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Differential effects of endocannabinoid catabolic inhibitors on opioid withdrawal in mice

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of
Philosophy at Virginia Commonwealth University.

By

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List of Abbreviations

%MPE	% Maximal Possible Effect
2-AG	2-arachidonoyl glycerol
AEA	<i>N</i> -arachidonylethanolamide; anandamide
AA	Arachidonic Acid
ABHD	alpha/beta hydrolase
ANOVA	Analysis of Variance
cAMP	Cyclic Adenosine Monophosphate
CB ₁	Cannabinoid receptor, subtype 1
CB ₂	Cannabinoid receptor, subtype 2
COX	Cyclooxygenase
CPA	Conditioned Place Avoidance
CPP	Conditioned Place Preference
CREB	cAMP Response Element Binding Protein
DAGL	Diacylglycerol lipase
DOR	Delta Opioid Receptor
DSE	Depolarization-induced suppression of excitation
DSI	Depolarization-induced suppression of inhibition
FAAH	Fatty Acid Amide Hydrolase
FLAT	FAAH-like AEA transporter
GABA	gamma-Aminobutyric acid
GDE	Glycerophosphodiesterase
GRK	G-protein receptor kinase

i.c.v.	Intracranial ventricular
i.p.	Intraperitoneal
JZL184	4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl) piperidine-1-carboxylate, selective MAGL inhibitor
LC	Locus Coeruleus
MAGL	Monoacylglycerol Lipase
MAPK	Mitogen-activated protein kinase
MOR	Morphine
NAc	Nucleus Accumbens
NAPE	N-acylphosphatidylethanolamine
NLX	Naloxone Hydrochloride
PAG	Periaqueductal Grey
PKA	Protein Kinase A
PKC	Protein Kinase C
PF-3845	N-(pyridin-3-yl)-4-(3-(5-(trifluoromethyl)pyridin-2-yloxy)benzyl) piperidine-1-carboxamide, selective FAAH inhibitor
PLC	Phospholipase C
PLD	Phospholipase D
POMC	Proopiomelanocortin
Rim	Rimonabant, CB1 antagonist
SAL	Saline (0.9% NaCl)
SA-57	2-(Methylamino)-2-oxoethyl 4-(4-chlorophenethyl)piperidine-1-Carboxylate; dual FAAH/MAGL inhibitor
s.c.	subcutaneous

THC	delta-9-tetrahydrocannabinol
VTA	Ventral Tegmental Area

Abstract

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By Thomas F. Gamage

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of

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Virginia Commonwealth University, 2013.

Director: Dr. Aron Lichtman, Professor, Department of Pharmacology & Toxicology

The effects of cannabinoids in reducing somatic signs of opioid withdrawal have been known for some time. In morphine dependent rodents, opioid withdrawal following precipitation with the mu opioid antagonist naloxone elicits robust withdrawal behaviors including jumps, paw flutters, head shakes, diarrhea and weight loss. Delta-9-tetrahydrocannabinol has been shown to reduce this opioid withdrawal in mice via activation of the cannabinoid type-1 (CB₁) receptor and recently it has been shown that inhibition of the catabolic enzymes for endocannabinoids also reduce somatic signs of opioid withdrawal. Specifically, inhibition the enzyme fatty acid amide hydrolase (FAAH), the catabolic enzyme for the endocannabinoid *N*-arachidonylethanolamide (AEA; anandamide) or inhibition of the enzyme monoacylglycerol lipase (MAGL), the catabolic enzyme for the endocannabinoid 2-arachidonoylglycerol (2-AG) has been shown to reduce opioid withdrawal in mice. However, FAAH inhibition only reduced a subset of withdrawal signs in mice and full MAGL inhibition which maximally reduced somatic withdrawal signs has been shown to produce THC-like effects and dependence potential. Additionally, the effects of endocannabinoid catabolic inhibitors on other aspects of withdrawal,

