 wk (50, 52, 54^{o}C) $t_{621}= 6.51, 5.85, 4.37$, $p<0.001$. In addition, the responses of morphine-treated mice were significantly different from baseline at all points where responses were different from those of saline-treated mice. These differences suggest that mice treated with 30, 56, or 100 mg/kg of morphine for 5.5 days and then withdrawn from morphine were more sensitive to the thermal stimulus than mice treated with saline.

It is also important to note significant differences in response latency between different morphine treated groups during the withdrawal period. Response latencies of mice treated with 56 mg/kg morphine were significantly different from those of mice treated with 30 mg/kg morphine at 8hrs and 24hrs (52^{o}C) $t_{621}=3.31, 3.70$, $p<0.001$, respectively and at 32 hrs (50^{o}C) $t_{621}=5.32$, $p<0.001$. Response latencies in mice treated with 56 mg/kg morphine were also significantly different from response latencies obtained in mice treated with 100 mg/kg morphine at 8hrs and 24hrs (52^{o}C) $t_{621}=4.08, 3.44$, $p<0.001$, respectively and at 32 hrs (50^{o}C) $t_{621}=4.43$, $p<0.001$. Finally, a significant difference in response latencies was apparent between mice treated with 100 mg/kg and 30 mg/kg morphine at 1 wk (50 and 52^{o}C) $t_{621}=5.66, 4.02$, $p<0.001$.

Taken together, these data suggest that 5.5 days of morphine treatment was sufficient to produce significant changes in thermal sensitivity compared to both within-subject baselines and saline controls. However, the dose of

morphine (30, 56, or 100 mg/kg) affected the extent and time course of this response, with the greatest changes in latency observed following 56 mg/kg morphine and at 32 hrs into the withdrawal period.

Fig. 2.2 shows the ET10 value at baseline, 8, 24, 32, 48 hrs and 1 wk following termination of the 5.5-day treatment period with either 30, 56, 100 mg/kg morphine or saline. The ET10 values were derived from the data shown in Fig. 2.1. They represent the theoretical temperature necessary to produce a 10 sec response on the hot plate. A 2-way repeated measures ANOVA revealed a main effect of time $F(5, 135) = 2.299$, $p<0.05$. Individual groups and time points were compared using appropriate follow up contrasts. For mice treated with 30 mg/kg morphine, a significant difference in ET10 value compared to baseline was apparent at 32 and 48 hrs, $t_{133}=3.42, 4.30$, $p<0.001$, respectively. For mice treated with 56 mg/kg morphine, a significant difference in ET10 value compared to baseline was apparent at 24, 32, 48 hrs and 1 wk, $t_{133}=5.45, 6.74, 4.97, 3.97$, $p<0.001$, respectively. At each of these time points (24, 32, 48 hrs and 1 wk), the ET10 values of mice treated with 56 mg/kg morphine were also significantly different from those of saline-treated mice, $t_{133}=4.46, 5.37, 3.91, 3.52$, $p<0.001$, respectively. For mice treated with 100 mg/kg morphine, a significant difference in ET10 value compared to baseline was apparent at 32, 48 hrs and 1 wk, $t_{133}=3.64, 6.89, 8.29$, $p<0.001$, respectively. The ET10 values of mice treated with 100 mg/kg morphine were also significantly different from those of saline-treated mice at 48 hrs and 1 wk, $t_{133}=5.16, 6.75$, $p<0.001$, respectively. There were no significant differences between the ET10 values of the groups at

baseline or between the ET10 values of saline-treated mice across time. Taken together, these data further support the hypothesis that 5.5 days of morphine treatment significantly increase thermal sensitivity during spontaneous morphine withdrawal.

Effects of buprenorphine on thermal sensitivity during spontaneous morphine withdrawal.

Buprenorphine is a partial mu-opioid receptor agonist and, like all mu-opioid agonists, it produces antinociception on the hot plate. Consequently, prior to determining whether buprenorphine would attenuate withdrawal induced increases in thermal sensitivity, a dose of buprenorphine that did not produce antinociception on its own was identified.

Fig. 2.3a presents the dose-effect curve of buprenorphine (0.01 mg/kg-0.32 mg/kg) at each of the temperatures used during the thermal sensitivity testing. Based on these data, a dose of 0.01 mg/kg buprenorphine was selected since this dose did not produce measurable antinociception on the hot plate at 50, 52, 54 or 56^{o}C

Fig. 2.3b shows the ET10 value at baseline, 8, 24, 32, and 48 hrs following termination of 5.5 days of twice daily morphine. As in Experiment I, ET10 values represent the theoretical temperature necessary to produce a 10 sec response on the hot plate. All mice in this experiment received 56 mg/kg morphine. During the withdrawal period, mice received saline or 0.01 mg/kg buprenorphine treatment 30 min prior to test sessions at 8, 24, 32 and 48 hrs.

Immediately following the final morphine injection (0 hr), response latencies were at the cut off value of 20 sec at all temperatures; consequently these data are not shown.

A 2-way repeated measures ANOVA revealed a time x group interaction $F(4, 56) = 3.739$, $p<0.01$, respectively. Individual groups and time points were compared using appropriate follow up contrasts. Significant differences were apparent between the buprenorphine-treated and saline-treated groups at 24 and 32 hours, $t_{56}=3.94, 3.56$, $p<0.001$, respectively. Additionally, response latencies of buprenorphine-treated mice showed no difference from baseline throughout the withdrawal period ($p>0.01$). However, significant differences were again apparent between the saline-treated group and baseline at all withdrawal time points (8, 24, 32, and 48 hrs), $t_{56}=3.66, 6.35, 6.65, 3.74$, $p<0.001$. These data suggest that buprenorphine can attenuate the decrease in response latency observed during morphine withdrawal.

Jumping behavior during spontaneous withdrawal from 30, 56, or 100 mg/kg morphine.

Experiment III assessed jumping responses during a 30-min period at baselines and at 0, 8, 24, 32, and 48 hrs following termination of the 5.5 day treatment period with either 30, 56, 100 mg/kg morphine or saline (s.c., twice daily). Jumping responses provide a measure of withdrawal for comparison to the thermal sensitivity data.

Fig. 2.4 shows the number of jumps obtained at the morning (10:00 am) and evening (6:00 pm) baselines. Jumping responses during the withdrawal period are presented and analyzed as jumps observed during the withdrawal period minus the average number of jumps that occurred during the corresponding baseline period (i.e., 0, 24, and 48 hrs minus AM baseline; 8 and 36 hrs minus PM baseline). This adjustment for AM and PM baseline measures was included since baseline differences were observed at the two time periods.

A 2-way repeated measures ANOVA revealed a time x group interaction $F(12, 108) = 2.87$, $p<0.01$, respectively. Individual groups and time points were compared using appropriate follow up contrasts. Significant differences in adjusted jumping between mice treated with 56 mg/kg morphine and saline were apparent at 24, 32 and 48 hrs, $t_{108}= 5.81, 3.61, 3.66$, $p<0.001$, respectively. A significant difference was seen in adjusted jumping between mice treated with 100 mg/kg morphine and saline at 24 hrs, $t_{108}= 4.43$, $p<0.001$. In addition, immediately following the final morphine injection (0 hr), no jumping was observed in any of the morphine treated mice.

Taken together, these data suggest that 5.5 days of morphine is sufficient to produce significant changes in jumping behavior compared to saline controls. However, as seen in Experiment I, the extent of this response varies with the dose of morphine (30, 56, or 100 mg/kg), with the greatest effects observed following 56 mg/kg.

DISCUSSION

The experiments yielded three main findings. First, the results from Experiment I supported the hypothesis that the measurement of changes in thermal sensitivity provides a reliable method for assessing spontaneous withdrawal from morphine in mice. Second, Experiment II demonstrated that buprenorphine could attenuate changes in thermal sensitivity as measured by latency to respond on the hot plate. Third, the results from Experiment III indicated that changes in thermal sensitivity during withdrawal were similar to changes in jumping behavior, a well-established measure of morphine withdrawal. Taken together, these data validate the thermal sensitivity procedure as a method for assessing spontaneous morphine withdrawal.

In the first experiment, an orderly temperature by latency relationship was observed at all time points, with increasing temperatures producing shorter response latencies. Treatment with all three of the morphine doses (30, 56, or 100 mg/kg) produced significant decreases in response latency on the hot plate following the cessation of morphine treatment. The downward displacement of the temperature-response curves was most prominent at 52 and 54^{o}C. At 56^{o}C, response times were so short that changes in response time were difficult to detect. The response latencies of saline-treated control groups were consistent across all time points. This illustrates that neither 1) repeated testing nor 2) time of day measurably affected responding on the hot plate. Finally, across all experimental groups there was little within-group variability as measured by

standard error. The observation that mice were more sensitive to a thermal stimulus during morphine withdrawal is consistent with previous research in both humans and animals reporting heightened sensitivity to thermal stimuli following termination of a regimen of morphine administration (Angst et al. 2003; Compton et al. 2003; Dunbar and Pulaj 1998; Rubovitch et al. 2009; Sweitzer et al. 2004).

The effect of dose and the time course of withdrawal are clearly apparent in the ET10 data, where a single latency score was generated for each time point. It is well established that dose of morphine is a factor in the severity of physical dependence (e.g., Papaleo and Contarino, 2006). In the experiment reported here, looking at the totality of the week-long withdrawal period, treatment with 56 mg/kg morphine produced a more pronounced increase in sensitivity than 30 mg/kg morphine; however, the time course during which the behavior was expressed was similar following both 30 and 56 mg/kg. For both groups, thermal sensitivity peaked in the second day following the cessation of morphine administration and showed a return toward baseline levels by one week.

The magnitude of the change in ET10 value in mice treated with 100 mg/kg morphine was similar to that of mice treated with 56 mg/kg; however, the time course of this decrease was shifted temporally. We speculate that treatment with 100 mg/kg morphine produced a more severe withdrawal syndrome and that a change in thermal sensitivity was only apparent as physical dependence eased during the spontaneous withdrawal period. It is possible that other symptoms of withdrawal such as sedation blocked the measurement of increases in thermal sensitivity or that this behavior is only apparent at a certain

magnitude of withdrawal severity. Taken together, these data suggest that a change in latency to respond on the hot plate is a sensitive measure of morphine withdrawal; however, time, dose and hot plate temperature are all critical variables to consider when using this measure.

The second experiment demonstrated that changes in thermal sensitivity during withdrawal could be attenuated by treatment with buprenorphine. Buprenorphine was selected because it is commonly used in agonist replacement therapy for opioid dependence (Kraus et al., 2011). Mice received either saline or a non-antinociceptive dose (0.01 mg/kg) of buprenorphine during the withdrawal period, following the cessation of 5.5 days of 56 mg/kg morphine. The response latency of buprenorphine-treated mice was attenuated compared to saline-treated mice at 24 and 32 hrs. Mice that received saline during the withdrawal period showed the same course of withdrawal as mice similarly treated with 56mg/kg morphine in Experiment I.

Experiment III examined jumping behavior as a measure of withdrawal severity. Jumping was selected for comparison because it is a well-established measure of opioid withdrawal (e.g. Saelens et al., 1971; Kest et al., 2002; and Papaleo and Contarino, 2006). In the current experiment, withdrawal severity, as measured by number of jumps in a 30-min period replicated the findings of the thermal sensitivity experiments. Termination of treatment with 56 mg/kg morphine produced the most pronounced increase in jumping compared to treatment with 30 mg/kg or 100 mg/kg morphine. Experiment III revealed two major limitations of using jumping to assess withdrawal severity. First, baseline

data indicate that time of testing (early or late in the dark-cycle) can affect responding. Second, within-group variability for the jumping response is relatively large. As a result, it is more difficult to determine whether differences between experimental groups are significant when jumping is used to measure withdrawal.

The most notable limitation of the thermal sensitivity procedure examined here is the difficulty in automating the measure since it is time intensive and requires observers who are well trained in the observation of hot plate responses. Nevertheless, the thermal sensitivity procedure could be adjusted for higher throughput screening by examining latencies at a single temperature ($52^{o}C$) and a single time point (24 or 32 hrs). Additionally, the procedure could be adapted for within subject (baseline v withdrawal period) or between subject (treatment group v untreated withdrawal group) designs.

In summary, the present study supports the use of thermal sensitivity, as measured by changes in response latency on the hot plate, as a reliable method for assessing spontaneous morphine withdrawal in mice. Response latencies on the hot plate show little variability within groups and little effect of repeated testing, maximizing sensitivity to subtle changes in withdrawal severity. The procedure is also well suited for examining withdrawal over longer periods, a distinct advantage over procedures in which withdrawal is precipitated by an antagonist and withdrawal behaviors are observed at a single time point. These characteristics make the thermal sensitivity procedure optimal for assessing the efficacy of medications and environmental interventions for alleviating opioid

withdrawal. In fact, our laboratory recently showed that two environmental interventions, i.e., access to a running wheel and group housing, could attenuate the increase in thermal sensitivity observed during spontaneous withdrawal from morphine (Balter and Dykstra, 2012).

FIGURES

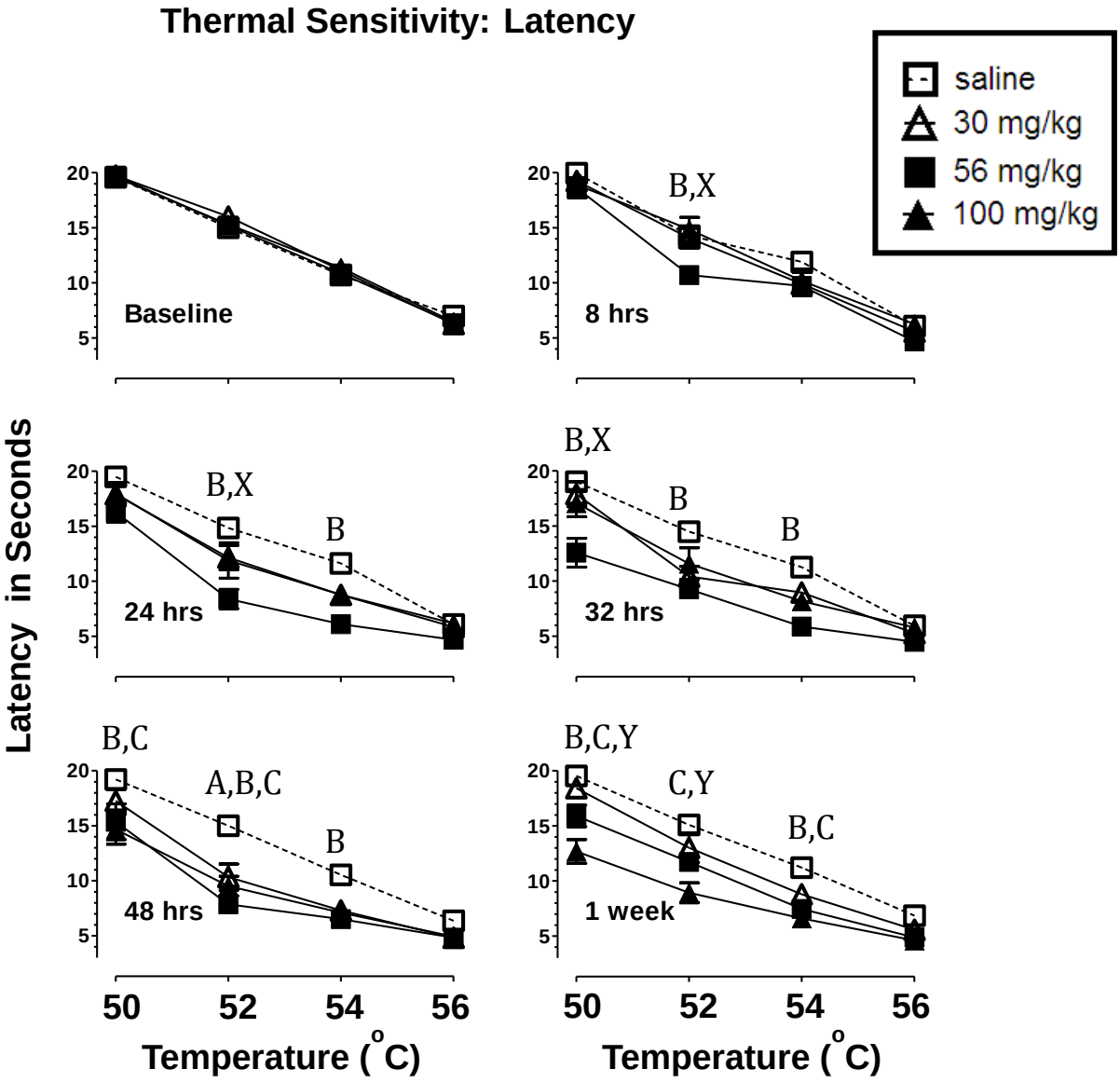


Fig. 2.1 **Effects of 30, 56 or 100 mg/kg morphine or saline treatment on latency (mean ±SEM) to respond on the hot plate at 50, 52, 54, and 56° C**. Morphine or saline treatment consisted of 5.5 days of twice daily injections (s.c.). Latency on the hot plate was determined at baseline and at 8, 24, 32, 48 hrs, and 1 wk after the final injection. Abscissa: hot plate temperature in ° C. Ordinate: latency to respond in seconds. N=7-8. Statistically significant differences ($p<0.001$) are indicated as follows: A= 30 mg/kg v. sal, B= 56 mg/kg v sal, C= 100 mg/kg v sal, X= 56 mg/kg v 30 and 100 mg/kg, Y= 100 mg/kg v 30 mg/kg.

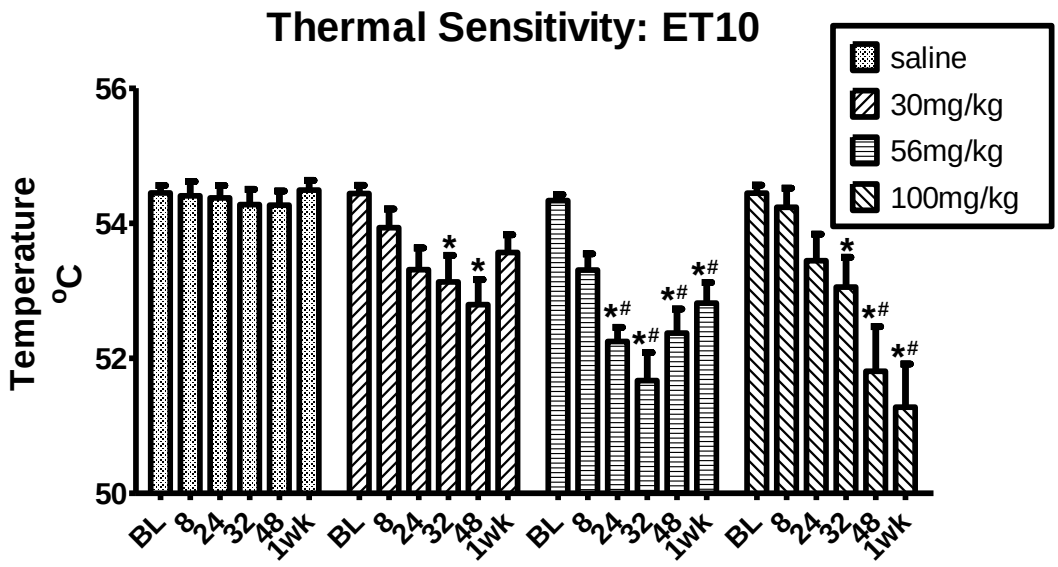


Fig. 2.2 **ET10 values (mean ±SEM) for mice following 5.5 days of 30, 56 or 100 mg/kg morphine or saline treatment**. ET10 values represent the temperature that would produce a 10 sec response on the hot plate. Response latency on the hot plate was determined at baseline and at 8, 24, 32, 48 hrs and 1 wk after the final injection. N=8. Statistically significant differences are indicated as follows: *= a difference from the group's baseline, # = a difference between morphine and saline treated mice at a particular time point. $p<0.001$

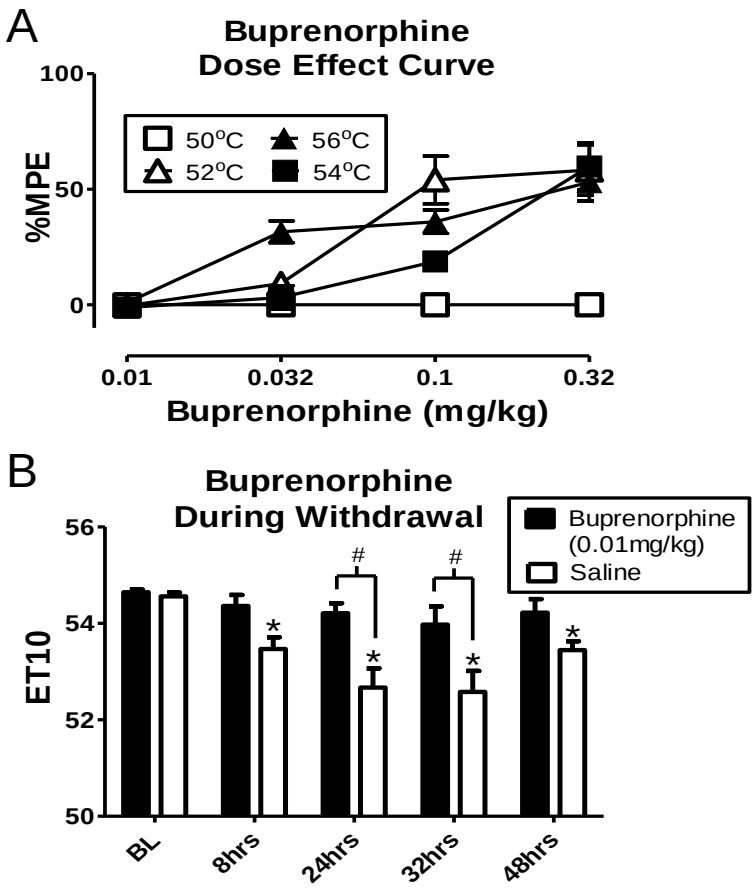


Fig. 2.3 **The effect of 0.01 mg/kg buprenorphine on withdrawal from 5.5 days of 56 mg/kg morphine.** A. Dose-effect curves for buprenorphine (0.01-0.32 mg/kg) at 50, 52, 54, and 56^{o}C. Mean latencies (±SEM) are presented as % maximum possible effect (%MPE). B. ET10 values (mean ±SEM) for mice treated with 0.01 mg/kg buprenorphine or saline following 5.5 days of 56 mg/kg morphine. ET10 values represent the temperature that would produce a 10 sec response on the hot plate. Response latency on the hot plate was determined at baseline and at 8, 24, 32, and 48 hrs after the final morphine injection. Mice received 0.01 mg/kg buprenorphine (s.c.) 30 min prior to each hot plate test session. N=8. Statistically significant differences are indicated as follows: *= a difference from the group's baseline, # = a difference between buprenorphine and saline treated mice. $p<0.001$

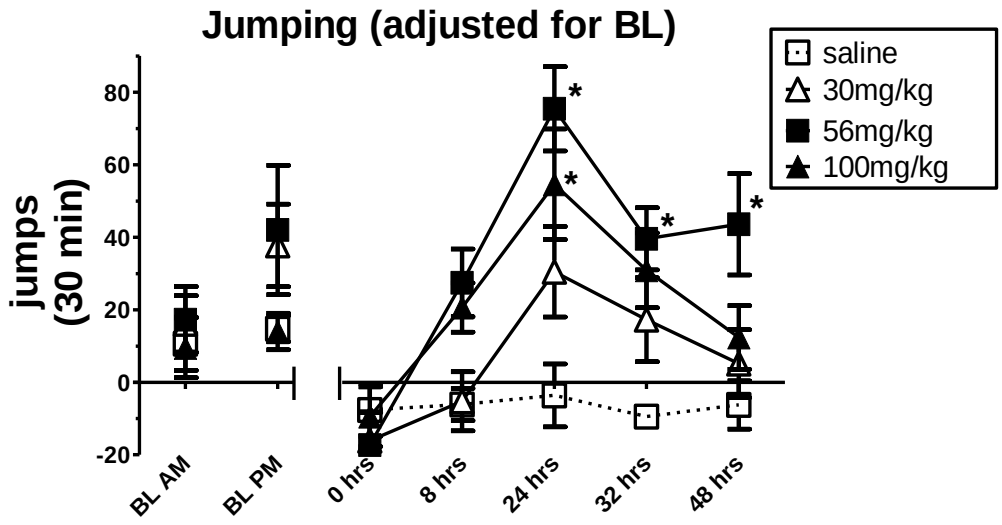


Fig. 2.4 **Jumps (mean ±SEM) adjusted for baseline following 30, 56 or 100 mg/kg morphine or saline.** Morphine or saline treatment consisted of 5.5 days of twice daily injections (s.c.). Jumping was determined at baseline and at 0, 8, 24, 32, and 48 hrs after the final injection. Baseline jumps indicate total jumping in 30 min at 10am and 6pm. Jumps at 0, 8, 24, 32, and 48 hrs indicate jumps observed during the 30-min withdrawal period minus the average number of jumps that occurred during the corresponding baseline period. Data obtained for the 0, 24, and 48 hrs withdrawal period fell in the AM; therefore, total jumps were adjusted using baseline measures from the AM period. Data obtained for the 8 and 36 hrs withdrawal period fell in the PM; therefore, total jumps were adjusting using baseline measures from the PM period. N=8. * = a statistically significant difference compared to saline treated mice. $p<0.001$

Chapter 3

The effect of wheel access on morphine withdrawal in C57BL/6J mice

INTRODUCTION

There is a growing body of literature examining the effects of voluntary exercise on responses to drugs of abuse. In humans, short periods of aerobic exercise have been shown to reduce the desire for alcohol and tobacco (Ussher et al. 2004; Taylor and Katomeri 2007). Exercise also reduces symptoms of nicotine withdrawal and aids in smoking cessation (Taylor and Ussher 2005; Taylor and Katomeri 2007). It is possible that such behavioral effects are due to increases in levels of endogenous opiates following aerobic exercise in the blood (Mahler et al. 2009; Cohen et al. 2010) as well as in the brain (Becker et al. 2008).

In animals, access to running wheels decreases oral self-administration of both amphetamine and alcohol (Kanarek et al. 1995; Hammer et al. 2010). Wheel running also reduces morphine self-administration and morphine conditioned place preference (Lett et al. 2002; Hosseini et al. 2009). Many studies have shown that wheel running attenuates morphine's antinociceptive

potency, suggesting that running may alter the functioning of the opiate system (Kanarek et al. 1998; Mathes and Kanarek 2001; Smith and Yancey 2003; Smith and Lyle 2006). Beyond altering acute drug effects, wheel access also reduces cognitive deficits and anxiety associated with spontaneous morphine withdrawal in rats (Miladi-Gorji et al., 2011, 2012).

Given the evidence suggesting that exercise can alter the effects of opioids, the present study examines the effect of access to running wheels in the home cage on spontaneous morphine withdrawal in mice. Withdrawal is assessed following the termination of a regimen in which mice receive injections of either 30 or 56 mg/kg morphine (s.c.) twice-daily over a period of six days.

Withdrawal severity is examined at multiple time points (8, 24, 32 and 48 hrs) following the termination of morphine administration. Unlike withdrawal that is precipitated by an opioid antagonist such as naloxone, spontaneous withdrawal takes place over an extended time period that allows mice to have access to running wheels throughout the withdrawal period. Additionally, spontaneous withdrawal, as opposed to antagonist precipitated withdrawal, more closely parallels the human experience.

Since it is well-documented that the termination of a regimen of chronic morphine administration often results in heightened sensitivity to sensory stimuli (Kaplan and Fields 1991; Simonnet and Rivat 2003; Sweitzer et al. 2004), including painful stimuli, withdrawal severity is quantified by determining sensitivity to a thermal stimulus on a hot plate analgesia meter. Measures of thermal sensitivity have been used previously to examine morphine withdrawal in

both humans (Angst et al. 2003; Compton et al. 2003) and rodents (Tilson et al. 1973; Dunbar and Pulai 1998; Crain and Shen 2007; Rubovitch et al. 2009).

In addition, this study examined the relationship between access to a running wheel, attenuation of opioid withdrawal and endogenous opioid activity. Specifically, the effect of wheel access on opioid withdrawal was examined in the presence of the opioid antagonist, naltrexone. Studies have also shown that naloxone, a similar opioid antagonist, can precipitate opiate-like withdrawal following aerobic activity and beta-endorphin administration (Kanarek et al., 2009; Park et al., 2012). In the present experiment naltrexone, as opposed to naloxone, was used because it has a higher potency (Verebey and Mulé, 1975) as well as a lower K_i for both mu opioid receptor binding and antagonist activity (Wang et al., 2007). Furthermore, naltrexone is selective for the mu and kappa opioid receptors as compared to the delta receptor (Wang et al., 2007).

For this study, we hypothesize 1) that morphine- treated mice will be more sensitive to a thermal stimulus during withdrawal than control mice treated with saline, 2) that this increase in thermal sensitivity will be attenuated in mice that have access to running wheels and 3) that acute naltrexone administration during withdrawal will block the effect of wheel access.

METHODS

Animals

All experiments were conducted in male C57BL/6J mice (Jackson Labs, Raleigh, NC), 10 weeks of age upon delivery. Male C57BL/6J mice were selected to allow comparison with other data collected in our laboratory as well as the extensive behavioral literature in these mice. Additionally, in comparison to other inbred strains, C57BL/6J mice are known to be highly sensitive across many behavioral assays. Specifically, they exhibit high sensitivity in measures of acute nociception (Mogil et al 1999), naloxone precipitated morphine withdrawal (Kest et al. 2002) and morphine self-administration (Elmer et al. 2009). Finally, C57BL/6J mice are known to exhibit high rates of voluntary wheel running (Clark et al. 2011).

Mice were individually housed in polycarbonate cages (floor area=335cm^2) with continuous access to food and water throughout the study. The colony room was maintained on a 12-hr, reverse, light/dark cycle (lights off at 7:00 am) and all behavioral testing was conducted during the dark cycle, between 9:00 am and 7:00 pm. Mice were habituated to handling and the colony room environment for two weeks prior to any experimental manipulation. Mice were also exposed to the testing environment for at least two days prior to initiation of an experiment and for 1 hr prior to all behavioral testing. Although a criterion was set such that mice <20 g or those that lost >20% of initial body weight would be removed from the study, it was not necessary to remove any mice from the study. Animal protocols

were approved by the Institutional Animal Care and Use Committee, and the methods were in accord with the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council, 2011).

Experimental Procedures

Experimental Groups: Mice were assigned to one of four groups during each of three, three-week experimental sessions (described below). New mice were used for each experiment.

Experiment I: 1) morphine treatment (30 mg/kg), no wheel access; 2) morphine treatment (30 mg/kg), wheel access; 3) saline, no wheel access and 4) saline, wheel access. N=8 for morphine treated mice, n=7 for saline treated mice.

Experiment II: 1) morphine treatment (56 mg/kg), no wheel access; 2) morphine treatment (56 mg/kg), wheel access; 3) saline, no wheel access and 4) saline, wheel access. N=8 for all groups.

Experiment III: All mice were treated with 56 mg/kg morphine. Naltrexone or saline was administered 32 hrs after the final morphine injection. 1) naltrexone (0.01 mg/kg), no wheel access; 2) saline, no wheel access; 3) naltrexone (0.01 mg/kg), wheel access and 4) saline, wheel access. N=8 for all groups.

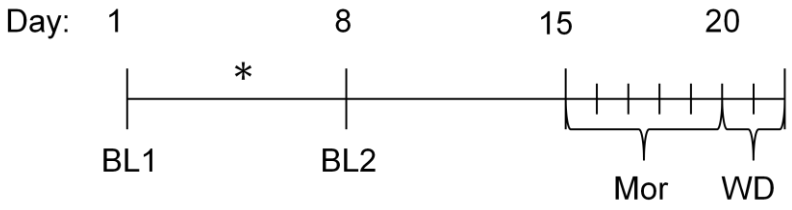
Wheel Access: Mice in wheel access groups had Med Associates Mouse Low-Profile Wireless Running Wheels in their home cages. Activity on the wheels

was monitored continuously (24 hr/day) via a computer equipped to record radio signals from the wheels.

Thermal Sensitivity: Thermal sensitivity was assessed using a hot plate analgesia meter (25.3 × 25.3 cm), Columbus Instruments, Columbus, OH. During each 1-hr hot plate testing period, a temperature-effect curve was determined for each mouse. Sensitivity was evaluated by recording the latency to lick or flutter the hind paw(s), or to jump from the hot plate surface at each of four temperatures presented in the following order: 50, 54, 52, 56^{o}C with 15-min intervals between temperatures. Response latency was measured to the nearest 0.1 sec. To prevent tissue damage, a predetermined cutoff time of 20 sec was defined as the maximal trial duration. Immediately following the termination of a trial, whether due to a mouse's response or elapsed cutoff time, mice were removed from the hot plate surface. Parameters were selected based on prior work in our laboratory regarding responses on the hot plate (e.g. Fischer et al. 2008).

Experimental Protocol: On day one, thermal sensitivity was assessed in all groups of mice (baseline 1). Immediately following baseline 1, running wheels were placed in the cages of mice in wheel access groups. One week later on day 8, a second baseline measure (baseline 2) was determined in all groups of mice and wheels were removed from the cages. The average of baseline 1 and baseline 2 was used for data analysis and is presented in all figures. One week

later on day 15, morphine or saline administration began as described below and continued for 5.5 days. Immediately following the final injection of morphine or saline (day 20), thermal sensitivity was assessed again, and wheels were returned to the cages of mice in the wheel access groups. Following the last dose of morphine, thermal sensitivity was assessed four more times in Experiment I and II: at 8 hrs after the final injection (6:00 pm on day 20), at 24 hrs (10:00 am on day 21), at 32 hrs (6:00 pm on day 21) and at 48 hrs (10:00 am on day 22). In Experiment III, thermal sensitivity was assessed three more times: at 24 hrs after the final injection (10:00 am on day 21), at 32 hrs (6:00 pm on day 21) and at 56 hrs (6:00 pm on day 22). This period (days 20-22) was designated the withdrawal period.



* = 1 week of wheel exposure for mice in wheel access groups
BL= Baseline assessment of thermal sensitivity on the hot plate. WD= 8, 24, 32 and 48hr hot plate test sessions after the final morphine injection.

Pharmacological Procedure: During the saline/morphine administration period, doses of saline, 30 mg/kg or 56 mg/kg of morphine were administered daily for 5.5 days, with injections occurring at 10:00 am and 8:00 pm daily (11 injections total). In Experiment III, 0.01mg/kg naltrexone was administered immediately prior to the 32 hr time point. 32 hrs (6:00pm) is at the end of the dark cycle maximizing the likelihood of wheel use prior to testing. Morphine sulfate and naltrexone hydrochloride, provided by the National Institute on Drug Abuse

(Bethesda, MD, USA), were dissolved in 0.9% saline to yield all concentrations. Doses were injected subcutaneously at a volume of 0.1 ml/10 g.

Data analysis

Data are presented as response latencies on the hot plate, expressed as means (±SEM) at each of the four temperatures. For each experimental group, a 2-way repeated measures ANOVA revealed no difference between baseline 1 and baseline 2; therefore, baselines were averaged for all analyses. Each experiment was first analyzed using a 3-way repeated measures ANOVA, with time and temperature as repeated measures factors and group as an independent factor. For the 3-way ANOVA, an alpha level of significance was set at $p<0.01$. Following the 3-way ANOVA, appropriate follow-up contrasts and Student's t-tests were performed using a fully saturated mixed model of the data. The model was a straight model of the means and included random intercepts for each animal. Statistical analyses were conducted with an alpha level of significance set at $p<0.001$. The alpha level was determined using Bonferoni corrections to account for the large number of comparisons. The ANOVA's were performed using SPSS for Windows software, version 9.0. All post hoc analysis was performed using SAS for Windows software, version 9.2. Figures were created with GraphPad Prism 5.

RESULTS

In general, three findings were consistent for experiments I and II. First, latency to respond on the hot plate decreased as a function of temperature. Response latencies in both saline and morphine-treated mice were at or close to the maximal value of 20 sec when the hot plate was set at 50^{o}C; at 52, 54 and 56^{o}C. Latency to respond on the hot plate decreased as a function of temperature. Second, response latencies at the 0 (data not shown), 8, 24, 32 and 48-hr time points for saline-treated mice were never significantly different from baseline, calculated as the average of baseline 1 and 2, indicating that repetition of testing did not produce measurable effects on response latency. Third, response latencies for all groups of saline-treated mice were nearly identical at all time points.

In addition, immediately following the final morphine injection (0 hr), response latencies were at the cut off value of 20 sec at all temperatures for morphine-treated mice; consequently these data are not shown. The failure to respond within in the 20 sec maximal trial duration indicates a full antinociceptive response to acute morphine exposure.

Experiment I: The effect of wheel access following 30 mg/kg morphine

Fig. 3.1 shows latency to respond on the hot plate as a function of temperature at baseline, 8, 24, 32 and 48 hrs following termination of the 5.5 day treatment period of either 30 mg/kg morphine or saline. A 3-way repeated

measures ANOVA revealed a time x temperature x group interaction $F(36, 312)= 1.93$, $p=0.002$. Follow up contrasts and Student's t-tests were then used to compare individual groups, time points, and temperatures. Although the temperature-effect curves revealed the same orderly relationship in all mice and at all time points, the curves of the mice in the morphine-treated/no wheel group were displaced downward from those obtained at baseline. This displacement was significant at 24, 32 and 48 hrs following the final morphine injection. $F(4,494)= 11.23, 11.51, 6.53$, respectively, $p<0.001$.

Significant differences between the morphine-treated/no wheel mice and saline-treated mice were apparent at a 24 hrs (52 and 54 ^{o}C), 32 hrs (52 and 54^{o}C) and at 48hrs (54 ^{o}C): $F(2,494)= 16.14, 10.30, 17.15, 9.36, 11.59$, respectively, $p<0.001$. These differences suggest that mice treated with 30 mg/kg of morphine for 5.5 days and then withdrawn from morphine were more sensitive to the thermal stimulus than mice treated with saline at 24, 32 and 48 hrs. Furthermore, response latencies of mice that were treated with morphine and given access to running wheels during the withdrawal period were significantly different from those of mice that did not have access to wheels during the withdrawal period at 24 hrs (52 ^{o}C), 32 hrs (52 and 54 ^{o}C) and at 48hrs (52 ^{o}C): $t_{494}= 4.13, 3.59, 3.80, 4.13$, respectively, $p<0.001$.

Moreover, response latencies of morphine-treated mice with wheel access were similar to those of saline-treated mice at all but one point (48 hrs, 52 ^{o}C. $F(2,494) =7.78$, $p<0.001$). Taken together, these data suggest that wheel access attenuated the increase in thermal sensitivity observed during withdrawal from 30

mg/kg of morphine and produced response latencies similar to those seen in saline controls.

Experiment II: The effect of wheel access following 56 mg/kg morphine

Fig. 3.2 shows latency to respond on the hot plate as a function of temperature at baseline, 8, 24, 32 and 48 hrs following termination of the 5.5 day treatment period of either 56 mg/kg morphine or saline. A 3-way repeated measures ANOVA revealed a time x group interaction $F(12, 112) = 4.50$, $p<0.001$ and a temperature x group interaction $F(9, 84)=4.75$, $p<0.001$. Follow-up contrasts and Student's t-tests were then used to compare individual groups, time points, and temperatures. The temperature-effect curves again revealed an orderly relationship in all mice and at all time points. Additionally, the curves of morphine-treated/no wheel mice were again displaced downward from those obtained at baseline and this displacement was significant at 8, 24, 32 and 48hrs following the final morphine injection $F(4, 532)= 6.70, 10.75, 12.60, 14.35$, respectively, $p<0.001$.

Significant differences between the morphine-treated/no wheel mice and saline-treated mice were apparent at 8 hrs (52 and 54 ^{o}C) $F(2,532)= 10.25, 7.12$, $p<0.001$; at 24hrs (52 and 54 ^{o}C) $F(2,532)=11.85, 16.82$, $p<0.001$; at 32hrs (50, 52, 54 ^{o}C) $F(2,532)= 9.72, 21.32, 17.09$, $p<0.001$; and at 48hrs (50, 52, 54 ^{o}C) $F(2,532)= 6.98, 24.56, 14.30$, $p<0.001$. These differences suggest that mice treated with 56 mg/kg of morphine for 5.5 days and then withdrawn from

morphine were more sensitive to the thermal stimulus at 8, 24, 32 and 48 hrs than mice treated with saline.

Furthermore, response latencies of mice that were treated with morphine and given access to running wheels during the withdrawal period were significantly different from those of mice that did not have access to wheels during the withdrawal period at 8 hrs (52 and 54 ^{o}C) t_{532}= 3.44, 3.53, $p<0.001$; at 24hrs (52 and 54 ^{o}C) t_{532}= 4.09, 4.97, $p<0.001$; at 32hrs (50, 52, 54 ^{o}C) t_{532}= 3.75, 4.78, 5.95, $p<0.001$; and at 48hrs (52 and 54 ^{o}C) t_{532}= 4.09, 4.97, $p<0.001$.

There were no significant differences in the response latencies of morphine-treated mice with wheel access and those of saline-treated mice. Taken together, these data further support the hypothesis that wheel access attenuated the increase in thermal sensitivity observed during withdrawal from 56 mg/kg of morphine and produced latencies similar to those seen in saline controls.

Experiment III: 0.01 mg/kg Naltrexone blocks the effect of wheel access

Fig. 3.3 shows latency to respond on the hot plate as a function of temperature at baseline, 24, 32 and 56 hrs following termination of the 5.5 day treatment period of 56 mg/kg morphine. A dose of 0.01mg/kg naltrexone or saline was administered immediately prior to the 32 hr time point. A 3-way repeated measures ANOVA revealed a time x group interaction $F(9, 84)= 10.63$, $p<0.001$, respectively. Follow-up contrasts and Student's t-tests were then used to compare individual groups, time points, and temperatures. The temperature-

effect curves again revealed an orderly relationship in all mice and at all time points. Prior to naltrexone treatment, at 24hrs, response latencies of mice in the naltrexone/wheel access group were significantly different from those of the naltrexone/no wheel access: at 50 and 52^{o}C, t_{420}= 1.75, 4.59, $p<0.001$. Significant differences were also apparent between the response latencies of mice in the saline/wheel access and the saline/no wheel access groups: at 52 and 54^{o}C, t_{420}= 4.36, 3.42, $p<0.001$. There were no differences between the responses of the two groups that had wheel access and the two groups that did not have wheel access.

At 32 hrs, a significant difference was apparent between the response latencies of the saline and naltrexone treated wheel access groups, $F(4, 420)=4.94$, $p<0.001$. Latencies of the wheel access/saline group were also significantly different from those of the no wheel access groups at 52^{o}C $F(2, 420)= 9.26$, $p<0.001$. Additionally, there was no significant difference between responses of the wheel access group treated with naltrexone and those of the no wheel access groups: latencies at 50, 52, 54, 56^{o}C $F(2, 420)= 0.32$, 0.31, 0.01, and 0.14, respectively, $p>0.7$.

At 56 hours, response latencies of mice in the naltrexone/wheel access group were again significantly different from those of the naltrexone/no wheel access: at 52 and 54^{o}C, t_{420}= 5.73, 3.74, $p<0.001$. Significant differences were also apparent between the response latencies of mice in the saline/wheel access and the saline/no wheel access groups: at 50 and 52^{o}C, t_{420}= 3.76, 4.08, $p<0.001$. There were no differences between the responses of the two groups with wheel

access and the two groups with no wheel access. A within subject analysis showed a significant difference in response latency of the naltrexone/wheel access group at 32 hrs as compared to both 24 and 56 hrs, $F(4,420)= 14.88, 15.67$, $p<0.001$. Conversely, the response latency of saline treated mice with wheel access was no different at 32 hrs compared to 24 or 56 hrs $F(4, 420)= 1.95, 0.82$, $p>0.1$.

First, data from Experiment III replicate the effects observed in Experiment II, i.e., withdrawal following 56 mg/kg of morphine was attenuated in mice that had access to a running wheel. Secondly, access to running wheels did not attenuate withdrawal, as observed at 32 hrs, in mice that were pretreated with 0.01 naltrexone at the 32 hr time point. Importantly, naltrexone pretreatment alone did not alter withdrawal in mice that did not have access to a running wheel.

When the data are compared across all three experiments, it can be seen that response latencies during withdrawal in morphine-treated mice without wheel access were consistently lower (indicating greater thermal sensitivity) than latencies observed during baseline conditions, as well as lower than those observed in saline-treated mice. Moreover, wheel access attenuated the increase in thermal sensitivity, as evidenced by the fact that response latencies of mice with wheel access during withdrawal from both 30 and 56 mg/kg of morphine were very similar to those observed in saline controls. Finally, administration of naltrexone (0.01mg/kg) at one point during the withdrawal period reversed the effect of wheel access.

DISCUSSION

The experiments conducted yielded three main findings. First, the results from all three experiments indicated that thermal sensitivity reliably increased during withdrawal following termination of a chronic regimen of morphine administration. These increases in thermal sensitivity were apparent for at least 48 hrs after morphine administration was terminated. Secondly, results from both Experiments I (30 mg/kg morphine) and II (56 mg/kg morphine) indicated that increases in thermal sensitivity during withdrawal were attenuated in mice with access to running wheels in their home cages. Thirdly, the attenuation by wheel access could be blocked by a dose of naltrexone.

The observation that mice are more sensitive to a thermal stimulus during morphine withdrawal is consistent with other research in both humans and animals reporting heightened sensitivity to thermal stimuli (Dunbar and Pulaj 1998; Angst et al. 2003; Compton et al. 2003; Sweitzer et al. 2004; Rubovitch et al. 2009), including painful stimuli, following termination of a regimen of morphine administration. Our findings expand on a study by Rubovich et al. (2009) demonstrating that thermal sensitivity also increased in a tail-flick procedure in mice at a single time point following termination of (or withdrawal following) 10 days of 10 mg/kg morphine (s.c.).

In the current experiment, both 30 and 56 mg/kg morphine given subcutaneously twice daily for 5.5 days produced significant decreases in response latency on the hot plate. These shifts were most prominent at 52 and

54°C. At 56°C, response times were so short, even in control groups, that decreases in response time were not significant. A decrease in response latency (increase in thermal sensitivity) occurred within the first 24 hours after the final morphine injection and remained stable for at least 48 hours. Looking at the totality of the 48-hour withdrawal period, treatment with 56 mg/kg morphine produced a more pronounced increase in sensitivity than 30 mg/kg morphine.

This study, like others that have used similar measures of withdrawal (Dunbar and Pulai 1998; Rubovich et al. 2009), indicates that increases in thermal sensitivity can be used as a reliable measure of spontaneous opioid withdrawal. Although robust signs of withdrawal can also be precipitated by the administration of an opioid antagonist (Tilson et al. 1973; Devillers et al. 1995; Crain and Shen 2007), there are several advantages to a measure of withdrawal that occurs spontaneously (without being precipitated). First, spontaneous withdrawal provides a more realistic parallel to the human condition in which withdrawal usually does not involve precipitation with an antagonist, but rather involves a period in which drug is no longer available. Second, the spontaneous withdrawal procedure provides a method for examining treatment interventions that take place over time, such as wheel running or administration of long acting opioid agonists.