such as the negative motivational effects, are not known. The objectives of this dissertation were to 1) assess the efficacy of dual inhibition of FAAH and MAGL on somatic signs of opioid withdrawal and 2) determine whether these treatments would produce cannabimimetic effects (hypomotility, catalepsy, antinociception and hypothermia); 3) develop other behavioral assays of opioid withdrawal; and 4) determine if endocannabinoid catabolic inhibitors would reduce the acquisition of opioid withdrawal induced conditioned place avoidance (CPA) as a measure of the negative motivational consequences of opioid withdrawal. We found that full inhibition of FAAH with the selective inhibitor PF-3845 and partial inhibition of MAGL with the selective inhibitor JZL184 reduced withdrawal-related jumps and the expression of diarrhea to a greater degree than either inhibitor alone and these effects were shown to be CB₁ mediated. Additionally, we tested the novel dual FAAH/MAGL inhibitor SA-57 which has greater potency at inhibiting FAAH over MAGL and found that it similarly reduced withdrawal signs at doses that only partially elevated 2-AG while fully elevating AEA; furthermore, SA-57 did not produce cannabimimetic effects at these doses. We next assessed the effects of morphine withdrawal in five behavioral assays: marble burying, novelty-induced hypophagia, the light/dark box, a novel procedure developed to assess “escape behavior” and the CPA procedure. From these studies we selected the CPA procedure to further evaluate the effects of endocannabinoid catabolic inhibitors to determine their ability to reduce the negative motivational aspect of opioid withdrawal. We found that naloxone (0.056 mg/kg) produced robust CPA in morphine-pelleted, but not placebo-pelleted, mice and that this dose elicited minimal somatic withdrawal signs. Morphine pretreatment was shown to block withdrawal CPA and withdrawal jumping in mice while clonidine only blocked withdrawal CPA and these served as positive controls. We found that THC, JZL184, and SA-57 significantly reduced the percentage of mice that jumped during

the conditioning session, demonstrating that these treatments blocked the somatic signs of withdrawal. However, none of these treatments significantly affected acquisition of the withdrawal CPA. These studies suggest that dual inhibition of FAAH/MAGL has enhanced effects on attenuating withdrawal-related jumps and diarrhea, but not the negative motivational aspects of morphine withdrawal as inferred by the Pavlovian CPA experiments.

Chapter 1: Introduction

1.1. Opioid dependence and addiction in the United States

While opioids are the most potent and efficacious drugs currently available for the treatment of pain, they possess an abuse potential and dependence liability that limit the safety of their application. Upon cessation of drug administration, opioid dependence manifests as a syndrome that includes both physical and psychological withdrawal symptoms. These include physical aspects such as diarrhea, emesis, dehydration, hypertension, hyperthermia, tachycardia and body aches as well as affective aspects such as anxiety, dysphoria, and panic attacks (Farrell, 1994; Gossop, 1988; Jasinski, 1981; Wesson *et al.*, 2003). These withdrawal symptoms are extremely aversive and are considered a primary driver of ongoing drug use (Bart, 2012).

In the United States, non-prescription opioid use has been increasing with approximately 6 million Americans reporting such use in 2009. Dispensed prescriptions for opioids increased by 50% from 2000 to 2009 (Governale, 2010) and studies suggest that anywhere from 12% to 34% of individuals receiving prescription opioids will abuse them (Chabal *et al.*, 1997; Fishbain *et al.*, 2008). Emergency room visits involving prescription opioids have more than doubled in the years between 2004 and 2009 increasing from 198,114 to 477,936 (DAWN, 2010). Additionally non-medical use of prescription opioids is increasing in the youth (Birnbaum *et al.*, 2011; McCabe *et al.*, 2005).