The second important finding of the current study is the observation that increases in thermal sensitivity during morphine withdrawal were attenuated in mice that had access to running wheels in their home cages. Comparisons between mice that received saline and either had or did not have access to

wheels indicated that wheel access alone did not alter thermal sensitivity. However, in mice undergoing morphine withdrawal, wheel access significantly attenuated one very prominent sign of withdrawal, i.e., increases in thermal sensitivity. These findings support our hypothesis that wheel access can reduce withdrawal severity. Interestingly, wheel access not only attenuated the increase in sensitivity observed during morphine withdrawal, but also fully returned thermal sensitivity to the same level observed in saline treated control mice.

Extensive research has shown that opiate agonists, specifically methadone and buprenorphine are highly effective substitution treatments for opioid dependence (e.g. Connock et al. 2007 and Tetrault and Fiellin 2012). In addition, buprenorphine has been shown to suppress spontaneous opioid withdrawal symptoms during the induction phase of treatment (Strain et al. 2011). Data from the current study indicate that access to a running wheel also attenuates increases in thermal sensitivity observed during withdrawal. One possible explanation for this finding is that wheel running leads to the release of endogenous opiates, thereby reducing withdrawal signs much the same way an opioid agonist might reduce withdrawal symptoms.

Findings from Experiment III provide support for the hypothesis that attenuation of withdrawal severity in mice with wheel access is dependent on opioid activity. Initially, withdrawal-induced thermal sensitivity was attenuated in mice that had access to a running wheel; however, this effect was blocked in mice that received naltrexone. Importantly, naltrexone did not alter thermal sensitivity in mice that did not have access to running wheels, demonstrating that

naltrexone was not simply precipitating further withdrawal. Additionally, 24 hours later, thermal sensitivity in mice previously treated with naltrexone was no different from mice treated with saline; a time course consistent with the metabolism of naltrexone (Verebey and Mulé, 1975).

These data are consistent with other findings that aerobic exercise activates the endogenous opiate system. Specifically, chronic wheel running in rats attenuates morphine's antinociceptive potency, suggesting that cross-tolerance develops between wheel running and morphine administration (Kanarek et al. 1998; Mathes & Kanarek 2001; Smith & Lyle 2006). Additionally, opiate antagonists have been shown to precipitate an opioid-like withdrawal following chronic exercise in rats (Smith and Yancey, 2003; Kanarek et al. 2009). Finally, in humans, aerobic exercise increases levels of endogenous opiates in the blood (Mahler et al. 2009; Cohen et al. 2010) as well as in the brain (Becker et al. 2008).

The current study is primarily limited by the use of a single, behavioral measure of morphine withdrawal. It will be critical to consider the effects of wheel access on other signs of opioid withdrawal such as jumping, wet dog shakes, conditioned aversion, and changes in schedule controlled responding. Despite limitations, the data presented here provide evidence that environmental manipulations such as access to running wheels can attenuate morphine withdrawal. This supports the suggestion that aerobic exercise may be a valuable addition to interventions designed to treat drug dependence.

FIGURES

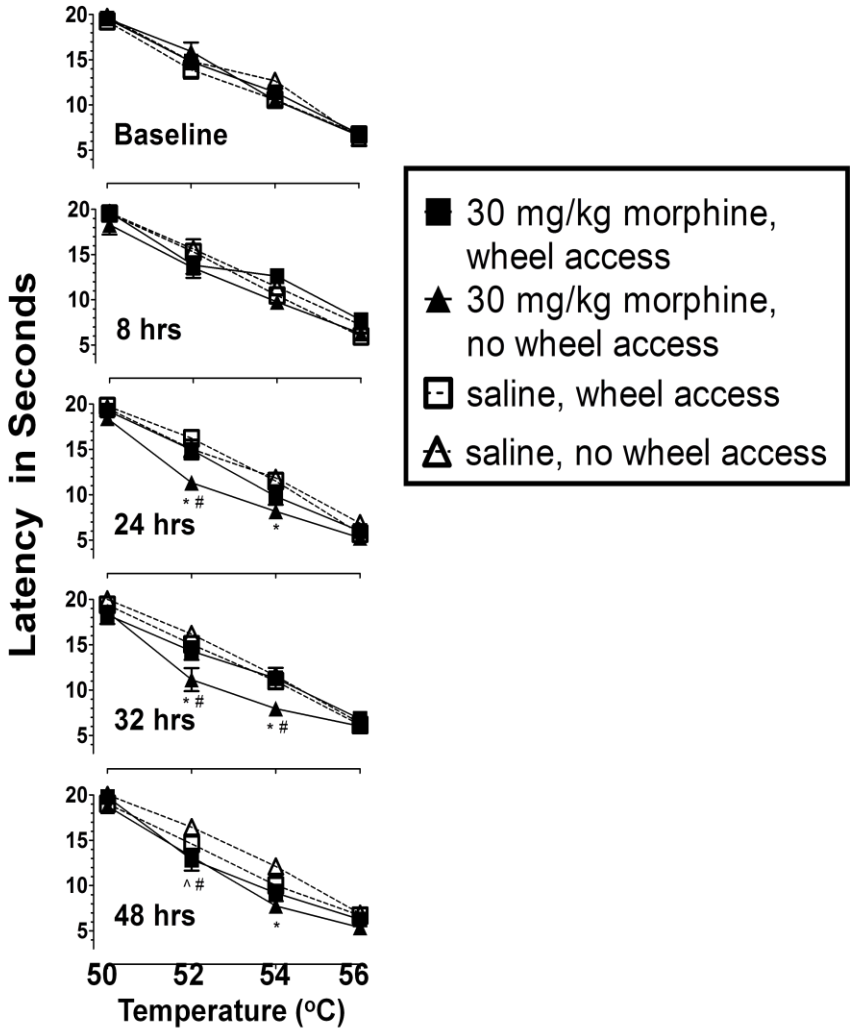


Fig. 3.1 **Effect of 30 mg/kg morphine or saline treatment on latency (mean ±SEM) to respond on the hot plate at 50, 52, 54, and 56° C**. Data are shown for mice that had access to wheels in their home cages as well as for mice that did not have access to wheels. Latency on the hot plate was determined at baseline and at 8, 24, 32, and 48 hrs after the final morphine injection. Abscissa: hot plate temperature in ° C. Ordinate: latency to respond in seconds. Statistically significant differences are indicated as follows: * = a difference between morphine- and saline-treated mice without wheel access, # = a difference between morphine-treated mice with and without wheel access, ^ = a difference between morphine-treated mice with wheel access and saline controls. $p<0.001$

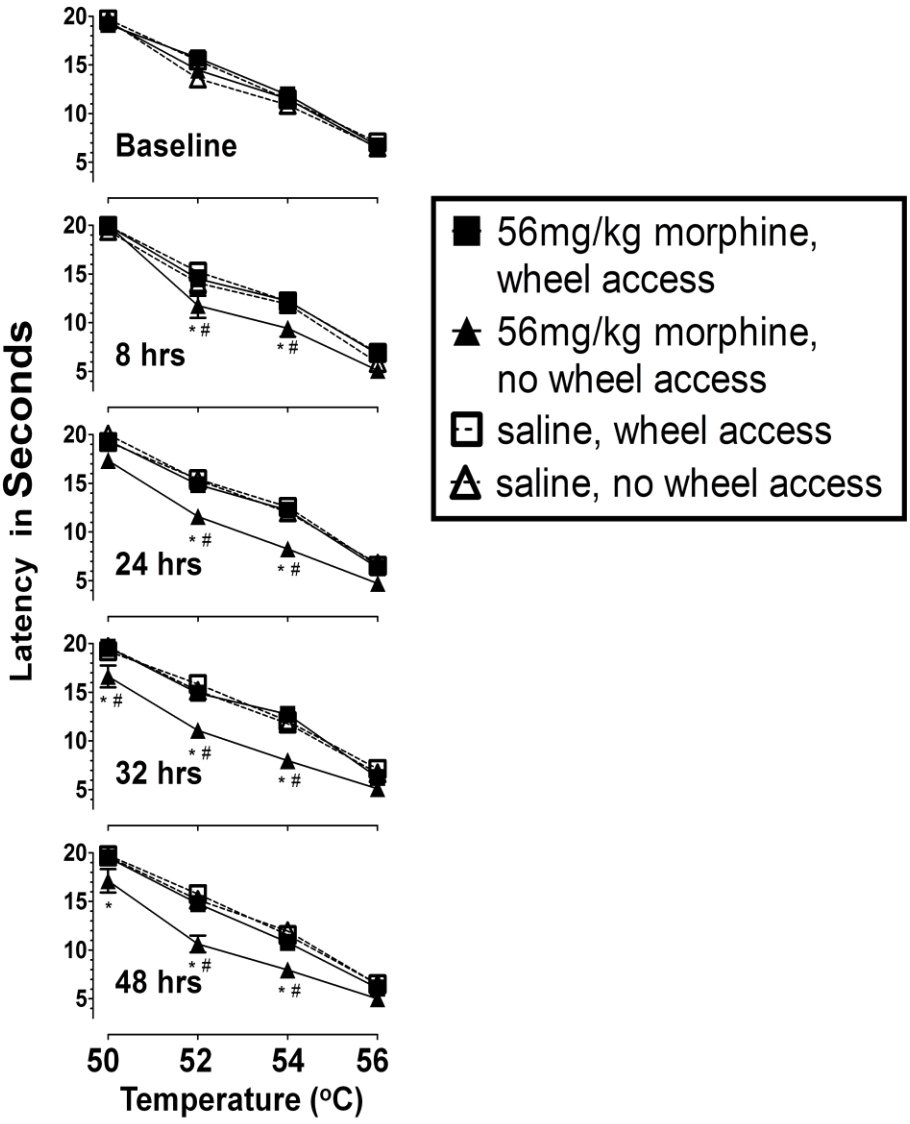


Fig. 3.2 **Effect of 56 mg/kg morphine or saline treatment on latency (mean ±SEM) to respond on the hot plate at 50, 52, 54, and 56^{o} C**. Data are shown for mice that had access to wheels in their home cages as well as for mice that did not have access to wheels. Latency on the hot plate was determined at baseline and at 8, 24, 32, and 48 hrs after the final morphine injection. Abscissa: hot plate temperature in ^{o} C. Ordinate: latency to respond in seconds. Statistically significant differences are indicated as follows: * = a difference between morphine- and saline-treated mice without wheel access, # = a difference between morphine-treated mice with and without wheel access. $p<0.001$

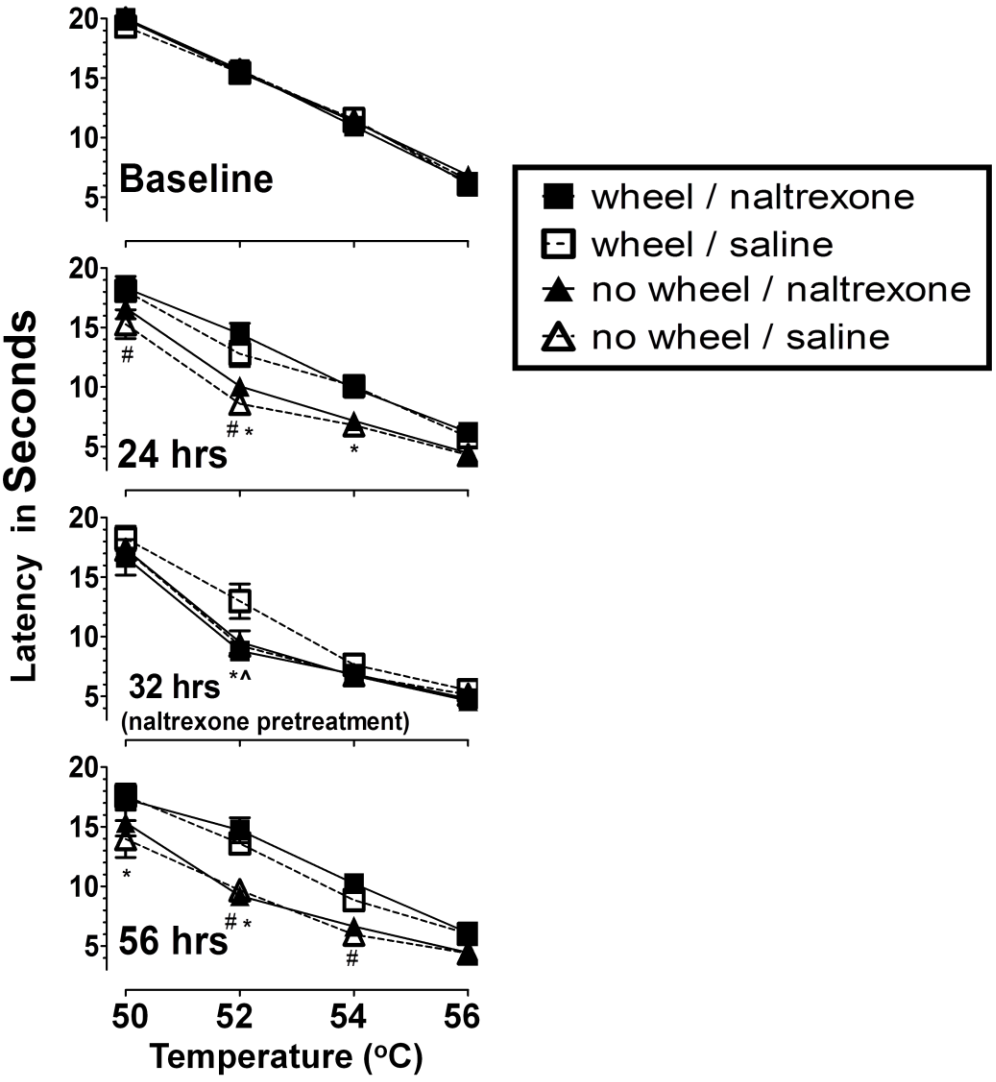


Fig. 3.3 **Effect of 0.01mg/kg naltrexone on latency (mean ±SEM) to respond on the hot plate in mice with and without wheel access**. All mice received 56 mg/kg morphine for 5.5 days. At 32 hrs following the final morphine injection mice received either naltrexone or saline. Data are shown for mice that had access to wheels in their home cages as well as for mice that did not have access to wheels. Latency on the hot plate (50, 52, 54, and 56^{o} C) was determined at baseline and at 24, 32, and 56 hrs after the final morphine injection. Abscissa: hot plate temperature in ^{o} C. Ordinate: latency to respond in seconds. Statistically significant differences are indicated as follows: * = a difference between saline-treated mice with and without wheel access, # = a difference between naltrexone-treated mice with and without wheel access, ^ =a difference between naltrexone- and saline-treated mice with wheel access. $p<0.001$.

Chapter 4

An expanded consideration of the wheel: locked wheel, correlation, and group housing

INTRODUCTION

The results of the previous chapter demonstrate that wheel access can attenuate increased thermal sensitivity observed during spontaneous morphine withdrawal. This chapter examines further the role that wheel access plays during the withdrawal period.

There is extensive research suggesting that environmental enrichment can have significant effects on various behavioral assays associated with drug use (e.g., review by Stairs and Bardo, 2009). Although there is no set standard for what constitutes an enriched environment, it often includes some combination of social (group housing), exploratory (toys) and exercise (running wheel) elements. Consequently, it is of interest to determine whether the effects of wheel access are dependent on wheel use (exercise) or simply the presence of a wheel in the cage (exploratory toy). Although many studies on the effects of wheel access do not include locked-wheel controls, the majority of those that do conclude that the presence of a locked wheel is not sufficient to replicate the effects of wheel access. For example, Pietropaolo et al., (2006) demonstrated

that access to unlocked but not locked wheels enhanced multiple measures of cognitive function. A locked wheel also failed to produce changes seen following wheel running in both defensive behaviors and antidepression assays (Burghardt et al., 2004; Sartori et al., 2011). Additionally, access to locked wheels had no effect on cocaine self-administration, extinction or reinstatement (Zlebnik et al., 2010 and 2012). Finally, the reduction of seizures during ethanol withdrawal in rats with wheel access did not occur in rats with locked wheel access (Devaud et al., 2012).

Although it appears that the effects of wheel access are dependent on wheel use, it is unclear whether the extent of wheel use is correlated with the strength of behavioral effects. Many studies report average running across wheel access groups; nevertheless, few of those studies present correlations between amount of running and behavioral outcomes. That said, a few studies have shown that the intensity of aerobic exercise is positively correlated with the release of beta-endorphin and other opioid peptides (Goldfarb et al., 1990; Mehl et al., 2000; Mougin et al., 1988). Thus, we hypothesize that there will be a correlation between amount of running and reduction in withdrawal severity. This hypothesis is supported by findings of the Smith laboratory, which showed positive correlations between individual running and acute opioid sensitivity as well as cocaine reinforcement in rats (Smith and Lyle, 2006 and 2008).

In addition to wheel access, the number of animals per cage is another variable examined in enrichment studies. For example, single housing as compared to group housing of rodents alters both morphine conditioned place

preference as well as morphine and heroin self-administration (Alexander et al. 1978; Bozarth et al. 1989; Bardo et al. 1997; Coudereau et al. 1997a; Raz and Berger 2010; Kennedy 2012). Coudereau et al. (1997b) and Broseta et al. (2005) both found that social isolation decreased symptoms of antagonist-precipitated opioid withdrawal.

Given observations suggesting that both wheel access and housing conditions can alter the effects of opioids, the present study examines the effect of 1) a locked wheel and 2) group versus single housing on spontaneous morphine withdrawal in mice. The correlation between wheel use and thermal sensitivity is also examined. As in previous experiments, withdrawal is assessed following the termination of a drug regimen in which mice receive twice-daily injections of 56 mg/kg morphine (s.c.) for 5.5 days. Withdrawal severity is examined at multiple time points (8, 24, 32 and 48 hrs) following the termination of morphine administration. Withdrawal severity is quantified by determining sensitivity to a thermal stimulus on a hot plate analgesia meter as described in previous chapters. The use of a spontaneous withdrawal procedure allows mice to have access to running wheels throughout the withdrawal period.

Based on data from previous studies in our laboratory and others, we hypothesize that increases in thermal sensitivity observed during withdrawal will be attenuated in mice that have access to running wheels. However, sensitivity will not be changed in mice with access to a locked wheel. We also propose to examine the effects of group housing on thermal sensitivity during spontaneous withdrawal.

METHODS

Animals

All experiments were conducted in male C57BL/6J mice (Jackson Labs, Raleigh, NC), 10 weeks of age upon delivery. Male C57BL/6J mice were selected to allow comparison with other data collected in our laboratory as well as the extensive behavioral literature in these mice. Additionally, in comparison to other inbred strains, C57BL/6J mice are known to be highly sensitive across many behavioral assays. Specifically, they exhibit high sensitivity in measures of acute nociception (Mogil et al 1999), naloxone-precipitated morphine withdrawal (Kest et al. 2002) and morphine self-administration (Elmer et al. 2009). Finally, C57BL/6J mice are known to exhibit high rates of voluntary wheel running (Clark et al. 2011).

Mice were either singly-housed (Experiment I) or group-housed, four per cage (Experiment II) in polycarbonate cages (floor area=335cm^2) with continuous access to food and water throughout the study. The colony room was maintained on a 12-hr, reverse, light/dark cycle (lights off at 7:00 am) and all behavioral testing was conducted during the dark cycle, between 9:00 am and 7:00 pm. Mice were habituated to handling and the colony room environment for two weeks prior to any experimental manipulation. Mice were also exposed to the testing environment for at least two days prior to initiation of an experiment and for 1 hr prior to all behavioral testing. Criterion was set such that mice <20 g or

those that lost >20% of initial body weight would be removed from the study. Animal protocols were approved by the Institutional Animal Care and Use Committee, and the methods were in accord with the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council, 2011).

Experimental Procedures

Pharmacological Procedure: During the saline/morphine administration period, doses of saline or 56 mg/kg of morphine were administered daily for 5.5 days, with injections occurring at 10:00 am and 8:00 pm daily (11 injections total). Morphine sulfate, provided by the National Institute on Drug Abuse (Bethesda, MD, USA), was dissolved in 0.9% saline to yield all concentrations. Doses were injected subcutaneously at a volume of 0.1 ml/10 g.

Experimental Groups: Mice were assigned to one of four groups during each three-week experimental session (described below). Morphine treated mice received 56mg/kg morphine. New mice were used for each experiment.

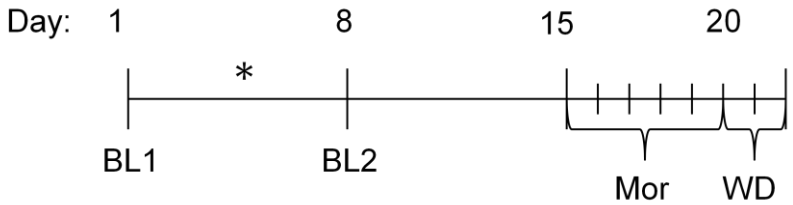
Experiment I, Locked Wheel: 1) wheel access, morphine treatment; 2) locked-wheel access, morphine treatment; 3) no wheel access, morphine treatment and 4) locked wheel access, saline. N=7 for locked wheel, morphine treatment group, n=8 for all other groups.

Experiment II, Group Housing: 1) singly-housed, morphine; 2) group-housed, morphine; 3) singly-housed, saline and 4) group-housed, saline. N=8 for singly-housed mice, n=7 for group-housed mice.

Wheel Access: Mice in wheel access groups had Med Associates Mouse Low-Profile Wireless Running Wheels in their home cages. Wheels were locked with a small metal peg connecting the wheel and the base. Activity on the wheels was monitored continuously (24 hr/day) via a computer equipped to record radio signals from the wheels.

Thermal Sensitivity: Thermal sensitivity was assessed using a hot plate analgesia meter (25.3 × 25.3 cm), Columbus Instruments, Columbus, OH. During each 1-hr hot plate testing period, a temperature-effect curve was determined for each mouse. Sensitivity was evaluated by recording the latency to lick or flutter the hind paw(s), or to jump from the hot plate surface at each of four temperatures presented in the following order: 50, 54, 52, 56^{o}C with 15-min intervals between temperatures. Response latency was measured to the nearest 0.1 sec. To prevent tissue damage, a predetermined cutoff time of 20 sec was defined as the maximal trial duration. Immediately following the termination of a trial, whether due to a mouse's response or elapsed cutoff time, mice were removed from the hot plate surface. Parameters were selected based on prior work in our laboratory regarding responses on the hot plate (e.g. Fischer et al. 2008).

Experimental Protocol: On day one, thermal sensitivity was assessed in all groups of mice (baseline 1). Immediately following baseline 1, running wheels were placed in the cages of mice in wheel access groups. One week later on day 8, a second baseline measure (baseline 2) was determined in all groups of mice and wheels were removed from the cages. The average of baseline 1 and baseline 2 was used for data analysis and is presented in all figures. One week later on day 15, morphine or saline administration began as described below and continued for 5.5 days. Immediately following the final injection of morphine or saline (day 20), thermal sensitivity was assessed again, and wheels were returned to the cages of mice in the wheel access groups. Following the last dose of morphine, thermal sensitivity was assessed four more times: at 8 hrs after the final injection (6:00 pm on day 20), at 24 hrs (10:00 am on day 21), at 32 hrs (6:00 pm on day 21) and at 48 hrs (10:00 am on day 22). This period (days 20-22) was designated the withdrawal period.