The trends are striking and the prognosis for public health in the United States in terms of opioid abuse and dependence appears even grimmer when other opiates such as heroin are factored in. While the late 1980s and early 1990s saw a decrease in heroin use, there has been a

recent upswing in abuse of heroin since the late 1990s and it still poses a serious threat to public health (Brown, 2004). Between 2008–2012, heroin use among young adults has remained at peak rates (0.5-0.6%) and prescription opioids, while still high (7.3%), have slightly declined from the peak in 2009 (9.2%) (Johnston *et al.*, 2012). Overall, opioid dependence and abuse are becoming increasingly prevalent and newer and better treatment pharmacotherapies are needed since currently available treatments (e.g. methadone, buprenorphine, clonidine) are not fully effective (see section 1.4).

1.2. The endogenous opioid system

Morphine, the prototypical opioid analgesic, and other opioids act upon an endogenous opioid system. Evidence supporting the existence of opioid receptors came from observations of stereospecific opiate binding in the central nervous system (Pert *et al.*, 1973). The existence of multiple subtypes was first suggested by Martin *et al.* (1976) after studies examining several opiates (morphine, ketocyclazocine, SKF-10,047) in neurophysiological and behavioral assays. They observed that the effects of all three drugs could be blocked by naltrexone yet all three drugs produced distinct physiological effects. While morphine and ketocyclazocine produced miosis, SKF-10,047 produced mydriasis. Additionally morphine produced bradycardia while SKF-10,057 produced tachycardia and ketocyclazocine did not affect heart rate. In morphine-dependent dogs, morphine would suppress the withdrawal syndrome while ketocyclazocine would not. From these studies, they proposed three different receptors named for their respective agonists: mu, kappa, and sigma (Martin *et al.*, 1976). Later, subtypes were discovered and cloned and included mu (Chen *et al.*, 1993; Min *et al.*, 1994), kappa (Meng *et al.*, 1993), and delta (Evans *et al.*, 1992; Kieffer *et al.*, 1992) with mu opioid receptors mediating the rewarding and

analgesic effects (Becker *et al.*, 2000; Dhawan *et al.*, 1996; Matthes *et al.*, 1996; Sora *et al.*, 1997) of opioids. Mu opioid receptors are members of the class A (rhodopsin) family of receptors that couple to the pertussis toxin sensitive, heterotrimeric $G_{i/o}$ proteins (Jordan *et al.*, 1998) and upon activation inhibit adenylyl cyclase (Sharma *et al.*, 1975b), inhibit N and P/Q type Ca^{2+} channels and activate inwardly rectifying K^+ channels (Darlison *et al.*, 1997).

Endogenous ligands for opioid receptors are post-translationally cleaved from peptide precursors and result in a wide number of peptide ligands. Opioid peptides include 1) endorphins (Cox *et al.*, 1976)($\mu = \delta > \kappa$) such as beta-endorphin; derived from proopiomelanocortin; 2) endomorphins (Zadina *et al.*, 1997)(μ selective); the peptide precursor is yet to be established; 3) enkephalins (Hughes *et al.*, 1975)($\delta \gg \mu$) derived from pro-enkephalin; and 4) dynorphins (Goldstein *et al.*, 1979)($\kappa \gg \mu, \delta$); derived from pro-dynorphin (Janecka *et al.*, 2004). Naloxone, the antagonist used for these studies, binds to mu, delta and kappa and has only slightly more affinity for mu opioid receptors. Since the focus of the present study is on morphine dependence and withdrawal, the rest of this section will focus on the mu opioid receptor since expression of naloxone-precipitated morphine withdrawal is dependent on this receptor (Matthes *et al.*, 1996).

1.3. Opioid tolerance, dependence and withdrawal

Opioid dependence encompasses multiple aspects of behavioral and physiological responses that accompany prolonged drug use and that occur during a specific time frame. The Diagnostic and Statistical Manual of Mental Disorders IV (American Psychiatric Association, 2000) lists seven primary aspects to drug dependence, of which the presence of three or more is considered to qualify a patient as dependent. These include: 1) tolerance or markedly increased amounts of the substance to achieve intoxication or desired effect or markedly

diminished effect with continued use of the same amount of substance; 2) withdrawal symptoms or the use of certain substances to avoid withdrawal symptoms; 3) use of a substance in larger amounts or over a longer period than was intended; 4) persistent desire or unsuccessful efforts to cut down or control substance use; 5) involvement in chronic behavior to obtain the substance, use the substance, or recover from its effects; 6) reduction or abandonment of social, occupational or recreational activities because of substance use; and 7) use of substances even though there is a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance.