* = 1 week of wheel exposure for mice in wheel access groups
BL= Baseline assessment of thermal sensitivity on the hot plate.
WD= 8, 24, 32 and 48hr hot plate test sessions after the final morphine injection.

Data analysis

ANOVA and correlation analysis was performed using SPSS for Windows software, version 9.0. All post hoc analysis was performed using SAS for Windows software, version 9.2. Figures were created with GraphPad Prism 5.

Thermal sensitivity: Data are presented as response latencies on the hot plate, expressed as means (±SEM) at each of the four temperatures. For each experimental group, a 2-way repeated measures ANOVA revealed no difference between baseline 1 and baseline 2; therefore, baselines were averaged for all analyses. Each experiment was first analyzed using a 3-way repeated measures ANOVA with time and temperature as repeated measures factors and group as an independent factor. For the 3-way ANOVA, an alpha level of significance was set at $p<0.01$. Following the 3-way ANOVA, appropriate follow-up contrasts and Student's t-tests were performed using a fully saturated mixed model of the data. The model was a straight model of the means and included random intercepts for each animal. Statistical analyses were conducted with an alpha level of significance set at $p<0.001$. The alpha level was determined using Bonferoni corrections to account for the large number of comparisons.

Correlations: A Pearson product-moment correlation was used to compare individual running (simple or % of baseline) with ET10 values. The alpha level for correlation coefficients was set at $p < 0.05$.

Running data: During the withdrawal period running was defined as the total number of wheel revolutions in the seven-hour period prior to each test session. This included the full inter-session interval for the 8 and 32 hr time points (period from 10:00 am until 5:00 pm when mice were taken into the testing room). For the 24 and 48 hr time points, revolutions during the seven-hour period from 2:00 am- 9:00 am were used. Baseline running was calculated using the average revolutions during the last two full days of the wheel pre-exposure period. Separate morning (2:00 am- 9:00 am) and afternoon (10:00 am until 5:00 pm) baselines were calculated. Running data are presented as either simple revolutions or revolutions as a percent of appropriate baseline (8 and 32 hr time points as percent of afternoon baseline, 24 and 48 hr time points as percent of morning baseline).

ET10: The ET10 represents the theoretical temperature required to produce a response latency of 10 sec (half the maximal response latency of 20 sec) and was derived using log-linear interpolation. Larger ET10 values indicate longer response latencies and lower thermal sensitivity.

RESULTS

In general, two findings were consistent for both experiments. First, latency to respond on the hot plate decreased as a function of temperature. Response latencies in both saline and morphine-treated mice were at or close to the maximal value of 20 sec when the hot plate was set at 50^{o}C; at 52, 54 and

56^{o}C, latency to respond on the hot plate decreased as a function of temperature. Second, response latencies at the 8, 24, 32 and 48-hr time points for saline-treated mice were never significantly different from baseline, calculated as the average of baseline 1 and 2, indicating that repetition of testing did not produce measurable effects on response latency.

In addition, immediately following the final morphine injection (0 hr), response latencies were at the cut off value of 20 sec at all temperatures for morphine-treated mice; consequently these data are not shown. The failure to respond within in the 20 sec maximal trial duration indicates a full antinociceptive response to acute morphine exposure.

Experiment I, Part I: The effect of a locked wheel following 56 mg/kg morphine

Fig. 4.1 shows latency to respond on the hot plate as a function of temperature at 8, 24, 32 and 48 hrs following termination of the 5.5 day treatment period of either 56 mg/kg morphine or saline. A 3-way repeated measures ANOVA revealed time x temperature x group interaction, $F(36, 324) = 1.51$, $p=0.035$. Main effects of time and temperature were $F(4, 108) = 60.62$, $p<0.001$ and $F(3, 81)= 2932.32$, $p<0.001$, respectively. Follow-up contrasts and Student's t-tests were then used to compare individual groups, time points, and temperatures.

At 8, 24, 32, and 48 hrs following the final morphine injection, the curves of morphine-treated mice without wheel access were displaced downward from those of saline treated mice, $F(4, 513) = 5.73, 27.96, 21.46, 23.16$, respectively,

$p<0.001$. Additionally the response curves of morphine-treated mice with wheel access were no different from those of the saline-treated mice ($p>0.1$). These data replicate the findings of the previous chapter: thermal sensitivity is increased during withdrawal form 56 mg/kg of morphine and attenuated by wheel access.

The response curves of mice with a locked wheel were never significantly different from the curves of mice with no wheel access ($p>0.1$). Furthermore, significant differences between the response latencies morphine-treated mice with a locked wheel and those with an unlocked wheel were apparent at 8 hrs (52, 54^{o}C) t_{513}= 3.30, 3.88; 24 hrs (50, 52^{o}C) t_{513} = 4.02, 5.73; 32 hrs (50, 52, 54^{o}C) t_{513} = 4.71, 7.20, 4.82; and 48 hrs (50, 52, 54^{o}C)t_{513} = 5.61, 6.95, 4.72, $p<0.001$. These differences suggest that the thermal sensitivity of mice with access to a locked wheel is increased during withdrawal similarly to that of mice with no wheel rather than attenuated like that of mice with an unlocked wheel.

Experiment I, Part II: Comparison between wheel use and thermal sensitivity

In addition to between-group comparisons, correlations between wheel running and thermal sensitivity were also examined. Fig. 4.2 shows average wheel revolutions during seven-hour periods throughout the experiment in the light and dark cycles. A seven-hour period was selected because it included the entire interval from 11:00am to 5:00pm (dark period) between behavioral testing sessions during the withdrawal period. To maintain consistency, seven-hour periods were sampled for the time prior to morning testing: 2:00 am to 9:00 am (light period). During the dark cycle in the baseline, pre-morphine period, mice

ran an average of 9388.8 rev/ 7hrs (3.57 km / 7hrs). Consistent with the nocturnal nature of mice, wheel use during the light cycle was substantially less. Though wheel use during the withdrawal period was greatly reduced, mice did use the wheels during this period.

Fig. 4.3 shows the correlation between individual wheel use and hot plate response during the withdrawal period. All panels present hot plate response as an ET10 value derived from the latency data shown in Fig. 4.1. ET10 values represent the theoretical temperature necessary to produce a 10 sec response on the hot plate. Thus, higher ET10 values indicate longer response times and lower thermal sensitivity. In panels A-D, individual running data are presented as revolutions during the prior seven-hour period. In panels E-H, running data are presented as a percent change in revolutions from matched baseline (light or dark cycle). Correlation coefficients failed to reach statistical significance ($p<0.05$). For correlations with $p<0.1$, r values were as follows: 8hrs (%BL), $r = -0.71$; 32 hrs (total revs), $r = -0.66$; 32 hrs (%BL), $r = -0.66$; 48hrs (total revs), $r = 0.67$; 48hrs (%BL), $r = 0.70$. These data suggest a trend towards a negative correlation between wheel use and thermal sensitivity at 32 hrs and a positive correlation at 48 hrs.

Experiment II: 56 mg/kg Morphine, Group Housing

In Experiment II mice were either housed in groups of 4 or singly housed. Fig. 4.4 shows latency to respond on the hot plate as a function of temperature at baseline, 8, 24, 32 and 48 hrs following termination of the 5.5 day treatment

period of either 56 mg/kg morphine or saline. A 3-way repeated measures ANOVA revealed a time x temperature x group interaction, $F(36, 312)=1.88$, $p=0.003$. Follow-up contrasts and Student's t-tests were then used to compare individual groups, time points, and temperatures.

In comparison to baseline, the temperature-effect curves revealed the same orderly relationships seen previously. The curves of the morphine-treated/singly-housed mice showed significant differences from baseline at 8, 24, 32, 48 hrs, respectively $F(4,494)= 7.15, 23.18, 23.04, 18.80$, $p<0.001$. In contrast, response latencies of morphine-treated mice that were group-housed were only different from baseline at 48 hrs. $F(4,494)= 5.59$, $p=0.0002$.

Throughout the withdrawal period, differences between the response latencies of morphine-treated/singly-housed mice and saline-treated mice were apparent at 8 hrs (54 ^{o}C) $F(2,494)= 7.72$, $p<0.001$; 24 hrs (50, 52, 54 ^{o}C) $F(2,494)= 10.40, 16.24, 14.77$, $p<0.001$; 32 hrs (50, 52, 54 ^{o}C) $F(2,494)= 9.06, 28.58, 8.90$, $p<0.001$; 48 hrs (52 and 54^{o}C) $F(2,494)= 23.46, 14.67$, $p<0.001$. These data replicate the data obtained in the 56 mg/kg morphine-treated/ no wheel access groups from Experiment I. That is, morphine-treated mice were more sensitive to the thermal stimulus than saline controls at 24, 32, and 48 hrs following termination of morphine administration.

The response latencies of morphine-treated, group-housed mice were similar to those observed in the saline controls, except at three points (32 hrs, 52 ^{o}C and 48 hrs, 52 and 54 ^{o}C). $F(2, 494)= 9.85, 11.73, 11.58$, respectively, $p>0.001$. Although there was some evidence that the decrease in response

latencies observed in morphine-treated/singly-housed mice was attenuated in group-housed mice (54 ^{o}C at 8, 24 hrs t_{494} =5.90, 3.54, p<0.001), this attenuation was not as robust as that observed in Experiment I, as the result of wheel access.

Taken together, these data replicate previous findings that 1) thermal sensitivity is increased during withdrawal in morphine-treated mice singly housed without wheel access and 2) this increase in sensitivity is attenuated with wheel access. In addition, these data suggest that withdrawal is not attenuated when access to the wheel is locked; however, attenuation of withdrawal is not correlated with the amount of wheel use. Although group housing also attenuated the increase in thermal sensitivity observed during withdrawal from 56 mg/kg morphine, the attenuation was not as robust as that observed as the result of wheel access.

DISSCUSION

The experiments conducted yielded three main findings. First, the presence of a locked wheel in the home cage is not sufficient to alter the severity of morphine withdrawal, as measured by thermal sensitivity. Second, although thermal sensitivity during withdrawal was generally lower in mice with wheel access, individual thermal sensitivity was not correlated with the amount of wheel running. Third, an alternative form of enrichment, group housing, produced moderate decreases in withdrawal severity. In addition, the experiments replicated two important findings. First, the results from both experiments

indicated that thermal sensitivity reliably increased during withdrawal following termination of a chronic regimen of morphine administration. Second, increases in thermal sensitivity during withdrawal were attenuated in mice with access to running wheels in their home cages.

In the current experiment, thermal sensitivity of mice without wheels was significantly decreased following the termination of chronic morphine. The sensitivity of mice with locked wheels was the same as that of mice without wheels and was significantly higher than the sensitivity of mice with unlocked wheels. The observation that access to a locked wheel did not produce the same behavioral effects as access to an unlocked wheel is consistent with the majority of published findings (e.g. Burghardt et al., 2004; Devaud et al., 2012; Pietropaolo et al., 2006; Sartori et al., 2011; Zlebnik et al., 2010 and 2012). These data suggest that the effect of wheel access is dependent on wheel use not simply the presence of the wheel acting as a toy to explore.

We hypothesized that mice that ran more would exhibit greater benefits (i.e. less thermal sensitivity during withdrawal) than mice that ran less. The data of experiment 1 did not support this hypothesis. Throughout the withdrawal period wheel revolutions in the hours prior to behavioral testing were not correlated with individual thermal sensitivity, as quantified by ET10. ET10 values also were not correlated with running adjusted for possible individual difference (revolutions as % baseline). The initial hypothesis is based on the assumption that wheel revolutions are an accurate measure of aerobic exercise. It is possible that expended effort was correlated with withdrawal severity but was

masked by variation in wheel tension such that different amounts of running produced similar aerobic effects. Alternatively, reduction in thermal sensitivity may simply require that a certain threshold of wheel use be exceeded. Wheel running beyond that point will provide no further effect. Considering the little variation in ET10 values between mice and the full recovery to baseline sensitivity, this may be a reasonable hypothesis. Finally, the lack of correlation does not diminish the significant effects of wheel access during spontaneous withdrawal.

Since wheel access is often placed under the umbrella of environmental enrichment, Experiment II examined the effect of a second environmental manipulation, group housing. Comparisons between mice that received saline and either were singly housed or group-housed indicated that group housing alone did not alter thermal sensitivity. However, in mice undergoing morphine withdrawal, group housing attenuated the withdrawal-induced increases in thermal sensitivity for at least two of the time points during the withdrawal period (i.e., 8 and 24 hrs, but not 32 and 48 hrs). Therefore, both wheel access and group housing attenuated the increases in thermal sensitivity observed during morphine withdrawal.

A comparison between the results from Experiment I and Experiment II suggests that the attenuation of thermal sensitivity during withdrawal was greater in mice that had access to wheels, than those that were group-housed. Specifically, with wheel access, significant attenuation was observed at 8, 24, 32

and 48 hours following the final morphine injection. Group-housed mice only showed clear attenuation during the earlier withdrawal period, at 8 and 24 hours.

The finding that one symptom of withdrawal, i.e., thermal sensitivity was attenuated in group-housed mice is in contrast to the findings of Coudereau et al. (1997b) and Broseta et al. (2005), which suggested that social isolation (single housing) decreased the symptoms of opioid withdrawal. There are a number of significant methodological differences between the Coudereau and Broseta studies and the current study. Perhaps most importantly, the Coudereau and Broseta studies both examined naloxone precipitated withdrawal rather than spontaneous withdrawal. Additionally, Broseta et al. (2005) only quantified physical symptoms of withdrawal after repeated naloxone exposure during conditioned place aversion. Coudereau et al. (1997b) used younger mice (5 as opposed to 12 weeks old) and housed them 6, rather than 4, per cage. Interestingly, in studies from Burghardt et al. (2004) and Pietropaolo et al. (2006) that considered the effect of group housing and wheel access on cogntion and anxiety, group housing produced an intermediate effect between locked and unlocked wheel access, similar to what was observed here.

To fully characterize the effects of group housing on morphine withdrawal, it is clear that additional studies examining a range of parameters (length of isolation/group housing, number of animals per cage, drug treatment of group-housed peers) would be needed. The current study is also limited by the use of a single, behavioral measure of morphine withdrawal. It will be critical to consider the effects of group housing as well as wheel access on additional signs

of physical withdrawal such as jumping, wet dog shakes, conditioned aversion, and changes in schedule controlled responding. It is possible that wheel running may correlate with individual changes in one of these other behavioral measures of withdrawal. Taken together, these experiments suggest that although environmental manipulations such as group housing may affect withdrawal severity, voluntary access to (unlocked) running wheel produces the most dramatic behavioral effect.

FIGURES

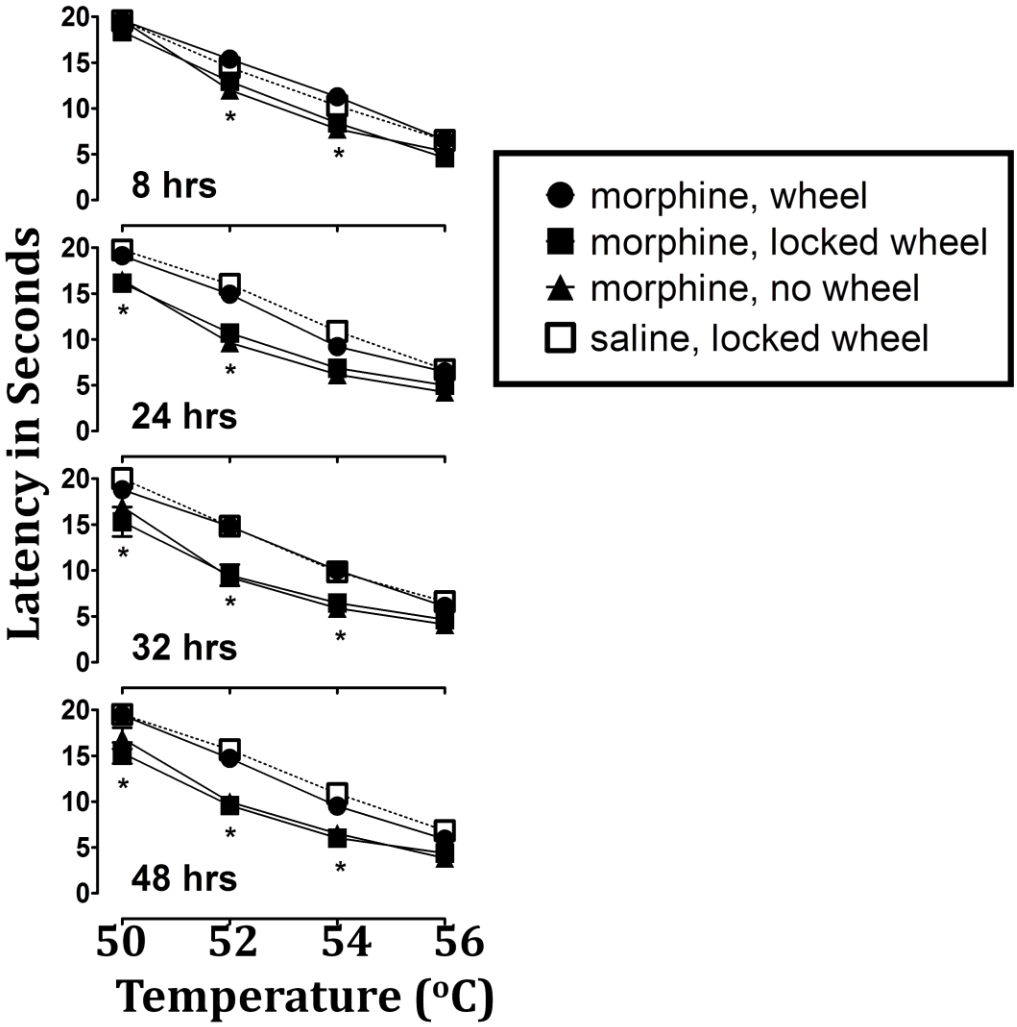


Fig. 4.1 **Effects of a locked, compared to unlocked, wheel on latency (mean ±SEM) to respond on the hot plate at 50, 52, 54, and 56^{o} C in saline- and morphine- (56 mg/kg) treated mice.** Data are shown for mice that had access to locked as well as unlocked wheels. Latency on the hot plate was determined at baseline and at 8, 24, 32, and 48 hrs after the final morphine injection. Abscissa: hot plate temperature in ^{o} C. Ordinate: latency to respond in seconds. *= statistically significant difference between morphine treated mice with access to locked as compared to unlocked wheels. $p<0.001$

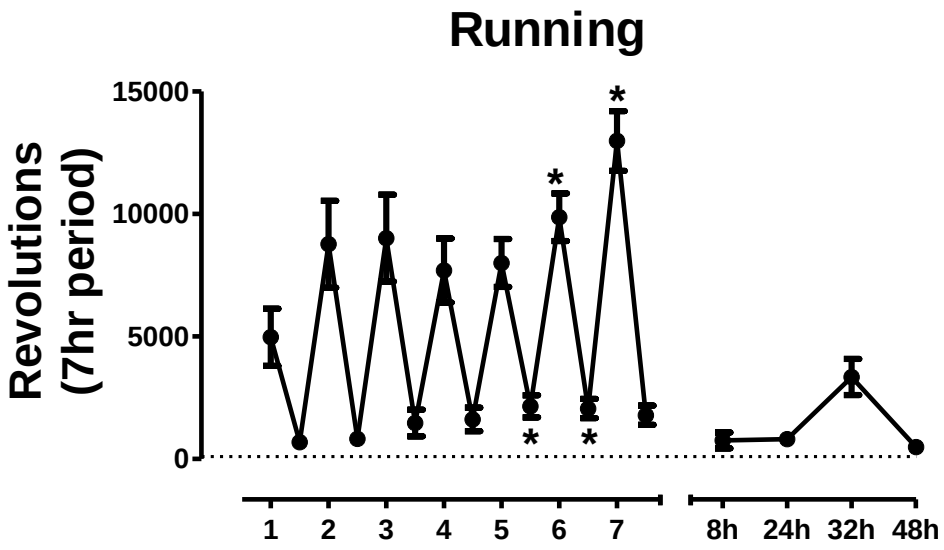


Fig. 4.2 **Total revolutions (mean ±SEM) during 7hr sample periods.** Dark cycle sample period= 10:00 am until 5:00 pm, light cycle sample period= 2:00 am- 9:00 am.

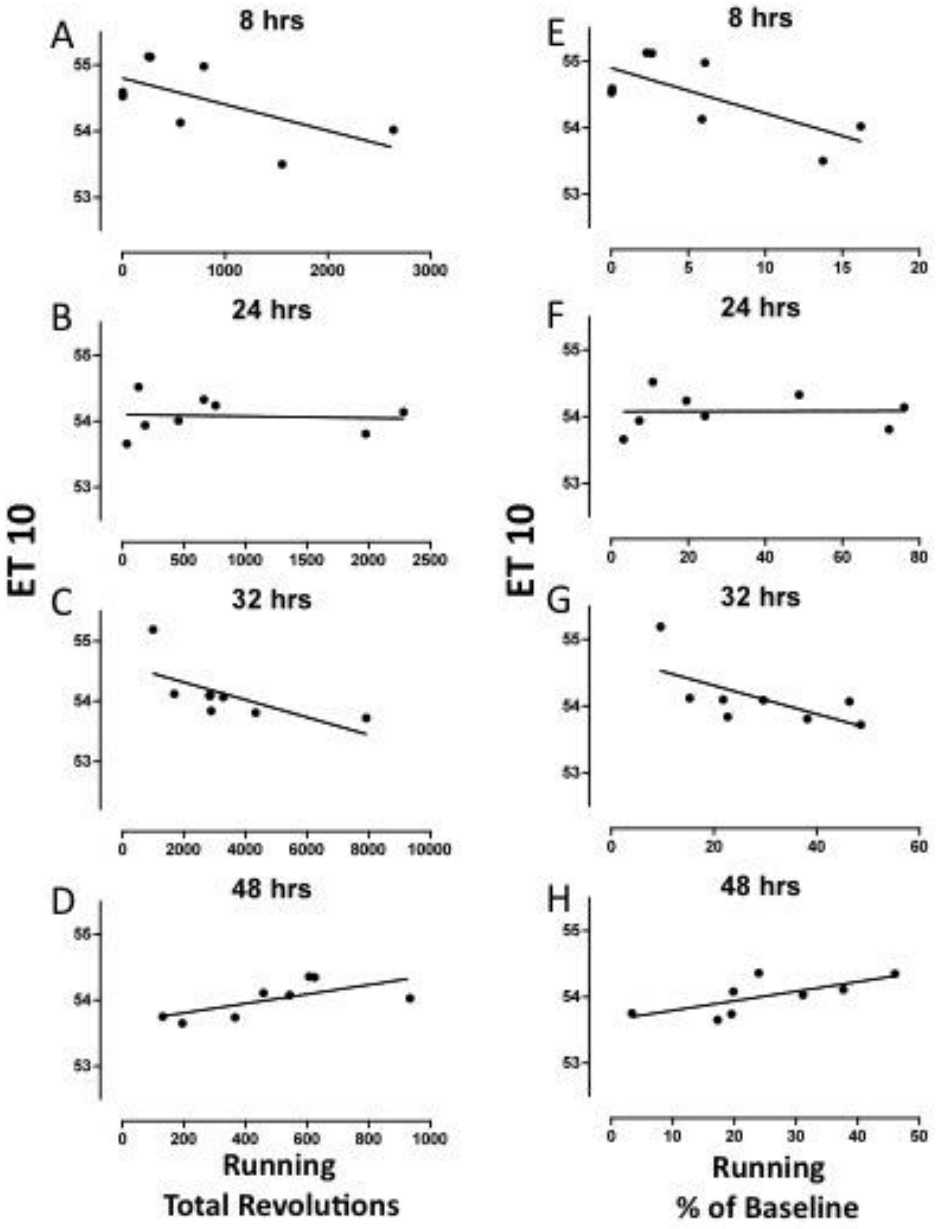


Fig. 4.3 **Correlation between running and response latency as quantified by ET10 during spontaneous withdrawal from 56 mg/k morphine**. ET10 values represent the temperature that would produce a 10 sec response on the hot plate; larger values represent lower sensitivity. Individual wheel use is presented as total revolutions in the prior 7hr period (A-D) or revolutions as a percent of individual baseline (E-H). Data are presented with linear regression line

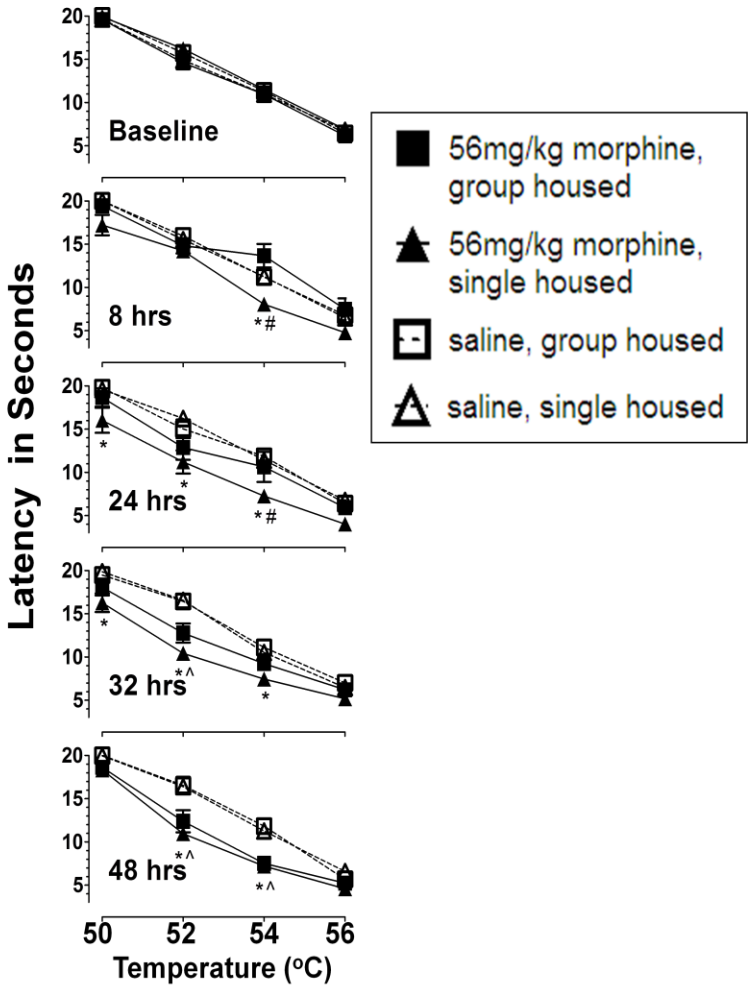


Fig. 4.4 **Effects of group housing (4/cage) on latency (mean ±SEM) to respond on the hot plate at 50, 52, 54, and 56° C in saline- and morphine-(56 mg/kg) treated mice**. Data are shown for mice that were group-housed as well as for mice that were singly-housed. Latency on the hot plate was determined at baseline and at 8, 24, 32, and 48 hrs after the final morphine injection. Abscissa: hot plate temperature in ° C. Ordinate: latency to respond in seconds. Statistically significant differences are indicated as follows: * = a difference between singly-housed mice treated with morphine and saline controls, # = a difference between group- and singly-housed mice treated with morphine, ^ = a difference between group-housed mice treated with morphine and saline controls. $p<0.001$

Chapter 5

Chronic wheel access can decrease morphine sensitivity and alter gene expression.

INTRODUCTION

Chronic administration of morphine and other opioids leads to the development of tolerance and physical dependence in both primates and rodents (e.g. Bhargava, 1978; Christie, 2008; Fernandes et al., 1977; Kamei et al., 1973; Sell et al., 2005). Furthermore, once tolerance to one opioid is established cross-tolerance to other opioids develops as well (e.g. Dumas et al., 2008; Eyler, 2013). The ability of two drugs to produce "cross-tolerance" suggests similar sites of cellular action.

Interestingly, multiple groups have found that chronic wheel access in rats can decrease morphine's potency similarly to decreases produced by chronic morphine (Kanarek et al. 1998; Mathes and Kanarek 2001, 2006; Smith and Yancey 2003; Smith and Lyle 2006). The fact that chronic wheel running also decreases morphine's antinociceptive potency is consistent with the hypothesis that wheel running activates the opioid system. This is further supported by data reviewed by Koltyn (2000), which describes a number of studies in both humans

and animals showing that aerobic exercise can produce analgesic effects across a number of pain assays.

The current experiment confirms the finding that extended wheel access decreases morphine potency. Specifically, we hypothesize that 6 weeks of access to a running wheel will produce a rightward shift in the morphine dose-effect curve as assessed by tail-flick latency. Changes in morphine's antinociceptive effects are used to assess tolerance as these effects are easily observable, dose-dependent and reliable (Fischer et al., 2005; 2008).

Tolerance to the antinociceptive effects of opioids is almost certainly concurrent with, if not caused by, various cellular changes resulting from chronic opioid receptor activity. Opponent process theory provides a heuristic model suggesting that the changes during tolerance are the opposite of the changes following acute drug exposure. As proposed, these "opposite" changes produce an allostatic state to balance the effects of the exogenous input. A great deal of experimental literature is devoted to identifying the cellular changes that occur during opioid tolerance. One subset of this body of literature considers changes in the expression of various genes.

Based on these observations, the following argument might hold: if chronic wheel access and chronic morphine administration produce similar behavioral changes, then it is possible that both produce similar changes in gene expression. The experiments described here use quantitative real time polymerase chain reaction (qRT-PCR) to compare gene expression following chronic wheel access and chronic morphine administration. The expression of five genes was

assessed: PENK, PDYN, POMC, MOR1, and BARR2. Proenkephalin (PENK), prodynorphin (PDYN), and proopiomelanocortin (POMC) were selected because they code for the precursor proteins that are post-translationally modified into the three major endogenous opioids: enkephalin, dynorphin, and beta-endorphin, respectively, (Aghajanian and Sanders-Bush 2002). The MOR1 gene codes for the mu-opioid receptor (Ammon-Treiber et al 2005). Beta-arrestin 2 (BARR2) is a protein which regulates mu-opioid receptor desensitization and internalization and plays a role in the onset of opioid tolerance (Bohn et al., 2004).

The mRNA level of each of these genes was assessed in four brain regions integral to the formation and expression of morphine tolerance: the striatum, nucleus accumbens, hypothalamus and periaqueductal grey. Furthermore, research has shown that chronic morphine can alter gene expression in each region: the striatum (Martin-Soelch et al., 2001; Gieryk et al., 2010), the nucleus accumbens (Leriche et al., 2007; Gieryk et al., 2010), the hypothalamus (Wang et al., 2011; Wei et al., 2009) and the periaqueductal grey (Fan et al. 2003; Folkesson et al.,1989; Maldonado et al.,1992; Stamford 1995; Wang et al., 2011).

METHODS

Animals

All experiments were conducted in male C57BL/6J mice (Jackson Labs, Raleigh, NC), 10 weeks of age upon delivery. Male C57BL/6J mice were

selected to allow comparison with other data collected in our laboratory regarding morphine's pharmacological effects as well as the extensive literature on the behavioral effects of opioids in C57BL/6 mice. Additionally, in comparison to other inbred strains, C57BL/6J mice are known to be highly sensitive across many behavioral assays. Specifically, they exhibit high sensitivity in measures of acute nociception (Mogil et al., 2000), naloxone precipitated morphine withdrawal (Kest et al. 2002) and morphine self-administration (Elmer et al. 2009).

Mice were individually housed in polycarbonate cages (floor area= 335cm^2) with continuous access to food and water throughout the study. The colony room was maintained on a 12-hr, reverse, light/dark cycle (lights off at 7:00 am) and all behavioral testing was conducted during the dark cycle, between 9:00 am and 7:00 pm. Mice were habituated to handling and the colony room environment for two weeks prior to any experimental manipulation. Mice were also exposed to the testing environment for four days prior to initiation of the experiment and for 1 hr prior to all behavioral testing. Although a criterion was set such that mice <20 g or those that lost >20% of initial body weight would be removed from the study, it was not necessary to remove any mice from the study. Animal protocols were approved by the Institutional Animal Care and Use Committee, and the methods were in accord with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council, 2011).

Experimental Procedures

Experimental Groups: Mice were assigned to one of three groups: wheel access (n=8), no wheel access (n=8), chronic morphine (n=7).

Mice in the wheel access group had access to Med Associates Mouse Low-Profile Wireless Running Wheels in their home cages for the six week period between determination of the first and second morphine dose-effect curves. Activity on the wheels was monitored continuously via a computer equipped to record radio signals from the wheels.

Experimental design: Following four days of training to acclimate the mice to the tail-flick procedure, morphine antinociception was assessed ("pre" time point). Immediately following the test session, running wheels were placed in the cages of mice in wheel access groups. Six weeks later a second morphine dose-effect curve was determined ("post" time point). Mice in the chronic morphine group received morphine injections for the final 5 days of the sixth week. During the last four days of the sixth week, mice were again acclimated to the tail-flick procedure. Immediately following the "post" dose-effect curve, the mice were decapitated and their brains were removed to examine gene expression.

Behavioral procedures

Tail-flick procedure: Mice were lightly restrained by hand and the animal's tail was positioned over a light source. The latency to remove (i.e. flick) the tail

was measured twice: when the light source was positioned 5 cm and 3 cm from the tip of the tail. The light was immediately removed from the tail when the mice exhibit a response or reached the predetermined cut off point of 10 sec.

A full dose-response curve was determined by measuring response latency at baseline and following 0.32, 1.0, 3.2, 10.0 and 32.0 mg/kg doses of morphine. A within subject, cumulative dosing procedure, with 30 min between doses, was used, according to an experimental protocol that has been used successfully in our laboratory to investigate morphine's antinociceptive effects.

Pharmacological Procedure: For mice in the chronic morphine group, doses of 56 mg/kg of morphine were administered daily for 5.5 days, with injections occurring at 10:00 am and 8:00 pm daily (11 injections total). Morphine sulfate was provided by the National Institute on Drug Abuse (Bethesda, MD, USA), was dissolved in 0.9% saline to yield all concentrations. Doses were injected subcutaneously at a volume of 0.1 ml /10 g.

Analysis of Tail-Flick Dose-Effect Curves: Data are presented as a percentage of the maximum possible effect (%MPE), expressed as means (±SEM) at each dose. Percentage of the maximum possible effect was calculated using the following formula:

$$\%MPE = \frac{[\text{Postdrug latency} - \text{baseline latency}]}{[\text{cutoff time (20sec)} - \text{baseline latency}]}$$

For each dose-effect determination, the dose of morphine (mg/kg) required to produce a 50% maximal antinociceptive effect (ED50) was derived mathematically (least-squares method) using log-linear interpolation, with at least three doses on the ascending limb of the dose-effect curve. Potency ratios (±95% confidence limits) were calculated using a procedure from Tallarida and Murray (1987), and were used for comparison of ED50 values.

Gene expression procedures

Tissue collection: Immediately following determination of the second dose-effect curve, mice were decapitated. Decapitation without anesthesia was necessary because anesthesia has been shown to alter RNA transcription patterns (Palotas et al., 2005; Quinones-Jenab et al., 1996). The brains were quickly removed from the skull, the hemispheres were separated and placed in RNAlater (an RNAse inhibitor) and stored at -80^{o}C.

Subsequently, brain hemispheres were removed from the freezer and the striatum, nucleus accumbens, hypothalamus and periaqueductal grey were dissected out. See Fig. 5.1 for details of dissection.

qRT-PCR procedure: Immediately following dissections, RNA was isolated from the tissue samples using the Qiagen RNeasy Lipid Tissue Mini Kit and quantified using the NanoDrop® ND-1000 Spectrophotometer. Sodium Acetate precipitation was used to reduce salt contamination. The RNA was reverse transcribed to cDNA using an Invitrogen SuperScript III First-Strand Synthesis

Super Mix Kit and run in a MJ Research PTC-225 Peltier Thermal Cycler. Quantitative PCR was then done to provide a measure of differential gene expression between samples. For the qPCR procedure, TaqMan® primers for one of the five genes of interest plus the TaqMan® Gene Expression Master Mix were added to the cDNA samples and run in an Applied Biosystems 7300 Real Time PCR. GAPDH was used as the reference gene. All samples were run in triplicate and threshold cycle values were averaged for calculations.

Primers:

TaqMan® primers for 5 genes were used: proenkephalin (PENK), prodynorphin (PDYN), proopiomelanocortin (POMC), mu-opioid Receptor 1 (MOR1), and beta-arrestin 2 (ARRB2). In addition to sense and antisense primers for the genes of interest, primers for the reference gene GAPDH was used.

TaqMan® Gene Expression Primers:

#Mm01212875_m1 (PENK)
Mm00457573_m1 (PDYN)
Mm00435874_m1 (POMC)
#Mm01188089_m1 (MOR1)
#Mm00520665_m1 (ArrB2)
#Mm99999915_G1 (GAPDH)

Analysis of results, q-PCR: Relative expression (mean ±SEM) of genes in the wheel and morphine groups compared to sedentary control was calculated using the threshold cycles (Ct) for the genes of interest and the reference gene. A one-

way ANOVA confirmed that there was no significant effect of group in the expression level of the reference gene GAPDH in any of the brain regions tested ($p>0.5$). Relative expression (fold change difference from control) was calculated with the formula: 2^(-(ΔΔCt) (Livak and Schmittgen, 2001). ΔCt is the gene of interest Ct minus GAPDH Ct and ΔΔCt is ΔCt (gene of interest) minus the average ΔCt of the sedentary control group for the same gene within the same brain region. To determine if the changes in expression for the wheel access and chronic morphine groups were significant, Student's t-tests compared the ΔΔCt values of the wheel access and chronic morphine groups to that of the no wheel control group.

RESULTS

Behavioral results: Morphine produced dose-dependent antinociception in the tail-flick procedure in all mice at all time points (Fig. 5.2). At baseline there was no significant difference between the three groups in their antinociceptive sensitivity to morphine as determined by the ED50, [ED50 (95%CL)= 2.92 mg/kg (2.55-3.33), 3.50 mg/kg (3.07-4.00), 3.07 mg/kg (2.62-3.5), for wheel, morphine, and no wheel groups, respectively].

Fig. 5.2a shows that the morphine dose-effect curves for mice before and after six weeks of wheel access. At both time points, latency to respond increased as a function of morphine dose. However, the dose-effect curve was shifted rightward at six weeks. The ED50 increased from 2.92 mg/kg (2.55-3.33)

at 0 weeks to 5.36 mg/kg (4.88-6.40) at six weeks, yielding a potency ratio of 1.98 (1.62-2.39). A similar pattern is seen in Fig. 5.2b. 5.5 days of twice daily injections of 56 mg/kg morphine produced a rightward shift in the morphine dose-effect curve. The ED50 increased from 3.50 mg/kg (3.07-4.00) at 0 weeks to 11.61 mg/kg (9.83-13.71) at six weeks, yielding a potency ratio of 3.22 (2.64-3.93). No such change in ED50 was apparent in mice control without wheel access (Table 1). Fig. 5.2c shows the second (post) dose-effect curve for each of the three groups. A significant difference (non-overlapping confidence intervals) is seen between the ED50's of all groups (see Table 5.1). Specifically the dose-effect curve of mice following 6 weeks of wheel access was shifted rightward compared to controls but was to the left of mice given chronic morphine. Taken together, these data suggest that both chronic wheel access and chronic morphine shift the morphine dose-effect curve to the right. However, these shifts were greater following chronic morphine than following wheel access.

qRT-PCR Results: Fig. 5.3 shows the relative expression (means $\pm$SEM) of proopiomelanocortin (POMC), proenkephalin (PENK), prodynorphin (PDYN), mu-opioid Receptor 1 (MOR1), and beta-arrestin 2 (ARRB2) in the hypothalamus, nucleus accumbens, striatum, and periaqueductal grey. Gene expression (mRNA levels) is presented as fold change compared to control (without wheel access or chronic morphine). A fold change of zero indicates gene expression in the experimental group is the same as the control group. Positive values indicate up regulation and negative values indicate down regulation of a gene. Only four

changes were significant. In the hypothalamus, POMC expression was significantly decreased in the both the wheel and morphine groups: t_{13}=3.068 and t_{14}=2.29 respectively, $p<0.05$. In the PAG, MOR and Barr expression in the morphine group were significantly increased and decreased, respectively: t_{13}=2.607and 2.90, $p<0.05$. General patterns of increases and decreases are summarized in Table 5.2. Taken together these data indicate that 1) six weeks of wheel access is sufficient to produce changes in gene expression and 2) the direction, if not magnitude, of these changes is the same as some of the changes following chronic morphine.

Discussion:

The experiments yielded two general findings. Both chronic wheel access and chronic morphine 1) shifted the morphine does-effect curve to the right and 2) altered gene expression. In the behavioral experiment, 6 weeks of wheel access and 5.5 days of twice daily injections of 56mg/kg morphine both produced rightward shifts in a morphine dose-effect curve. Such a shift is suggestive of tolerance to the antinociceptive effects of morphine. These data are consistent with previous reports that chronic wheel running in rats decreases sensitivity to multiple opioid agonists (Kanarek et al. 1998; Mathes and Kanarek 2001; Smith and Lyle, 2006; Smith and Yancey 2003). However, it must be noted that the shift in the morphine curve was significantly greater in mice given chronic morphine as compared to those given access to running wheels. The behavioral

tolerance to morphine is likely due to desensitization of opioid receptors following agonist binding. Further, it is generally accepted that different mu opioid agonists differentially desensitize the opioid receptors. In particular, morphine produces significant receptor desensitization but fails to promote efficient internalization and consequent resensitization (Bohn et al., 2004). By contrast, DAMGO (a synthetic endorphin), triggers strong internalization (Connor et al., 2004). In general, it seems that the relative likelihood of opioids to induce endocytosis is inversely correlated with their potential to induce opioid tolerance (Williams et al., 2013). This cellular model may explain why chronic morphine produced a greater shift in the dose-effect curve. Although the shift in the wheel access group was smaller, it is still a striking behavioral demonstration of running altering the function of the opioid system.

Following behavioral testing, the brains of the mice were collected for genetic testing. The striatum, nucleus accumbens, hypothalamus and periaqueductal grey (PAG) were dissected from both hemispheres. Quantitative real time PCR was used to quantify changes in gene expression in the wheel and morphine groups as compared to control mice. In general a complex picture emerged in which changes were sometimes similar and sometimes different between experimental groups and in comparison to published findings.

The striatum and nucleus accumbens are both implicated in the reinforcing properties of opioids (Pettit et al.1984; Shippenberg et al. 1992; Spyraki et al. 1983; Stinus et al. 1989). Although tolerance to the antinociceptive effects of morphine was examined here, chronic wheel access also has been

shown to decrease the rewarding properties of morphine as measured by conditioned place preference (Lett et al., 2002). In the striatum, chronic morphine but not wheel access increased proenkephalin expression. This is in contrast to findings of Georges et al. (1999) and Turchan et al. (1997) who reported decreases following chronic morphine. Prodynorphin expression increased in the morphine group, consistent with the findings of Turchan et al. (1997). Expression in the striatum also increased in the wheel access group, which is consistent with Werme et al. (2000) who also reported increased striatal prodynorphin following cocaine. In addition, POMC expression decreased in both the wheel and morphine groups in the stratum and in the wheel access group in the nucleus accumbens.

In the hypothalamus, the largest changes were decreases in expression of POMC in both the wheel access and morphine groups. This is consistent with finding from both Wei et al. (2009) and Garcia de Yebenes and Pelletier (1993) which report decreases in hypothalamic POMC following chronic morphine. Expression of the mu opioid receptor is generally not altered following morphine tolerance so the increase in expression in the both groups was surprising (Castelli et al., 1997). An in vitro study from Zarnegar et al. (2006) reported increases in mu opioid receptor mRNA following exposure to the high affinity ligand DAMGO. In addition, hypothalamic mu opioid receptors play a complex role in the regulation of blood pressure and heart rate (Barnes et al., 2003; Feuerstein and Siren, 1988). It is possible that up regulation of the receptor in the

hypothalamus is a response to the cardiovascular effects of chronic running rather than changes in neural circuitry directly mediating antinociception.

The most notable effect in the PAG was the decrease in expression of POMC and increase in the expression of the mu opioid receptor in both wheel and morphine groups. Stimulation of the mu opioid receptors in the PAG is predominantly responsible for the analgesic activity of the opioids (Ossipov et al., 2010). Therefore it is interesting to see a reduction in expression of the endorphin precursor, POMC, and perhaps compensatory increase in receptor expression in animals that display antinociceptive tolerance. Beta-arrestin expression was also decreased in chronic morphine exposed mice. Although this is somewhat surprising considering beta-arrestin's role in receptor desensitization it is consistent with the findings of Fan et al. (2003) which reported a 40% decrease in mRNA in the PAG following chronic morphine.

Although the gene expression data are intriguing there are limitations to consider. To begin, changes were subtle, a less than 1-fold difference from baseline in all but one case. It is possible that this reflects limits of the gross dissection method used. The genes of interest are all widely expressed in many brain regions and dissecting errors could have caused the inclusion of regions with opposing expression patterns (McDonald and Lambert, 2005). It is also worth noting that the periaqueductal grey dissection likely included parts of the locus coeruleus. Although qRT-PCR does allow for subtle changes to be measured, it is simply a measure of mRNA in the sample. Changes in mRNA may not be perfectly correlated with changes in protein levels and certainly do

not address the location of protein within the cell. Future experiments using Western blots and *in situ* hybridization could potentially address these questions. Finally, since mRNA levels can change quite rapidly, the changes observed could reflect how different groups responded to the morphine and the process of obtaining of the dose-effect curve rather than differences that arose over the six-week period.

Despite limits, these data do support the hypothesis that wheel access can produce changes to the opioid system that have both behavioral and cellular consequences. However, it is clear that much more research is necessary to fully characterize and understand the significance of the cellular changes following aerobic exercise.

TABLES

Table 5.1. Tail-flick ED50 values and potency ratio for each group.

	Week 0 ED50 (95%CL)	Week 6 ED50 (95%CL)	Potency Ratio (95%CL)
Wheel access	2.92 (2.55-3.33)	5.36 (4.48-6.40)	1.98 (1.62-2.39)
56mg/kg morphine	3.50 (3.07-4.00)	11.61 (9.83-13.71)	3.22 (2.64-3.93)
No wheel	3.07 (2.62-3.5)	2.76 (2.40-3.19)	0.91 (0.74-1.11)

Potency ratios greater than 1 are considered significant. ED50 values with 95% confidence limits that do not overlap are considered significantly different

Table 5.2. Significant increases and decreases in gene expression as compared to control.