Of these, tolerance and withdrawal have been intensely studied in mice and are two separate phenomena, which are produced by similar antecedents (i.e. prolonged exposure to an opioid agonist). Furthermore, tolerance and withdrawal are distinct phenomena both in how they are expressed behaviorally as well as in the cellular and molecular changes that underlie those individual responses. Tolerance to opioids is defined as a loss of effect following repeated treatments such that a higher dose is required for equivalent effects (Bailey *et al.*, 2005). Withdrawal manifests as a set of symptoms that occurs following cessation of drug administration or treatment with an antagonist that abruptly halts receptor activation (Bailey *et al.*, 2005). In humans, opioid withdrawal symptoms include anxiety, dysphoria, panic attacks, diarrhea, emesis, dehydration, hypertension, hyperthermia, tachycardia and body aches (Farrell, 1994; Gossop, 1988; Jasinski, 1981; Wesson *et al.*, 2003).

In rodents, opioid withdrawal produces a number of somatic signs including jumping, paw fluttering, head/body shaking/twitching, ptosis, teeth chattering, weight loss and diarrhea (Way *et al.*, 1969). Additionally, opioid withdrawal has been shown to produce changes in behavioral responses in assays described in Chapter 3 of this thesis, used to infer aspects of

motivation and emotionality including anxiety, aversion, and anhedonia. In rats, opioid withdrawal has been shown to elicit unconditioned avoidance behaviors in tests such as the elevated plus maze (Schulteis *et al.*, 1998b; Zhang *et al.*, 2008) where further decreases in the time spent in the open-arms are observed during withdrawal. These responses are often inferred to reflect “anxiogenic-like” behavior. Withdrawal from opioids has also been well established to produce conditioned place avoidance (CPA). In this procedure, injection of naloxone into opioid-dependent animals serves as an unconditioned stimulus and the subsequent opioid withdrawal serves as an unconditioned response. The pairing of naloxone in the opioid-dependent animal (unconditioned stimulus) with specific contextual cues (conditioned stimulus) leads to a conditioned response called conditioned place avoidance. It is then inferred from this conditioned avoidance response that the withdrawal state is aversive (Broseta *et al.*, 2005; Frenois *et al.*, 2002; Maldonado *et al.*, 2004; Sato *et al.*, 2005; Stinus *et al.*, 2000; Stinus *et al.*, 1990; Watanabe *et al.*, 2002). CPA is a sensitive measure of opioid withdrawal and is discussed in further detail in Chapter 3.

Additionally, other behaviors have been assessed during morphine withdrawal, which involve brain regions that are likely important for affective withdrawal symptoms in humans. Intracranial self-stimulation (ICSS) is a behavioral procedure in which operant responding by animals is maintained by electrical stimulation of the brain, typically the medial forebrain bundle. Drugs of abuse will typically facilitate ICSS and this increase in operant responding for brain stimulation has been suggested to be a rewarding effect. On the other hand, experimental manipulations, which suppress operant responding for ICSS are interpreted to indicate a loss of reward function reflecting anhedonia (Stoker *et al.*, 2011). Rats undergoing opioid withdrawal exhibit decreases in operant responding for ICSS (Altarifi *et al.*, 2011a; Liu *et al.*, 2004;