		Wheel	Morphine
Striatum	proenkephalin		↑
	prodynorphin	↑	↑
	POMC	↓	↓
	MOR1		
	beta-arrestin		
Nucleus accumbens	proenkephalin	↓	
	prodynorphin	↓	
	POMC	↓	
	MOR1		↑
	beta-arrestin		
Hypothalamus	proenkephalin		
	prodynorphin	↓	↓
	POMC	↓*	↓*
	MOR1	↑	↑
	beta-arrestin		
PAG	proenkephalin		
	prodynorphin		↑
	POMC	↓	↓
	MOR1	↑	↑*
	beta-arrestin		↓*

FIGURES

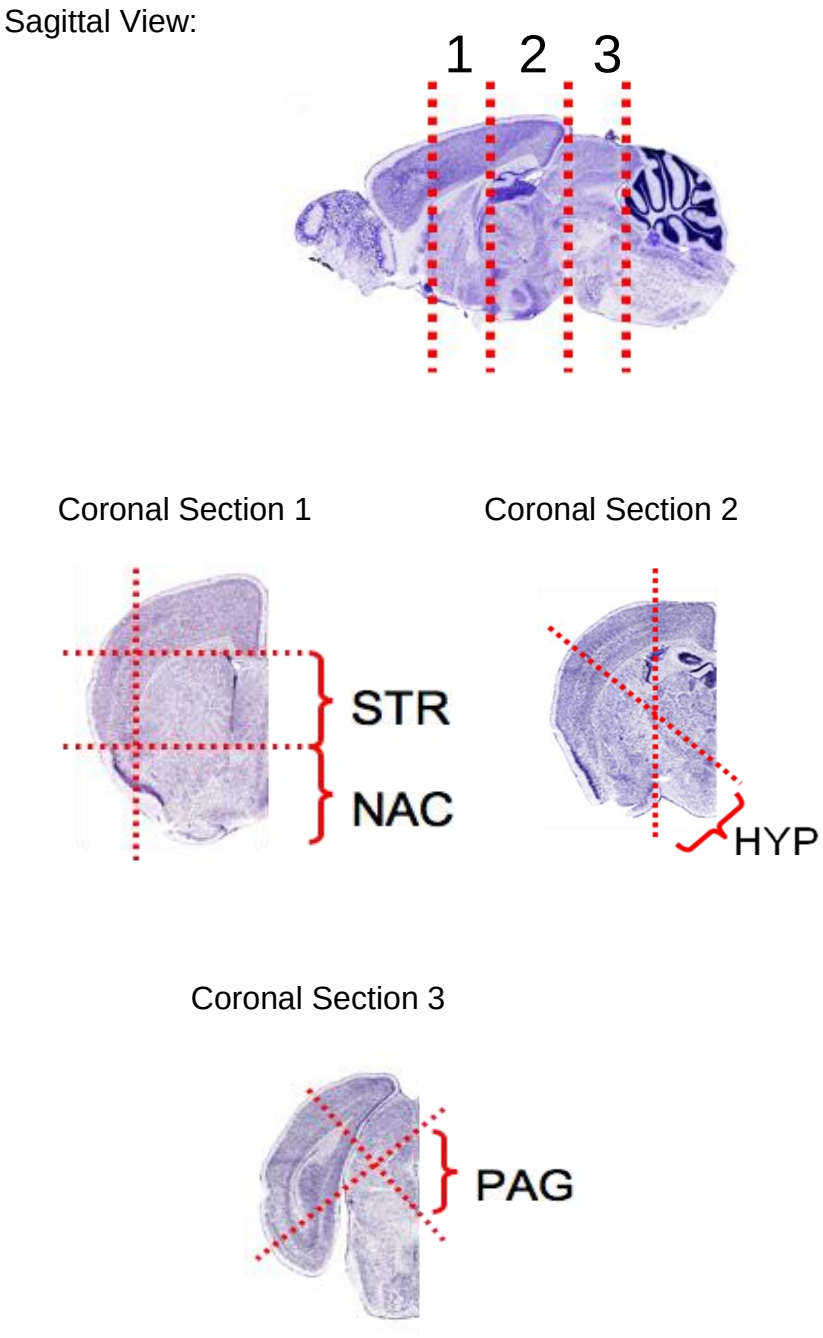


Fig. 5.1 **Dissections.** Starting from a sagittal orientation, four cuts are made to produce three coronal sections. Additional cuts are made, as indicated, to dissect out the striatum (STR), nucleus accumbens (NAC), hypothalamus (HYP), and periaqueductal grey (PAG) from coronal sections 1, 2 and 3.

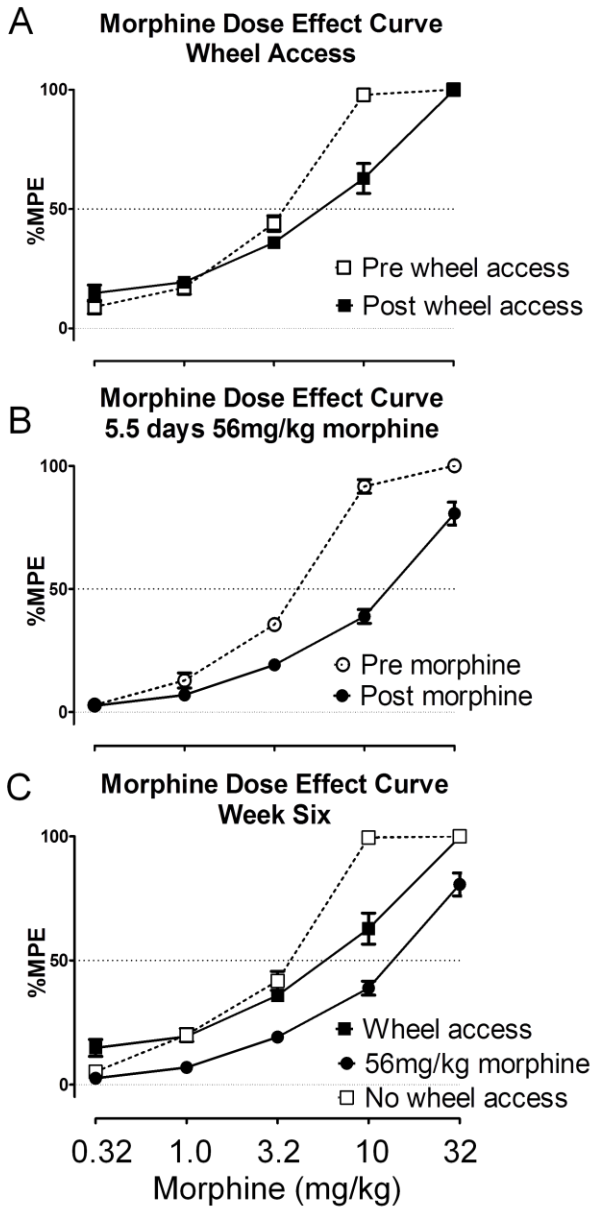


Fig. 5.2 **Six weeks of wheel access and chronic morphine both produced rightward sifts in the morphine dose-effect curve compared to pre exposure and controls**. Morphine treatment consisted of 5.5 days of twice daily injections (56 mg/kg, s.c.). The dose-effect curves for morphine (0.32-32 mg/kg) were assessed before and after wheel access (A) and chronic morphine (B) using the tail-flick. Tail-flick latencies of mice without wheel access were also compared to those of mice following wheel access and morphine (C). Mean latencies to respond (±SEM) are presented as % maximum possible effect (%MPE). N=7-8.

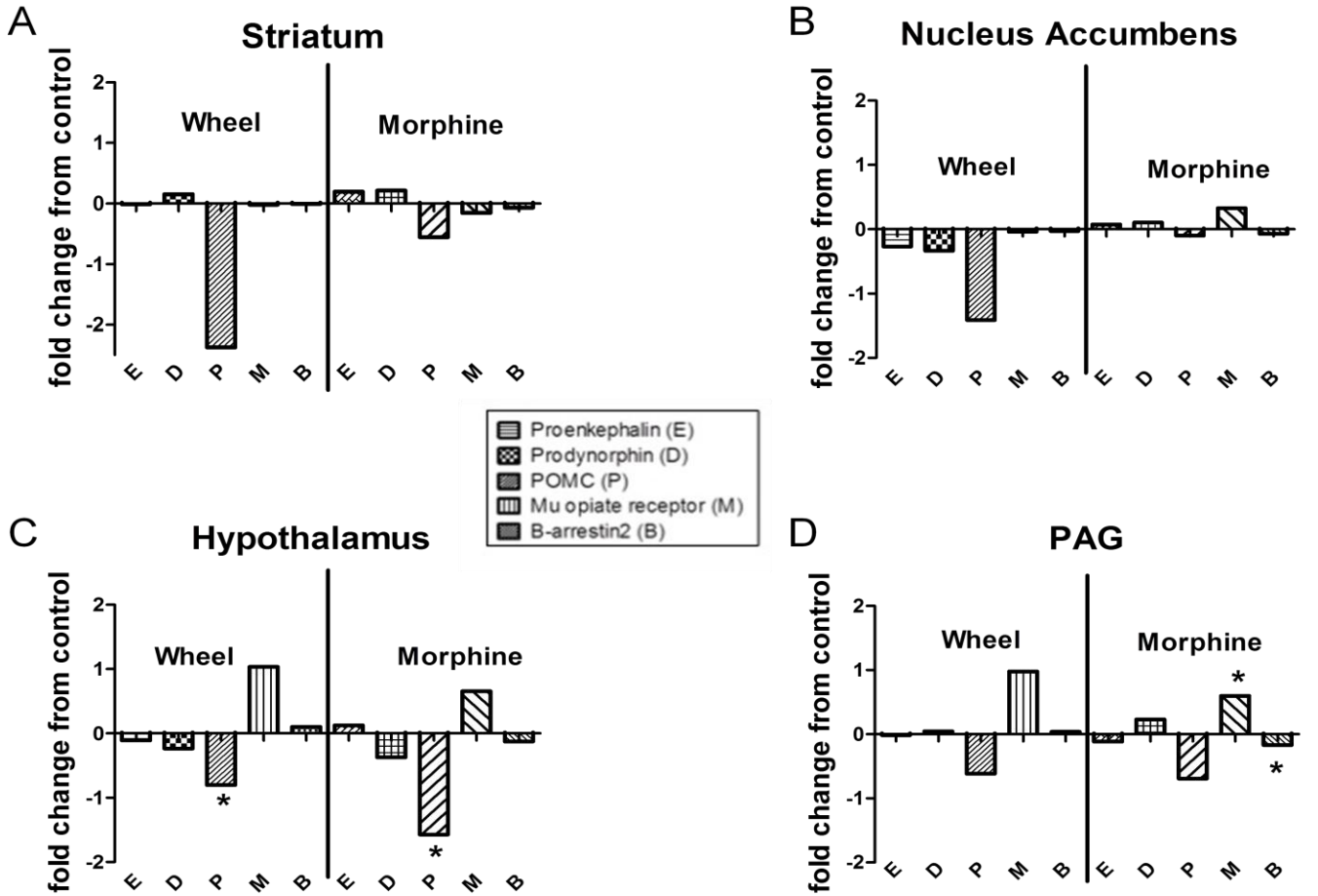


Fig. 5.3 **Fold change in gene expression as compared to control mice with no wheel access.** Changes in gene expression are calculated using the ΔΔCt method with GAPDH as the reference gene and mice without wheel access as the control group. Fold change from control (0) is presented as the group mean. *=significant change from control ($p<0.05$)

Chapter 6

General Discussion

EXPERIMENTAL RESULTS

The experiments included in this dissertation were designed with two goals in mind. The primary goal was to examine the effect of running wheel access on spontaneous morphine withdrawal, as well as to investigate possible mechanisms of the effect. A secondary goal was to assess the use of thermal sensitivity as a measure of spontaneous morphine withdrawal.

This second goal, the validation of procedure, was the aim of the first set of experiments described in Chapter 2. The data indicate that thermal sensitivity provides a sensitive and replicable measure of spontaneous morphine withdrawal. This is consistent with previous research in both humans and animals reporting heightened sensitivity to thermal stimuli following termination of a regimen of morphine administration (Angst et al. 2003; Compton et al. 2003; Dunbar and Pulaj 1998; Rubovitch et al. 2009; Sweitzer et al. 2004).

The current experiments are the first to systematically characterize and validate the use of thermal sensitivity as a measure of spontaneous morphine withdrawal in mice. Specifically, latency to respond on the hot plate at multiple temperatures was assessed at multiple time points throughout the first week

following the cessation of 5.5 days of twice-daily injections of 30, 56 and 100 mg/kg morphine. The greatest change in response latency during the first days of the withdrawal period was seen in mice that were administered 56 mg/kg morphine. Latencies returned to baseline by one week. Demonstration of a dose dependent response and recovery to baseline are critical for the validation of thermal sensitivity as a measure of withdrawal. In addition, data from saline-treated control groups confirmed that neither repeated testing nor time of day had significant effects on the withdrawal measures. In conclusion, response latencies on the hot plate showed little variability within groups and little effect of repeated testing, maximizing sensitivity to subtle changes in withdrawal severity. This suggests that the thermal sensitivity procedure is optimal for assessing subtle effects of medications and environmental interventions throughout spontaneous opioid withdrawal.

The second section of Chapter 2 found that buprenorphine, a partial opioid agonist that is often used in the treatment of withdrawal, attenuated the observed increase in thermal sensitivity. It will be of interest to further enrich the pharmacological validity of this assay using other opioid drugs. Additionally, it may be of interest to develop withdrawal assays that utilize other measures of hypersensitivity such as the tail-flick or von Frey tests and examine those during spontaneous withdrawal. Ultimately, it will be necessary for this assay to be replicated in other labs and optimized for other strains of mice.

The thermal sensitivity procedure was used to examine withdrawal throughout the majority of the following experiments. Chapter 3 tested the

primary hypothesis of this dissertation: The severity of spontaneous morphine withdrawal, as measured by hypersensitivity to a thermal stimulus, is reduced in mice that are given access to running wheels in their home cages. Thermal sensitivity was measured on the hot plate at baseline and at 8, 24, 32, and 48 hours after the final morphine injection to assess withdrawal severity.

Thermal sensitivity was significantly attenuated in mice with access to a running wheel during the withdrawal period. Furthermore, in mice with wheel access, thermal sensitivity was fully returned to control levels. That is, thermal sensitivity in mice with access to running wheels was identical to that observed in saline-treated mice, not in withdrawal. Importantly, wheel access alone (saline + wheel) had no effect on hot plate responding. Thus, it would be of interest to further explore the effect of wheel access following additional maintenance doses of morphine and at longer time points. Additionally, these experiments are limited by the use of a single behavioral measure of withdrawal. Future experiments should test whether wheel running can attenuate the many other measures of morphine withdrawal used in rodents. Despite limitations, these data do support the hypothesis that wheel running can reduce the severity of spontaneous morphine withdrawal.

The majority of prior animal literature considering wheel running and drugs of abuse (amphetamine, heroin, alcohol, and morphine) has focused on reductions in drug taking and drug seeking in rats with wheel access (Hammer et al., 2010; Kanarek et al., 1995; Lett et al., 2002; Smith and Pitts, 2012). To the best of our knowledge, only one other group has published data on the effect of

wheel running during spontaneous opioid withdrawal. Miladi-Gorji et al., (2011, 2012) reported reductions in cognitive deficits and anxiety during spontaneous morphine withdrawal in rats.

To date, no studies have been published on the effect of aerobic exercise during morphine withdrawal in humans. However, it is known that, during periods of abstinence, aerobic exercise can decrease the desire for alcohol and cannabis and reduce symptoms of nicotine withdrawal (Buchowski et al., 2011; Taylor and Ussher, 2005; Taylor and Katomeri, 2007; Ussher et al., 2004). We feel that the current findings are consistent with and provide an important addition to the literature.

Opioid dependence is often, and effectively, treated with substitution by methadone or buprenorphine (Connock et al. 2007; Strain et al. 2011; Tetrault and Fiellin 2012). Furthermore, studies from the Kanarek and Smith laboratories suggest that, in rodents, wheel running can alter the opioid system as measured by changes in morphine's potency (Kanarek et al., 1998; Mathes and Kanarek, 2001; Smith and Yancey 2003; Smith and Lyle, 2006). Thus, I speculate that the behavioral effects of wheel access are, at least partially, related to wheel running-induced release of endogenous opioids that are able to reduce withdrawal severity in much the same way as exogenous opioid agonists can reduce withdrawal symptoms.

The second section of Chapter 3 provides support for the possibility that the observed effects of wheel access during withdrawal are dependent on activity of the opioid system. Specifically, pretreatment with naltrexone, a high affinity

mu and kappa opiate receptor antagonist, blocked the effect of wheel access in mice 32 hours into spontaneous morphine withdrawal. Twenty-four hours later, following the metabolism of naltrexone, a full recovery of the effect of wheel access was observed. Importantly, naltrexone had no effect on thermal sensitivity in mice that were not in withdrawal or did not have wheel access. Although these data are intriguing, it will be of interest to consider the effect of multiple doses of naltrexone given at various time points. Furthermore, though these data support a role of the opioid system in the observed behavioral effect, they in no way prohibit a role for other mechanisms.

The experiments of Chapter 4 provide one approach to addressing the question of *how* wheel access impacts the opioid system. Specifically they test the hypothesis that the use of the wheel, not simply access to a wheel, is necessary for the behavioral effect seen during withdrawal. The current experiments indicated that a locked wheel (access without use) was insufficient to replicate the effects of an unlocked wheel on thermal sensitivity during withdrawal. This finding, that access to a locked wheel did not produce the same behavioral effects as access to an unlocked wheel, is consistent with the majority of published findings (e.g. Burghardt et al., 2004; Devaud et al., 2012; Pietropaolo et al., 2006; Sartori et al., 2011; Zlebnik et al., 2010 and 2012).

Although these data do support the hypothesis that the ability to run on the wheel is a critical part of the wheel effect, a clear correlation between the amount of running and the magnitude of effect was not apparent. This finding was contrary to our hypothesis that thermal sensitivity would be lowest in individual

mice that ran the most. This hypothesis was based on two assumptions 1) that wheel access acts like a pharmacological intervention such that larger “doses” produce greater effects and 2) that wheel revolutions are an accurate measure of aerobic exercise. A few studies do support the assumption that the intensity of aerobic exercise is positively correlated with greater release of beta-endorphin and other opioid peptides (Goldfarb et al., 1990; Mehl et al., 2000; Mougin et al., 1988). That said, it is quite possible that there are individual differences in the “dose” of running necessary to produce a maximal effect such that running beyond a certain amount fails to produce greater effect. This is somewhat supported by the finding that, despite differences in wheel revolutions, there was little variation in thermal sensitivity (which was fully returned to that of saline-treated controls).

In addition, it is possible that the measure of the “dose” of exercise was confounded by variations in the tension of wheels over time and between mice or by the way the wheels were used (multiple short or fewer long bouts of use within each period). It would be of interest to further explore individual differences in wheel use to identify which features (amount, amount up to a ceiling, intensity, etc.) are correlated with maximal behavioral effect. It seems reasonable to speculate that, once variations in the wheel and wheel use are controlled for, there is a strong ceiling effect such that a correlation emerges below a certain amount of running but once a certain level is reached (which may differ between individuals) a full behavioral effect is achieved. The complexity of the relationship between individual running and magnitude of behavioral effect may explain why

so few papers that include wheel use also report correlation data. In fact, the Smith laboratory is one of the few groups to report positive correlations (Smith and Lyle, 2006 and Smith et al., 2008) and yet, in a recent paper, they acknowledge a failure to see a correlation between heroin self-administration and wheel running during the study (Smith and Pitts, 2012). Finally, the lack of correlation does not diminish the significance of the effects of wheel access during spontaneous withdrawal.

The second section of Chapter 4 provided comparison between the effect of wheel access and that of an alternate environmental intervention, group housing. The current experiment indicated that group-housed mice only showed significant attenuation of morphine withdrawal at 8 and 24 hours. Although this finding is in contrast with Coudereau et al. (1997b) and Broseta et al. (2005), who reported that opioid withdrawal was attenuated in single- rather than group-housed mice, significant methodological differences make direct comparison of results difficult. In order to fully characterize the effects of group housing on morphine withdrawal, further studies are necessary to examine a range of parameters (length of isolation/group housing, number of animals per cage, drug treatment of group-housed peers).

In conclusion, the experiments of Chapter 4 replicate the finding from the previous chapter that wheel access can reduce the severity of morphine withdrawal as measured by thermal sensitivity. Although this effect requires access to an unlocked wheel, there is not a simple correlation between amount of running and attenuation of withdrawal. Finally, group housing, an alternative

form of enrichment, produced an intermediary effect between that of locked or no wheels and unlocked running wheels. Interestingly, although the dependent measure was different, studies from Burghardt et al. (2004) and Pietropaolo et al. (2006) also found that group housing produced an intermediate effect on cognition and anxiety between locked and unlocked wheel access.

The final experiments used genetic techniques to investigate a secondary behavioral effect of wheel running. Specifically, extended access to running wheels shifts the antinociceptive potency of morphine in rodents (Kanarek et al. 1998; Mathes and Kanarek 2001; Smith and Lyle, 2006; Smith and Yancey 2003). This behavioral effect was used because it is a simple and established model that demonstrates the consequence of wheel running on the opioid system. The behavioral data of Chapter 5 demonstrate that cross-tolerance between six weeks of wheel use and acute morphine can develop in mice as measured by a rightward shift in the morphine dose-effect curve. This shift, though significant, was less than the potency shift seen following traditional tolerance induced by chronic morphine (5.5 days of twice daily 56 mg/kg injections).

The second section of Chapter 5 used quantitative real-time PCR to assess changes in gene expression that might underlie the behavioral effect. In general, both chronic wheel and chronic morphine exposure produced changes in gene expression, in comparison to controls. However, parallel changes between groups were minimal and not consistent within a particular gene or brain region. This is not surprising because, although both interventions produce similar changes in one behavior, it is quite reasonable to expect that the body

responds and adapts differently to endogenous versus exogenous changes. For example, one striking finding was the large increase in expression of the mu opioid receptor in the hypothalamus of the wheel access mice. This change may be correlated with the measured behavior considering that limited evidence suggests that DAMGO, a synthetic opioid peptide, can also increase expression (Zarnegar et al., 2006). However, considering the role of the hypothalamus in regulation of the cardiac system, it seems likely that this is an example of one of the many effects of wheel running largely unrelated to the opioid tolerance and withdrawal that these experiments are concerned with (Barnes et al., 2003; Feuerstein and Siren, 1988). It is also worth noting that the hypothalamus plays a major role in the regulation of appetite and stress response, both of which are affected by aerobic exercise.

That said, expression in the periaqueductal grey of the mu opioid receptor and POMC, the precursor mRNA for its ligand, endorphin, were both changed, in a consistent manner, in the wheel and morphine groups. These genes are of interest considering that stimulation of the mu opioid receptor in this region is predominantly responsible for the antinociceptive potency of opioids (Ossipov et al., 2010).

In future experiments, it will be important to over or under express genes in order to determine whether the observed expression changes following wheel running are necessary and/or sufficient for a shift in morphine potency. In addition, future studies should measure changes in protein, as opposed to mRNA level, (Western blots) and consider more precise spatial data (*in situ*

hybridization). Despite limitation, this data set does provide novel data on, and direct comparison between, the expression of a range of genes associated with the opioid system in wheel and morphine exposed mice. Further, taken as a whole, mice with wheel access exhibited both a reduction in morphine sensitivity (tolerance) and a general down regulation of opioid system genes.

GENERAL LIMITATIONS AND FURTHER EXPERIMENTS

The current experiments found that access to a running wheel can reduce the increase in thermal sensitivity seen during spontaneous morphine withdrawal in mice. As with all experiments, results often lead to more questions than answers. One major limitation of the current research was the use of single measure of withdrawal. Thus, it would be of great interest to investigate the effect of wheel running in any of the many other assays of spontaneous withdrawal.