Schaefer *et al.*, 1986; Schaefer *et al.*, 1983; Schulteis *et al.*, 1994). Schulteis *et al.* (1994) reported that morphine-pelleted rats (2 x 75 mg subcutaneous morphine pellets) responding for stimulation of the lateral hypothalamus had marked elevations in current intensity-response thresholds from baseline following naloxone (0.01 – 0.03 mg/kg) while placebo-pelleted rats exhibited no such shifts from baseline even when administered a high dose of naloxone (1 mg/kg). In another study, rats trained to respond for electrical stimulation of the left medial forebrain bundle and receiving daily injections of morphine (3.2 - 18 mg/kg/day over the course of 4 weeks) became tolerant to the rate decreasing effects of chronic morphine by day 7 of week 4, the week in which they received the highest dose (18 mg/kg/day). The following day, prior to receiving the daily morphine dose, rats exhibited decreased responding for brain-stimulation reward as compared to baseline suggesting spontaneous withdrawal produced anhedonic effects. Furthermore, prior to the sessions, rats did not display somatic withdrawal signs (i.e., diarrhea, teeth chattering, wet dog shakes), suggesting alterations in ICSS were not due to overt withdrawal signs (Altarifi *et al.*, 2011a). Alterations in brain-stimulation reward thresholds were also demonstrated in rats that received repeated injections of morphine (5.6 mg/kg/day) and tested during spontaneous and naloxone-precipitated withdrawal. Increases in brain-stimulation reward thresholds were observed in spontaneously withdrawing rats on days 3 and 4, which were 23.5 h following the previous morphine injection. Additionally, 5 min after naloxone (1 mg/kg) treatment in these same rats, elevations over baseline thresholds were observed on days 1-4, while naloxone (0.1 and 0.33 mg/kg) resulted in elevated thresholds on day 4. Furthermore, naloxone (0.1 mg/kg) did not elevate thresholds in mice receiving repeated vehicle injections suggesting that its effects in morphine-treated mice reflect reward deficits accompanying precipitated withdrawal. In the case of naloxone (0.1 mg/kg) on day 1, these deficits reflect acute

withdrawal since the only previous exposure to morphine (5.6 mg/kg) occurred only 4 hours earlier (Liu *et al.*, 2004). Thus, CPA and ICSS assays are sensitive to morphine withdrawal.

Complex neuroadaptive changes underlie opioid tolerance and dependence (for reviews see: Bailey *et al.*, 2005; Williams *et al.*, 2001; Williams *et al.*, 2013). The level of tolerance that develops appears to depend on the intrinsic activity (i.e. relative propensity for a given drug-receptor complex to produce a maximum functional response) of the agonist whereby highly efficacious mu opioid receptor agonists produce less antinociceptive tolerance than those of low efficacy. Efficacy of opioids has been calculated from dose ratios following multiple dose-response curves for the test compounds after increasing doses of beta-funaltrexamine (β -FNA), the irreversible mu antagonist, in order to reduce the number of spare receptors incrementally. Mjanger and Yaksh (1991) determined that morphine's intrathecal antinociceptive dose-ratios (ED_{50} post β -FNA / ED_{50} post vehicle) was much larger than that of sufentanil and DAMGO (morphine \gg sufentanil = DAMGO) suggesting that morphine must have a higher fractional receptor occupancy to produce a similar effect and thus possesses less intrinsic activity. The differential effects of intrinsic activity on tolerance have been reported. In a study where 7 days of continuous intrathecal drug infusion of equieffective doses, as measured by antinociception in the hot plate test, of either morphine or sufentanil in rats, the antinociceptive tolerance to morphine resulted in an increase in morphine ED_{50} value from 0.65 to 30.0 nmol, a roughly 44 fold increase. In contrast, sufentanil ED_{50} value increased only 3-fold, from 0.07 to 0.21 nmol. Additionally, when cross tolerance was assessed, sufentanil ED_{50} value shifted from 0.07 nmol in saline treated mice to 0.68 nmol in morphine treated mice, roughly 3 fold higher than that observed in sufentanil treated rats (Sosnowski *et al.*, 1990). These data suggested that morphine caused greater desensitization and/or internalization of mu opioid receptors than sufentanil,

which would be consistent with the number of receptors necessary for each to produce their relative antinociceptive effects, i.e. sufentanil exhibiting properties of a full agonist thus requiring fewer receptors to produce a given effect than morphine which is a partial agonist. Continuous infusion of opioid agonists in mice also elicits similar effects on the magnitude of tolerance depending on the intrinsic activity of the agonist used, however this disparity in level of tolerance is abolished when equieffective doses are administered intermittently once a day over a period of 72 h or 7 days which may be due to the phasic stimulation of receptors which allows for resensitization (Duttaroy *et al.*, 1995; Madia *et al.*, 2009).