Although the most common method used to assess opioid withdrawal in rodents is a global scale, such scales are best suited to assess the severe withdrawal precipitated by opioid antagonists (Gellert and Holtzman, 1978; Kest et al., 2002). When used to assess spontaneous withdrawal, symptoms tend to appear with too much variability (jumping and paw flutters) or at such low rates (shakes, diarrhea) that assessing change, as opposed to presence/absence, of withdrawal is difficult (Papaleo and Contarino, 2006; unpublished pilot data). Miladi-Gorji et al. (2011, 2012) did report that wheel running had positive effects on measures of cognition (Morris water maze) and anxiety (elevated plus maze and light/dark box) during spontaneous withdrawal. However, these experiments

only considered a single dose of morphine and single time point during withdrawal. Thus, it would be interesting to assess the impact of wheel running on the cognitive effects of withdrawal. In addition, a few studies have reported reductions in intracranial self-stimulation (ICSS) during morphine withdrawal (Altarifi and Negus, 2011; Easterling and Holtzman, 1997; Liu and Schulteis, 2004; Schaefer and Michael, 1983). ICSS responding during spontaneous withdrawal appears to be sensitive and stable through repeated testing and could be an ideal measure to assess the effect of wheel access.

Although the experiments described here provide strong behavioral data on the effect of wheel access, they are limited in their ability to address why or how running has its effects. One line of thinking posits that running acts through activation of the opiate system. This hypothesis of mechanism is most directly addressed in the experiments of Chapter 3, which found that naltrexone pretreatment could temporarily block the effects of wheel access, although a full range of doses and time points were not tested. Since antagonism of the opiate system is sufficient to disrupt the behavioral effect, it would be of interest to test whether enhancement of the system could potentiate the effects.

The drug RB101, an enkephalinase inhibitor that increases synaptic levels of beta-endorphins, enkephalins and dynorphins, has previously been shown to decrease spontaneous morphine withdrawal in rats (Ruiz et al., 1996; Thanawala et al., 2008). If wheel running reduces withdrawal severity through the substitution of morphine with endogenous opioids, then RB101 should be able to potentiate wheel effects, especially at time points or following morphine doses

where wheel effects are more subtle.

Although this line of thinking is built on the idea that wheel running has its effects through the opiate system, it does not exclude the possibility of additional pathways of action. Signaling via the hypothalamic peptide galanin is one particularly intriguing alternative. The galanin receptor, GalR1, is expressed throughout the brain including in regions implicated in opioid withdrawal, the locus coeruleus and periaqueductal grey (Burgevin et al., 1995). Systemic administration of the galanin receptor agonist, galnon, has been shown to attenuate morphine withdrawal in mice. Furthermore, targeted expression of galanin to noradrenergic neurons of the locus coeruleus is sufficient to attenuate morphine withdrawal signs (Zachariou et al., 2003). In the locus coeruleus, galanin is known to decrease firing rates of norepinephrine releasing neurons. (Pieribone et al., 1995; Seutin et al., 1989; Sevcik et al., 1993). It is hypothesized that galanin attenuates morphine withdrawal by decreasing the release of norepinephrine in the locus coeruleus through activation of its G_i coupled receptors which decrease cAMP in a manner similar to that of prior opiate receptor activation (Zachariou et al., 2003).

Though no research is published on running induced changes in galanin during withdrawal, it has been shown that wheel access in rodents can increase galanin expression in the locus coeruleus, leading to suppression of neuronal activity and adaptive responses to stress (Sciolino and Holmes, 2012; Sciolino et al., 2012). Considering these two lines of study, we hypothesize that wheel running may decreases the severity of morphine withdrawal by (also) triggering

the release of galanin in the locus coeruleus.

In addition to probing the cellular mechanisms by which wheel running produces behavioral effects during withdrawal, it would also be of interest to identify the brain regions and circuits that play a role in these effects. To begin, there is extensive research implicating both the locus coeruleus and periaqueductal grey in opioid withdrawal (e.g. Christie et al., 1997). However, data from Chapter 5 highlights wheel access induced changes in multiple other brain regions as well. Considering the widespread effects of wheel running on the brain, it is likely that many brain regions play a role and it may be difficult to disrupt the effect of wheel running without disrupting the expression of withdrawal. Finally, it is possible that wheel running attenuates different signs of withdrawal via different mechanisms. For example, attenuation of thermal sensitivity (a pain response) may be mediated by endogenous opiate activity in the PAG while running induced decreases in anxiety as shown by Miladi-Gorji et al. (2011) are mediated by galanin activity in the locus coeruleus.

CONTRIBUTIONS AND SIGNIFICANCE

The first contribution of this dissertation is the development and validation of thermal sensitivity as an assay of spontaneous morphine withdrawal in mice (Balter and Dykstra, 2013). Currently, there are many assays of morphine withdrawal in rodents, each with strengths and weaknesses, ranging from somatic symptom scales to conditioned place aversion and disrupted operant responding. Thermal sensitivity is a valuable addition because 1) it provides a

reliable preclinical model for the hyperalgesia seen in humans and 2) is optimal for assessing subtle effects of chronic interventions during spontaneous withdrawal.

The second contribution of this work is the finding that aerobic exercise can reduce withdrawal severity in mice. Although it certainly will be of interest to fully explore the mechanisms by which wheel access attenuates withdrawal, the translational potential of the effect should not be ignored. To date, few studies have been published on the effect of exercise in the treatment of substance abuse in humans. That said, this is an area of active research. Dr. Richard De La Garza at Baylor University and Dr. Richard Rawson at UCLA both have NIDA funded grants to investigate the ability of exercise, in an inpatient setting, to improve outcomes of treatment for cocaine and methamphetamine dependence, respectively. Dr. Nancy Petry at the University of Connecticut has developed an outpatient procedure that uses contingency management to encourage exercise. She is currently funded to study the effects of this procedure on cocaine users. Looking forward, it will be of great interest to study the clinical benefits of aerobic exercise, in combination with current interventions, in the treatment of opioid dependence.

REFERENCES

Aghajanian GK, Sanders-Bush E. (2002). Opioid Peptides And Their Receptors: Overview And Function In Pain Modulation . In K. Davis, D. Charney, J. Coyle, C. Nemeroff (Eds) Neuropsychopharmacology: The Fifth Generation of Progress (35-46). Philadelphia, PA: Lippincott, Williams & Wilkins.

Almela P, Navarro-Zaragoza J, García-Carmona JA, Mora L, Hidalgo J, Milanés MV, Laorden ML. (2012). Role of corticotropin-releasing factor (CRF) receptor-1 on the catecholaminergic response to morphine withdrawal in the nucleus accumbens (NAc). PLoS One, 7(10), e47089.

Altarifi AA, Negus SS. (2011). Some determinants of morphine effects on intracranial self-stimulation in rats: dose, pretreatment time, repeated treatment, and rate dependence. Behav Pharmacol, 22(7), 663-73.

Ammon-Treiber S, Hollt. (2005). Morphine-induced changes of gene expression in the brain. Addict Biol,10(1), 81-9.

Angst MS, Koppert W, Pahl I, Clark DJ and Schmeiz M. (2003). Short-term infusion of the mu-opioid agonist remifentanil in humans causes hyperalgesia during withdrawal. Pain, 106, 49-57.

Balter RE, Dykstra LA. (2012). The effect of environmental factors on morphine withdrawal in C57BL/6J mice: running wheel access and group housing. Psychopharmacology, 224(1), 91-100.

Balter RE, Dykstra LA. (2013). Thermal sensitivity as a measure of spontaneous morphine withdrawal in mice. J Pharmacol Toxicol Methods, [Epub ahead of print].

Barnes MJ, Lapanowski K, Conley A, Rafols JA, Jen KL, Dunbar JC. (2003). High fat feeding is associated with increased blood pressure, sympathetic nerve activity and hypothalamic mu opioid receptors. Brain Res Bull, 61(5),511-9.

Becker GL, Gerak LR, Koek W, France CP. (2008). Antagonist-precipitated and discontinuation-induced withdrawal in morphine-dependent rhesus monkeys. Psychopharmacology (Berl), 201(3), 373-82.

Bhargava HN. (1978).The effects of naltrexone on the development of physical dependence on morphine. Eur J Pharmacol, 50(3),193-202.

Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, Caron MG. (2000). Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. Nature, 408, 720-723.

Bohn LM, Dykstra LA, Lefkowitz RJ, Caron MG, Barak LS. (2004). Relative opioid efficacy is determined by the complements of the G protein-coupled receptor desensitization machinery. Mol Pharmacology, 66(1),106-12.

Bozarth MA, Wise RA. (1984). Anatomically distinct opiate receptor fields mediate reward and physical dependence. Science, 224(4648), 516-7.

Brandt MR, France CP. (1998). Chronic l-alpha acetylmethadol in rhesus monkeys: discriminative stimulus and other behavioral measures of dependence and withdrawal. J Pharmacol Exp Ther, 287,1029–1037.

Brodal, Per. (2004). The Central Nervous System: Structure and Function (3 ed.). Oxford University Press US. pp. 369–396.

Broseta I, Rodriguez-Arias M, Aguilar MA, Minarro J. (2005). Isolation decreases physical and motivational aspects of morphine withdrawal. Behav Pharmacol, 16(3),131-8.

Buchowski MS, Meade NN, Charboneau E, Park S, Dietrich MS, Cowan RL, Martin PR. (2011). Aerobic exercise training reduces cannabis craving and use in non-treatment seeking cannabis-dependent adults. PLoS One, 6(3), e17465.

Burgevin MC, Loquet I, Quarteronet D, Habert-Ortoli E. (1995). Cloning, pharmacological characterization, and anatomical distribution of a rat cDNA encoding for a galanin receptor. J Mol Neurosci, 6(1),33-41.

Burghardt PR, Fulk LJ, Hand GA, Wilson MA. (2004). The effects of chronic treadmill and wheel running on behavior in rats. Brain Res, 1019(1-2):84-96.

Caillé S, Espejo EF, Reneric JP, Cador M, Koob GF, Stinus L. (1999). Total neurochemical lesion of noradrenergic neurons of the locus ceruleus does not alter either naloxone-precipitated or spontaneous opiate withdrawal nor does it influence ability of clonidine to reverse opiate withdrawal. J Pharmacol Exp Ther, 290(2), 881-92.

Carlezon WA Jr, Wise RA. (1996). Rewarding actions of phencyclidine and related drugs in nucleus accumbens shell and frontal cortex. J Neurosci, 16(9), 3112-22.

Castelli MP, Melis M, Mameli M, Fadda P, Diaz G, Gessa GL. (1997). Chronic morphine and naltrexone fail to modify mu-opioid receptor mRNA levels in the rat brain. Brain Res Mol Brain Res, 45, 149 – 153.

Childers SR. (1991). Opioid receptor-coupled second messenger systems. Life Sci, 48(21),1991-2003.

Christie MJ, Williams JT, Osborne PB, Bellchambers CE. (1997). Where is the locus in opioid withdrawal? Trends Pharmacol Sci,18(4),134-40.

Christie MJ. (2008). Cellular neuroadaptations to chronic opioids: tolerance, withdrawal and addiction. Br J Pharmacol, 154(2), 384-96.

Chu LF, Liang DY, Li X, Sahbaie P, D'arcy N, Liao G, Peltz G, David Clark J. (2009). From mouse to man: the 5-HT3 receptor modulates physical dependence on opioid narcotics. Pharmacogenet Genomics,19(3),193-205.

Clark PJ, Kohman RA, Miller DS, Bhattacharya TK, Brzezinska WJ, Rhodes JS. (2011). Genetic influences on exercise-induced adult hippocampal neurogenesis across 12 divergent mouse strains. Genes Brain Behav, 10(3), 345-53.

Cohen EE, Ejsmond-Frey R, Knight N, Dunbar RI. (2010). Rowers' high: behavioural synchrony is correlated with elevated pain thresholds. Biol Lett, 6(1),106-8.

Compton P, Athanasos P, Elashoff D. (2003). Withdrawal hyperalgesia after acute opioid physical dependence in non addicted humans: a preliminary study. J. Pain, 4, 511-519.

Connock M, Juarez-Garcia A, Jowett S, Frew E, Liu Z, Taylor RJ, Fry-Smith A, Day E, Lintzeris N, Roberts T, Burls A, Taylor RS. (2007). Methadone and buprenorphine for the management of opioid dependence: a systematic review and economic evaluation. Health Technol Assess,11(9), 1-171, iii-iv.

Connor M, Osborne PB, Christie MJ. (2004). Mu-opioid receptor desensitization: is morphine different? Br J Pharmacol,143(6), 685-96.

Contarino A, Papaleo F. (2005). The corticotropin-releasing factor receptor-1 pathway mediates the negative affective states of opiate withdrawal. Proc Natl Acad Sci, 102(51), 18649-54.

Coudereau JP, Debray M, Monier C, Bourre JM, Frances H. (1997a). Isolation impairs place preference conditioning to morphine but not aversive learning in mice. Psychopharmacology, 130(2),117-23.

Couderau JP, Monier C, Mourre JM, Frances H. (1997b). Effect of isolation on pain threshold and on different effects of morphine. Prog Neuropsychopharmacol Biol Psychiatry, 21, 997-1018

Crain SM, Shen KF. (2007). Naloxone rapidly evokes endogenous kappa opioid receptor-mediated hyperalgesia in naïve mice pretreated briefly with GM1 ganglioside or in chronic morphine-dependent mice. Brain Res, 1167, 31-4.

Curtis AL, Bello NT, Valentino RJ. (2001). Evidence for functional release of endogenous opioids in the locus ceruleus during stress termination. J Neurosci, 21(13), RC152.

Curtis AL, Florin-Lechner SM, Pavcovich LA, Valentino RJ. (1997). Activation of the locus coeruleus noradrenergic system by intracoerulear microinfusion of corticotropin-releasing factor: effects on discharge rate, cortical norepinephrine levels and cortical electroencephalographic activity. J Pharmacol Exp Ther, 281, 163–172.

Deneau GA, Seevers MH. (1963). Evaluation of new compounds for morphine-like physical dependence capacity. Proceedings of the Committee on Problems of Drug Dependence, National Academy of Sciences/National Research Council, Addendum 25.

Seevers MH. (1936). Opiate addiction in the monkey. I. Methods of study. J Pharmacol Exp Ther, 56,147-156.

Daunais JB, Letchworth SR, Sim-Selley LJ, Smith HR, Childers SR, Porrino LJ. (2001). Functional and anatomical localization of mu opioid receptors in the striatum, amygdala, and extended amygdala of the nonhuman primate. J Comp Neurol, 433(4), 471-85.

Devulder J, Bohyn P, Castille F, De Laat M, Rolly G. (1996). A case of uncommon withdrawal symptoms after a short period of spinal morphine administration. Pain, 64(3), 589-91.

Devaud LL, Walls SA, McCulley WD 3rd, Rosenwasser AM. (2012). Voluntary wheel running attenuates ethanol withdrawal-induced increases in seizure susceptibility in male and female rats. Pharmacol Biochem Behav, 103(1), 18-25.

Devillers JP, Boisserie F, Laulin JP, Larcher A, Simonnet G. (1995). Simultaneous activation of spinal antiopioid system (neuropeptide FF) and pain facilitatory circuitry by stimulation of opioid receptors in rats. Brain Res, 700,173-181.

Ding YQ, Kaneko T, Nomura S, Mizuno N. (1996). Immunohistochemical localization of mu-opioid receptors in the central nervous system of the rat. J Comp Neurol, 367(3), 375-402.

Dunbar SA, Pulai IJ. (1998). Repetitive opioid abstinence causes progressive hyperalgesia sensitive to N-methyl-D-aspartate receptor blockade in the rat. J Pharmacol Exp Ther, 284, 678–686.

Dykstra LA, Fischer BD, Balter RE, Henry FE, Schmidt KT, Miller LL. (2011). Opioid antinociception, tolerance and dependence: interactions with the N-methyl-D-aspartate system in mice. Behav Pharmacol, 22(5-6), 540-7.

Easterling KW, Holtzman SG. (1997). Intracranial self-stimulation in rats: sensitization to an opioid antagonist following acute or chronic treatment with mu opioid agonists. J Pharmacol Exp Ther, 281, 88–99.

Einstein EB, Asaka Y, Yeckel MF, Higley MJ, Picciotto MR. (2013). Galanin-induced decreases in nucleus accumbens/striatum excitatory postsynaptic potentials and morphine conditioned place preference require both galanin receptor 1 and galanin receptor 2. Eur J Neurosci, [Epub ahead of print].

Elmer GI, Pieper JO, Hamilton LR, Wise RA. (2010). Qualitative differences between C57BL/6J and DBA/2J mice in morphine potentiation of brain stimulation reward and intravenous self-administration. Psychopharmacology, 208(2),309-21.

Esmaeili-Mahani S, Ebrahimi Z, Noraie T, Sheibani V, Hajializadeh Z. (2010). Exercise-induced morphine insensitivity is accompanied with a decrease in specific G-protein subunits gene expression in rats. Pharmacol Biochem Behav, [Epub ahead of print].

Fan XL, Zhang JS, Zhang XQ, Yue W, Ma L.(2003). Differential regulation of beta-arrestin 1 and beta-arrestin 2 gene expression in rat brain by morphine. Neuroscience,117(2), 383-9.

Fareed A, Vayalapalli S, Stout S, Casarella J, Drexler K, Bailey SP. (2011). Effect of methadone maintenance treatment on heroin craving, a literature review. J Addict Dis, 30(1):27-38.

Fernandes M, Kluwe S, Coper H. (1977). Quantitative assessment of tolerance to and dependence on morphine in mice. Arch Pharmacol, 297(1), 53-60.

Feuerstein G, Sirén AL. (1988). Hypothalamic mu-opioid receptors in cardiovascular control: a review. Peptides, 9 Suppl 1:75-8.

Fiellin DA, O'Connor PG. (2002). Office-based treatment of opioid-dependent patients. New Eng J Med, 347, 817-23.4.

Fischer BD, Carrigan KA, and Dykstra LA. (2005). Effects of N-methyl-D-aspartate receptor antagonists on acute morphine-induced and l-methadone-induced antinociception in mice. J. Pain, 6, 425-433.

Fischer BD, Zimmerman EI, Picker MJ, Dykstra LA. (2008). Morphine in combination with metabotropic glutamate receptor antagonists on schedule-controlled responding and thermal nociception. , 324(2), 732-9.

Folkesson R, Monstein HJ, Geijer T, Terenius L. (1989). Modulation of proenkephalin A gene expression by cyclic AMP. Mol Brain Res, 5, 211-217.

France CP, Woods JH. (1989). Discriminative stimulus effects of naltrexone in morphine-treated rhesus monkeys. J Pharmacol Exp Ther, 250:937–943.

Froger-Colléaux C, Rompion S, Guillaume P, Porsolt RD, Castagné V, Moser P. (2011). Continuous evaluation of drug withdrawal in the rat using telemetry: effects of morphine and chlordiazepoxide. J Pharmacol Toxicol Methods, 64(1), 81-8.

Fukunaga Y, Kishioka S. Enkephalinergic neurons in the periaqueductal gray and morphine withdrawal. (2000). Enk suppress WD by inhibiting GABA transmission During Morphine WD see phosphorylation of CREB in LC. Jpn J Pharmacol, 82(3), 175-80.

Garcia de Yebenes E, Pelletier G. (1993). Opioid regulation of proopiomelanocortin (POMC) gene expression in the rat brain as studied by in situ hybridization. Neuropeptides, 25, 91-94.

Gellert VF, Holtzman SG. (1978). Development and maintenance of morphine tolerance and dependence in the rat by scheduled access to morphine drinking solutions. J Pharmacol Exp Ther, 205(3), 536-46.

Georges F, Stinus L, Bloch B, Le Moine C. (1999). Chronic morphine exposure and spontaneous withdrawal are associated with modifications of dopamine receptor and neuropeptide gene expression in the rat striatum. Eur J Neurosci, 11, 481 – 490.

Gieryk A, Ziolkowska B, Solecki W, Kubik J, Przewlocki R. (2010). Forebrain PENK and PDYN gene expression levels in three inbred strains of mice and their relationship to genotype-dependent morphine reward sensitivity. Psychopharmacology, 208(2),291-300.

Goldfarb AH, Hatfield BD, Armstrong D, Potts J. (1990). Plasma beta-endorphin concentration: response to intensity and duration of exercise. Med Sci Sports Exerc, 22(2), 241-4.

Gómez-Milanés I, Almela P, García-Carmona JA, García-Gutiérrez MS, Aracil-Fernández A, Manzanares J, Milanés Maquilón MV, Laorden ML. (2012). Accumbal dopamine, noradrenaline and serotonin activity after naloxone-conditioned place aversion in morphine-dependent mice. Neurochem Int, 61(3):433-40.

Gossop, M. (1990). The development of a short opiate withdrawal scale (SOWS). Addict. Behav, 15, 487–490.

Grilly DM, Gowans GC. (1986). Acute morphine dependence: effects observed in shock and light discrimination tasks. Psychopharmacology, 88(4), 500-4.

Hack SP, Vaughan CW, Christie MJ. (2003). Modulation of GABA release during morphine withdrawal in midbrain neurons in vitro. Neuropharmacology, 45(5), 575-84.

Hammer SB, Ruby CL, Brager AJ, Prosser RA, Glass JD. (2010). Environmental modulation of alcohol intake in hamsters: effects of wheel running and constant light exposure. Alcohol Clin Exp Res, 34(9),1651-8.

Handelsman L, Cochrane KJ, Aronson MJ, Ness R, Rubinstein KJ, Kanof PD. (1987).Two new rating scales for opiate withdrawal. Am J Drug Alcohol Abuse,13(3), 293-308.

Heinrichs SC, Menzaghi F, Schulteis G, Koob GF, Stinus L. (1995). Suppression of corticotropin-releasing factor in the amygdala attenuates aversive consequences of morphine withdrawal. Behav Pharmacol, 6(1),74-80.

Himmelsbach CK. (1942). Clinical studies of drug dependence. Physical dependence, withdrawal and recovery. Arch Intern Med, 69,766–772.

Holtzman SG, Villarreal JE (1969) Morphine dependence and body temperature in rhesus monkeys. J Pharmacol Exp Ther 166:125–133.

Holtzman SG, Villarreal JE. (1973). Operant behavior in the morphine-dependent rhesus monkey. J Pharmacol Exp Ther, 184(3), 528-41.

Hosseini M, Alaei HA, Naderi A, Sharifi MR, Zahed R. (2009). Treadmill exercise reduces self-administration of morphine in male rats.Pathophysiology,16(1), 3-7.

Huidobro F, Huidobro-Toro JP, Leong Way E. (1976). Studies on tolerance development to single doses of morphine in mice.J Pharmacol Exp Ther, 198(2), 318-29.

Jin H, Li YH, Xu JS, Guo GQ, Chen DL, Bo Y. (2012). Lipoxin A4 analog attenuates morphine antinociceptive tolerance, withdrawal-induced hyperalgesia, and glial reaction and cytokine expression in the spinal cord of rat. Neuroscience, 208, 1-10.

Johnson SM, Duggan AW. (1981). Tolerance and dependence of dorsal horn neurones of the cat: the role of the opiate receptors of the substantia gelatinosa. Neuropharmacology, 20(11), 1033-8.

Johnson SW, North RA. (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. J Neurosci, 12(2), 483-8.

Jutkiewicz EM, Roques BP. (2012). Endogenous opioids as physiological antidepressants: complementary role of δ receptors and dopamine. Neuropsychopharmacology, 37(1), 303-4.

Kamei C, Shimomura K, Ueki S. (1973). Significance of withdrawal jumping response in predicticing physical dependence in mice. Japan J. Pharmacol, 23, 421-426.

Kanarek RB, Marks-Kaufman R, D'Anci KE, Przypek J. (1995). Exercise attenuates oral intake of amphetamine in rats. Pharmacol Biochem Behav, 51(4), 725-9.