Mechanisms of morphine tolerance have not been fully elucidated, however phosphorylation of the receptor and binding of arrestin are components of this process (Williams *et al.*, 2001). Opioid agonist-induced desensitization of mu opioid receptors occurs through phosphorylation of the C terminus primarily by G-protein receptor kinases (GRKs), though protein kinase C (PKC) has also been suggested as a possible mediator of receptor phosphorylation (Williams *et al.*, 2013). Phosphorylation of the C-terminus leads to recruitment of arrestin and uncoupling of the receptor from G-proteins (Bailey *et al.*, 2005). Subsequently, receptors can be trafficked from the membrane via clathrin-dependent pathway and can then be either degraded/downregulated or recycled back up to the surface (Bailey *et al.*, 2005; Connor *et al.*, 2004; Ferguson, 2001; Van Bockstaele *et al.*, 2001; Zastrow *et al.*, 2003).

Phosphorylation and regulation of mu opioid receptors by morphine, on the other hand, diverges from other agonists such as DAMGO in that phosphorylation is less efficient than full agonists and morphine leads to minimal receptor internalization (Bailey *et al.*, 2005; Borgland *et al.*, 2003). Morphine can lead to sustained phosphorylation of the serine-375 residue intracellular C terminus without the receptor undergoing endocytosis (Schulz *et al.*, 2004) unlike in the case

of other high efficacy agonists such as DAMGO which causes marked phosphorylation of mu opioid receptors and subsequent internalization (Zhang *et al.*, 1998). Morphine also differs from DAMGO in that it recruits only beta-arrestin 2 whereas DAMGO recruits both beta-arrestin 1 and beta-arrestin 2 (Groer *et al.*, 2011). Resensitization of mu opioid receptors is thought to require receptor endocytosis which occurs with high efficacy agonists such as DAMGO but not with morphine. The lack of receptor internalization following morphine treatment is thought to underlie the marked and protracted tolerance that is observed following morphine treatment (Bailey *et al.*, 2005). This is supported by an increase in the ability of morphine to cause receptor endocytosis and a reduction in the development of morphine tolerance when animals are also treated with DAMGO, which itself is known to cause internalization of receptors. Specifically, spinal cord from rats treated with a low dose of DAMGO (0.01 nmol) and a high dose of morphine (30 nmol) intrathecally, neither of which produce endocytosis on their own, caused significant internalization of mu opioid receptors as determined by immunohistochemical staining of lamina II neurons in spinal cord. Additionally, the same dose of DAMGO (0.01 nmol) administered twice daily to mice receiving continuous morphine (6 nmol/h) prevented the development of tolerance to morphine which was suggested to likely be due to its actions on facilitating internalization of mu opioid receptors (He *et al.*, 2002).

Protein kinase C (PKC) has also been implicated in morphine tolerance. In HEK293 cells expressing mu opioid receptors and inward rectifying potassium channel (Kir3.1 and Kir3.2 subunits), overexpression of dominant negative mutants of GRK2 reduced the desensitization (as measured by changes in current amplitude as a percentage of the initial peak current) to DAMGO (10 μ M) but not to morphine (30 μ M) suggesting GRK2 is involved in desensitization of mu opioid receptors following DAMGO, but not morphine. However, exposure of cells to the