Kanarek RB, Gerstein AV, Wildman RP, Mathes WF, D'Anci KE. (1998). Chronic running-wheel activity decreases sensitivity to morphine-induced analgesia in male and female rats. Pharmacol Biochem Behav, 61(1),19-27.

Kanarek RB, D'Anci KE, Jurdak N, Mathes WF. (2009). Running and addiction: precipitated withdrawal in a rat model of activity-based anorexia. Behav Neurosci,123(4), 905-12.

Kaplan H, Fields HL. (1991). Hyperalgesia during acute opioid abstinence: evidence for a nociceptive facilitating function of the rostral ventromedial medulla. J Neuroscience, 11(5),1433-9.

Kolb, L. & Hi mmelsbach, C.K. (1938). Clinical studies of drug addiction. III. A critical review of withdrawal treatments with methods of evaluating abstinence syndromes. American Journal of Psychiatry, 94, 759-97.

Kelly MJ, Loose MD, Ronnekleiv OK. (1990). Opioids hyperpolarize beta-endorphin neurons via mu-receptor activation of a potassium conductance. Neuroendocrinology, 52(3), 268-75.

Kennedy BC, Panksepp JB, Runckel PA, Lahvis GP (2012) Social influences on morphine-conditioned place preference in adolescent BALB/cJ and C57BL/6J mice. Psychopharmacology 219 (3): 923-32

Kest, B., Palmese, C.A., Hopkins, E., Adler, M., Juni, A.J., Mogil, J.S. (2002). Naloxone-precipitated withdrawal jumping in 11 inbred mouse strains: evidence for common genetic mechanisms in acute and chronic morphine physical dependence. Neuroscience, 115, 463–469.

Koob GF, Le Moal M. (2001). Drug addiction, dysregulation of reward, and allostasis. Neuropsychopharmacology, 24(2)97-129.

Koob GF, Volkow ND. (2010). Neurocircuitry of addiction. Neuropsychopharm, 35(1), 217-38.

Knoll AT, Muschamp JW, Sillivan SE, Ferguson D, Dietz DM, Meloni EG, Carroll FI, Nestler EJ, Konradi C, Carlezon WA Jr. (2011). Kappa opioid receptor signaling in the basolateral amygdala regulates conditioned fear and anxiety in rats. Biol Psychiatry, 70(5), 425-33.

Koch T, Höllt V. (2008). Role of receptor internalization in opioid tolerance and dependence. Pharmacol Ther, 117(2),199-206.

Koltyn KF. (2000). Analgesia following exercise: a review. Sports Med, 29(2), 85-98.

Kraus ML, Alford DP, Kotz MM, Levounis P, Mandell TW, Meyer M, Salsitz EA, Wetterau N, Wyatt SA. (2011). American Society Of Addiction Medicine: Statement of the American Society Of Addiction Medicine Consensus Panel on the use of buprenorphine in office-based treatment of opioid addiction. J Addict Med, 5(4), 254-63.

Laulin JP, Larcher A, Célèrier E, Le Moal M, Simonnet G. (1998). Long-lasting increased pain sensitivity in rat following exposure to heroin for the first time. Eur J Neurosci, 10(2), 782-5.

Laulin JP, Maurette P, Corcuff JB, Rivat C, Chauvin M, Simonnet G. (2002). The role of ketamine in preventing fentanyl-induced hyperalgesia and subsequent acute morphine tolerance. Anesth Analg, 94(5),1263-9.

Law PY, Wong YH, Loh HH. (2000). Molecular mechanisms and regulation of opioid receptor signaling. Annu Rev Pharmacol Toxicol, 40, 389-430.

Le Moal M, Koob GF. (2007). Drug addiction: pathways to the disease and pathophysiological perspectives. Eur Neuropsychopharmacol, 17(6-7), 377-93.

Leriche M, Cote-Vélez A, Méndez M. (2007). Presence of pro-opiomelanocortin mRNA in the rat medial prefrontal cortex, nucleus accumbens and ventral tegmental area: studies by RT-PCR and in situ hybridization techniques. Neuropeptides, 41(6), 421-31.

Lett BT, Grant VL, Koh MT, Flynn G. (2002). Prior experience with wheel running produces cross-tolerance to the rewarding effect of morphine. Pharmacol Biochem Behav, 72(1-2), 101-5.

Li X, Angst MS, Clark JD. (2001). Opioid-induced hyperalgesia and incisional pain. Anesth Analg, 93(1), 204-9.

Li T, Hou Y, Cao W, Yan CX, Chen T, Li SB. (2010). Naloxone-precipitated withdrawal enhances ERK phosphorylation in prefrontal association cortex and accumbens nucleus of morphine-dependent mice. Neurosci Lett, 468(3), 348-52.

Liu J, Schulteis G. (2004). Brain reward deficits accompany naloxone-precipitated withdrawal from acute opioid dependence. Pharmacol Biochem Behav, 79, 101–8.

Lipman JJ, Blumenkopf B. (1989). Comparison of subjective and objective analgesic effects of intravenous and intrathecal morphine in chronic pain patients by heat beam dolorimetry. Pain, 39(3), 249-56.

Livak KJ, Schmittgen TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta] CT method. Methods, 25, 402-408.

Mahler DA, Murray JA, Waterman LA, Ward J, Kraemer WJ, Zhang X, Baird JC. (2009). Endogenous opioids modify dyspnoea during treadmill exercise in patients with COPD. Eur Respir J, 33(4),771-7.

Maldonado R, Negus S, Koob GF. (1992). Precipitation of morphine withdrawal syndrome in rats by administration of mu-, delta- and kappa-selective opioid antagonists. Neuropharmacology, 31(12), 1231-41.

Mansour A, Fox CA, Akil H, Watson SJ. (1995). Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends Neurosci,18(1), 22-9.

Martin WR, Jasinski DR. (1969). Physiological parameters of morphine dependence in man-tolerance, early abstinence, protracted abstinence. J Psychiatr Res, 7,9–17.

Martin WR, Gilbert PE, Jasinski DR, Martin CD. (1987). An analysis of naltrexone precipitated abstinence in morphine-dependent chronic spinal dogs. J Pharmacol Exp Ther, 240(2), 565-70.

Martín F, Mora L, Laorden M, Milanés M. (2011). Protein kinase C phosphorylates the cAMP response element binding protein in the hypothalamic paraventricular nucleus during morphine withdrawal. Br J Pharmacol, 163(4), 857-75.

Martin-Soelch C, Leenders KL, Chevalley AF, Missimer J, Künig G, Magyar S, Mino A, Schultz W. (2001). Reward mechanisms in the brain and their role in dependence: evidence from neurophysiological and neuroimaging studies. Brain Res Brain Res Rev, 36(2-3),139-49.

Martini L, Whistler JL. (2007). The role of mu opioid receptor desensitization and endocytosis in morphine tolerance and dependence. Curr Opin Neurobiol, 17(5), 556-64.

Mathes WF, Kanarek RB. (2001). Wheel running attenuates the antinociceptive properties of morphine and its metabolite, morphine-6-glucuronide, in rats. Physiol Behav, 74(1-2):245-51.

Mathes WF, Kanarek RB. (2006). Chronic running wheel activity attenuates the antinociceptive actions of morphine and morphine-6-glucouronide administration into the periaqueductal gray in rats. Pharmacol Biochem Behav, 83(4), 578-84.

McClung CA. (2006). The molecular mechanisms of morphine addiction. Rev Neurosci,17(4), 393-402.

McDonald J, Lambert DG. (2005). Opioid Receptors. Contin Educ Anaesth Crit Care Pain, 5 (1), 22-25.

McMahon LR, Li JX, Carroll FI, France CP. (2009). Some effects of dopamine transporter and receptor ligands on discriminative stimulus, physiologic, and directly observable indices of opioid withdrawal in rhesus monkeys. Psychopharmacology (Berl), 203(2), 411-20.

McNally GP, Akil H. (2002). Role of corticotropin-releasing hormone in the amygdala and bed nucleus of the stria terminalis in the behavioral, pain modulatory, and endocrine consequences of opiate withdrawal. Neuroscience, 112(3), 605-17.

Mehl ML, Schott HC 2nd, Sarkar DK, Bayly WM. (2000). Effects of exercise intensity and duration on plasma beta-endorphin concentrations in horses. Am J Vet Res, 61(8), 969-73.

Miladi-Gorji H, Rashidy-Pour A, Fathollahi Y, Akhavan MM, Semnanian S, Safari M. (2011). Voluntary exercise ameliorates cognitive deficits in morphine dependent rats: the role of hippocampal brain-derived neurotrophic factor. Neurobiol Learn Mem, 96(3), 479-91.

Miladi-Gorji H, Rashidy-Pour A, Fathollahi Y. (2012). Anxiety profile in morphine-dependent and withdrawn rats: Effect of voluntary exercise. Physiol Behav, 105(2),195-202.

Mogil JS, Wilson SG, Bon K, Lee SE, Chung K, Raber P, Pieper JO, Hain HS, Belknap JK, Hubert L, Elmer GI, Chung JM, Devor M. (1999). Heritability of nociception I: responses of 11 inbred mouse strains on 12 measures of nociception. Pain, 80(1-2),67-82.

Mogil JS, Chesler EJ, Wilson SG, Juraska JM, Sternberg WF. (2000). Sex differences in thermal nociception and morphine antinociception in rodents depend on genotype. Neurosci Biobehav Rev, 24(3), 375-89.

Mougin C, Henriet MT, Baulay A, Haton D, Berthelay S, Gaillard RC. (1988). Plasma levels of beta-endorphin, prolactin and gonadotropins in male athletes after an international nordic ski race. Eur J Appl Physiol Occup Physiol, 57(4), 425-9.

Narita M, Narita M, Mizoguchi H, Tseng LF. (1995). Inhibition of protein kinase C, but not of protein kinase A, blocks the development of acute antinociceptive tolerance to an intrathecally administered mu-opioid receptor agonist in the mouse. Eur J Pharmacol, 280(2), R1-3.

Nestler EJ, Aghajanian GK. (1997). Molecular and cellular basis of addiction. Science, 278(5335), 58-63.

Nicholls L, Bragaw L, Ruetsch C. (2010). Opioid dependence treatment and guidelines. J Manag Care Pharm, 16(1 Suppl B), S14-21.

Ossipov MH, Dussor GO, Porreca F. (2010). Central modulation of pain. J Clin Invest, 120(11), 3779-87.

Palotás M, Palotás A, Bjelik A, Pákáski M, Hugyecz M, Janka Z, Kálmán J. (2005). Effect of general anesthetics on amyloid precursor protein and mRNA levels in the rat brain. Neurochem Res, 30(8),1021-6.

Papaleo F, Contarino A. (2006). Gender- and morphine dose-linked expression of spontaneous somatic opiate withdrawal in mice. Behav Brain Res, 170(1),110-8.

Park SH, Sim YB, Kang YJ, Kim CH, Kwon MS, Suh HW. (2012). The differential profiles of withdrawal symptoms induced by morphine and beta-endorphin administered intracerebroventricularly in mice. Neuroscience, 218, 216-25.

Pennock RL, Hentges ST. (2011). Differential expression and sensitivity of presynaptic and postsynaptic opioid receptors regulating hypothalamic proopiomelanocortin neurons. J Neurosci, 31(1), 281-8.

Pettit HO, Ettenberg A, Bloom FE, Koob GF. (1984). Destruction of dopamine in the nucleus accumbens selectively attenuates cocaine but not heroin self-administration in rats. Psychopharmacology (Berl), 84(2), 167-73.

Pieribone VA, Xu ZQ, Zhang X, Grillner S, Bartfai T, Hökfelt T. (1995). Galanin induces a hyperpolarization of norepinephrine-containing locus coeruleus neurons in the brainstem slice. Neuroscience, 64(4), 861-74.

Pietropaolo S, Feldon J, Alleva E, Cirulli F, Yee BK. (2006). The role of voluntary exercise in enriched rearing: a behavioral analysis. Behav Neurosci, 120(4), 787-803.

Quiñones-Jenab V, Zhang C, Jenab S, Brown HE, Pfaff DW. (1996). Anesthesia during hormone administration abolishes the estrogen induction of preproenkephalin mRNA in ventromedial hypothalamus of female rats. Brain Res Mol Brain Res, 35(1-2), 297-303.

Radke AK, Rothwell PE, Gewirtz JC. (2011). An anatomical basis for opponent process mechanisms of opiate withdrawal. J Neurosci, 31(20), 7533-9.

Raz S, Berger BD. (2010). Social isolation increases morphine intake: behavioral and psychopharmacological aspects. Behav Pharmacol, 21(1), 39-46.

Rubovitch V, Pick CG, Sarne Y. (2009). Is withdrawal hyperalgesia in morphine-dependent mice a direct effect of a low concentration of the residual drug? Addict Biol, 14(4), 438-46.

Ruiz F, Fournié-Zaluski MC, Roques BP, Maldonado R. (1996). Similar decrease in spontaneous morphine abstinence by methadone and RB 101, an inhibitor of enkephalin catabolism. Br J Pharmacol, 119(1), 174-82.

Saelens JK, Granat FR, Sawyer WK. (1971). The mouse jumping test—a simple screening method to estimate the physical dependence capacity of analgesics Arch Int Pharmacodyn Ther, 190, 213–218.

Sartori CR, Vieira AS, Ferrari EM, Langone F, Tongiorgi E, Parada CA. (2011). The antidepressive effect of the physical exercise correlates with increased levels of mature BDNF, and proBDNF proteolytic cleavage-related genes, p11 and tPA. Neuroscience, 180, 9-18.

Schaefer GJ, Michael RP. (1983). Morphine withdrawal produces differential effects on the rate of lever-pressing for brain self-stimulation in the hypothalamus and midbrain in rats. Pharmacol Biochem Behav, 18(4), 571-7.

Sciolino NR, Dishman RK, Holmes PV. (2012).Voluntary exercise offers anxiolytic potential and amplifies galanin gene expression in the locus coeruleus of the rat.Behav Brin Res, 233(1),191-200.

Sciolino NR, Holmes PV. (2012). Exercise offers anxiolytic potential: a role for stress and brain noradrenergic-galaninergic mechanisms. Neurosci Biobehav Rev, 36(9),1965-84.

Seevers MH. (1936). Opiate addiction in the monkey. I. Methods of study. J Pharmacol Exp Ther, 56,147-156.

Sell SL, McMahon LR, Koek W, France CP. (2005). Monoaminergic drugs and directly observable signs of LAAM-withdrawal in rhesus monkeys. Behav Pharmacol, 16, 53–58.

Seutin V, Verbanck P, Massotte L, Dresse A. (1989). Galanin decreases the activity of locus coeruleus neurons in vitro. Eur J Pharmacol, 164(2), 373-6.

Sevcik J, Finta EP, Illes P. (1993). Galanin receptors inhibit the spontaneous firing of locus coeruleus neurones and interact with mu-opioid receptors. Eur J Pharmacol, 230(2), 223-30.

Sharma SK, Klee WA, Nirenberg M. (1975). Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. Proc Natl Acad Sci, 72(8), 3092-6.

Shippenberg TS, Herz A, Spanagel R, Bals-Kubik R, Stein C. (1992). Conditioning of opioid reinforcement: neuroanatomical and neurochemical substrates. Ann N Y Acad Sci, 654, 347-56.

Simonnet G, Rivat C. (2003). Opioid-induced hyperalgesia: abnormal or normal pain? Neuroreport ,14(1),1-7.

Smith MA, Gergans SR, Iordanou JC, Lyle MA. (2008). Chronic exercise increases sensitivity to the conditioned rewarding effects of cocaine. Pharmacol Rep, 60(4), 561-5.

Smith MA, Lyle MA. (2006). Chronic exercise decreases sensitivity to mu opioids in female rats: correlation with exercise output. Pharmacol Biochem Behav, (1),12-22.

Smith MA, Pitts EG. (2012). Wheel running decreases the positive reinforcing effects of heroin. Pharmacol Rep, 64(4), 960-4.

Smith MA, Yancey DL. (2003). Sensitivity to the effects of opioids in rats with free access to exercise wheels: mu-opioid tolerance and physical dependence. Psychopharmacology, 168(4),426-34.

Spyraki C, Fibiger HC, Phillips AG. (1983). Attenuation of heroin reward in rats by disruption of the mesolimbic dopamine system. Psychopharmacology (Berl), 79(2-3), 278-83.

Stamford JA. (1995). Descending control of pain. Br J Anaesth. 75(2), 217-27.

Stafford J, Degenhardt E, Black R, Bruno R, Buckingham K, Fetherston J et al. (2004). Australian Drug trends 2004. Findings from the Illicit Drug Reporting System (IDRS) National Drug and Alcohol Centre (NDARC) Monograph No. 55.

Sterling P, Eyer J. (1988). Allostasis: A new paradigm to explain arousal pathology. In Fisher S, Reason J (eds), Handbook of Life Stress, Cognition.

Stinus L, Nadaud D, Deminière JM, Jauregui J, Hand TT, Le Moal M. (1989). Chronic flupentixol treatment potentiates the reinforcing properties of systemic heroin administration. Biol Psychiatry, 26(4), 363-71.

Strain EC, Harrison JA, Bigelow GE. (2011). Induction of opioid-dependent individuals onto buprenorphine and buprenorphine/naloxone soluble-films. Clin Pharmacol Ther, 89(3), 443-9.

Sun HL. (1998). Naloxone-precipitated acute opioid withdrawal syndrome after epidural morphine. Anesth Analg, 86(3), 544-5.

Sweitzer SM, Allen Cp, Zissen MH and Kendig JJ. (2004). Mechanical allodynia and thermal hyperalgisia upon acute opioid withdrawal in the neonatual rat. Pain, 110, 269-280.

Taylor A, Katomeri M. (2007). Walking reduces cue-elicited cigarette cravings and withdrawal symptoms, and delays ad libitum smoking. Nicotine Tob Res, 9(11),1183-90

Taylor A, Ussher M. (2005). Effects of exercise on smoking cessation and coping with withdrawal symptoms and nicotine cravings. In: G. Faulkner, and A.H.Taylor (Eds.) Exercise, health and mental health: Emerging relationships. Routledge, London,135-158.

Tetrault JM, Fiellin DA. (2012). Current and potential pharmacological treatment options for maintenance therapy in opioid-dependent individuals. Drugs, 72(2), 217-28.

Thanawala V, Kadam VJ, Ghosh R. (2008). Enkephalinase inhibitors: potential agents for the management of pain. Curr Drug Targets. 9(10):887-94.

Thompson T, Schuster CR. (1964). Morphine self-administration, food-reinforced, and avoidance behaviors in rhesus monkeys. Psychopharmacologia, 5:87-94.

Tilson HA, Rech RH, Stolman S. (1973). Hyperalgesia during Withdrawal as a Means of Measuring the Degree of Dependence in Morphine Dependent Rats. Psychopharmacologia, 28(3), 287-300.

Tompkins DA, Bigelow GE, Harrison JA, Johnson RE, Fudala PJ, Strain EC. (2009). Concurrent validation of the Clinical Opiate Withdrawal Scale (COWS) and single-item indices against the Clinical Institute Narcotic Assessment (CINA) opioid withdrawal instrument. Drug Alcohol Depend, 105(1-2),154-9.

Turchan J, Lasoń W, Budziszewska B, Przewłocka B. (1997). Effects of single and repeated morphine administration on the prodynorphin, proenkephalin and dopamine D2 receptor gene expression in the mouse brain. Neuropeptides, 31(1), 24-8.

Ueda H, Miyamae T, Hayashi C, Watanabe S, Fukushima N, Sasaki Y, Iwamura T, Misu Y. (1995). Protein kinase C involvement in homologous desensitization of delta-opioid receptor coupled to Gi1-phospholipase C activation in Xenopus oocytes. J Neurosci, 15(11), 7485-99.

Umbricht A, Hoover DR, Tucker MJ, Leslie JM, Chaisson RE, Preston KL. (2003) Opioid detoxification with buprenorphine, clonidine, or methadone in hospitalized heroin-dependent patients with HIV infection. Drug Alcohol Depend, 69(3), 263-72.

Ussher M, Sampuran AK, Doshi R, West R, Drummond DC. (2004). Acute effect of a brief bout of exercise on alcohol urges. Addiction, 99(12), 1542-7.

Verebey K, Mulé SJ. (1975). Naltrexone pharmacology, pharmacokinetics, and metabolism: current status. Am J Drug Alcohol Abuse, 2(3-4), 357-63.

Wang D, Sun X, Sadee W. (2007). Different effects of opioid antagonists on mu-, delta-, and kappa-opioid receptors with and without agonist pretreatment. J Pharmacol Exp Ther, 321(2),544-52.

Wang GB, Wu LZ, Yu P, Li YJ, Ping XJ, Cui CL. (2011). Multiple 100 Hz electroacupuncture treatments produced cumulative effect on the suppression of morphine withdrawal syndrome: Central preprodynorphin mRNA and p-CREB implicated. Peptides, 32(4),713-21.

Wang WS, Kang S, Liu WT, Li M, Liu Y, Yu C, Chen J, Chi ZQ, He L, Liu JG. (2012). Extinction of aversive memories associated with morphine withdrawal requires ERK-mediated epigenetic regulation of brain-derived neurotrophic factor transcription in the rat ventromedial prefrontal cortex. J Neuroscience, 32(40),13763-75.

Watts VJ, Neve KA. (2005). Sensitization of adenylate cyclase by Galpha i/o-coupled receptors. Pharmacol Ther, 106(3), 405-21.

Way EL, Loh HH, Shen F. (1968). Morphine tolerance, physical dependence, and synthesis of brain 5-hydroxytryptamine. Science, 162(3859),1290-2.

Werme M, Thorén P, Olson L, Brené S. (2000). Running and cocaine both upregulate dynorphin mRNA in medial caudate putamen. Eur J Neurosci,12(8), 2967-74.

Wesson DR, Ling W. (2003). The Clinical Opiate Withdrawal Scale (COWS). J Psychoactive Drugs, 35(2), 253-9.

Wei YM, Xu Y, Yu CX, Han J., Sheng Li Xue Bao. (2009). Melatonin enhances the expression of beta-endorphin in hypothalamic arcuate nucleus of morphine-dependent mice. Acta Physiologica Sinica, 61(3), 255-62.

Whistler JL, Chuang HH, Chu P, Jan LY, von Zastrow M. Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. Neuron. 1999 Aug;23(4):737-46.

Williams JT, Ingram SL, Henderson G, Chavkin C, von Zastrow M, Schulz S, Koch T, Evans CJ, Christie MJ. (2013). Regulation of μ-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. Pharmacol Rev, 65(1), 223-54.

Xu GP, Van Bockstaele E, Reyes B, Bethea T, Valentino RJ. (2004). Chronic morphine sensitizes the brain norepinephrine system to corticotropin-releasing factor and stress. J Neurosci, 24(38), 8193-7.

Zachariou V, Brunzell DH, Hawes J, Stedman DR, Bartfai T, Steiner RA, Wynick D, Langel U, Picciotto MR. (2003). The neuropeptide galanin modulates behavioral and neurochemical signs of opiate withdrawal. Proc Natl Acad Sci, 100(15), 9028-33.

Zarnegar P, Persson AI, Ming Y, Terenius L. (2006). Opioid-induced regulation of gene expression in PC12 cells stably transfected with mu-opioid receptor. Neurosci Lett, 396(3), 197-201.

Zlebnik NE, Anker JJ, Gliddon LA, Carroll ME. (2010). Reduction of extinction and reinstatement of cocaine seeking by wheel running in female rats. Psychopharmacology (Berl), 209(1),113-25.

Zlebnik NE, Anker JJ, Carroll ME. (2012). Exercise to reduce the escalation of cocaine self-administration in adolescent and adult rats. Psychopharmacology, 224(3), 387-400.