PKC pseudosubstrate inhibitory peptide PKC (19-31) 10 min prior to exposure to morphine or DAMGO was able to reduce the desensitization caused by morphine (~50%), but not DAMGO. Additionally, pretreatment of HEK293 cells with the PKC inhibitor GF109203X (1 μ M) for 15 min reduced the small but significant increase in mu opioid receptor phosphorylation caused by 30 min exposure morphine, but not DAMGO. Arrestin translocation was also observed following DAMGO, but not morphine (Johnson *et al.*, 2006). A similar role for PKC was observed in horizontal slices containing locus coeruleus (LC) neurons in which morphine was shown to produce desensitization of mu opioid receptors when PKC was activated by either a phorbol ester or indirectly via activation of M3 muscarinic receptor Gq proteins, as this effect was completely reversed by PKC inhibition (Bailey *et al.*, 2004). Studies using genetically altered mice also suggested a role of PKC in antinociceptive tolerance to morphine whereby PKC γ (-/-) mice, which exhibit similar baseline tail-flick latencies as wild-type mice, showed a decreased degree of tolerance 96 h following subcutaneous implantation of 75 mg morphine pellets. While the PKC γ (-/-) and (+/+) mice exhibited similar antinociceptive dose-response curves for morphine prior to dependence, the ED₅₀ values for morphine following induction of dependence were 6.4 mg/kg (4.8 – 8.4 mg/kg) for PKC γ (-/-) and 14 mg/kg (11 – 18 mg/kg) for PKC γ (+/+) mice. The role of PKC in desensitization of mu opioid receptors following morphine remains under investigation.

While phosphorylation/downregulation of mu opioid receptors is believed to play major contributory roles to the development of tolerance, the mechanisms of dependence involve compensatory changes of downstream effectors that maintain normal function in the presence of opioid agonists (Williams *et al.*, 2001). Following mu opioid receptor activation, G_{i/o} effector proteins produce the following actions: 1) inhibition of adenylyl cyclase resulting in a decrease

in cAMP production (Childers *et al.*, 1992) and subsequently a reduction in protein kinase A (PKA) activity and phosphorylation of cAMP response element binding protein (CREB); 2) activation of potassium conductance; 3) inhibition of calcium conductance; 4) inhibition of neurotransmitter release; 5) activation of protein kinase C (PKC); 6) activation of mitogen-activated protein kinase (MAPK) (Williams *et al.*, 2001). Multiple adaptations to these effects of opioids occur following prolonged activation of mu opioid receptors including increased activity of adenylyl cyclase (Matthes *et al.*, 1996; Sharma *et al.*, 1975a), upregulation of CREB (Widnell *et al.*, 1994) and PKA catalytic and regulatory subunits (Lane-Ladd *et al.*, 1997).

Withdrawal occurs following either cessation of opioid exposure (spontaneous withdrawal) or abrupt halting of signaling via administration of a mu opioid receptor antagonist (precipitated withdrawal). The resulting reduction in activation of inhibitory G-proteins leads to a superactivation of upregulated adenylyl cyclase, consequent overshoot of cAMP production (Sharma *et al.*, 1975a; Watts *et al.*, 2005) and increased signaling downstream of this pathway. The end result of upregulation of adenylyl cyclase is an increased hyperexcitability of LC neurons (Aghajanian, 1978; Lane-Ladd *et al.*, 1997). In an early study, recording of LC neurons in rats implanted with 75 mg morphine pellets exhibited initial reductions in firing rates which recovered to baseline 48-72 h later. Upon continuous microiontophoretic administration of naloxone into LC or i.v. administration (0.02 mg/kg), LC neuron firing rates dramatically increased over baseline in morphine-dependent, but not non-dependent, rats (Aghajanian, 1978). In a later study using the same dependence induction procedure, LC firing rates were increased 3 min after naltrexone challenge (100 mg/kg, s.c.) roughly 6-fold over baseline and 4-fold over control animals receiving naltrexone. Furthermore, behavioral time course measurements corresponded with changes in rates of LC firing. The naltrexone dose used in this study

(100 mg/kg) was much higher than typically used to precipitate opioid withdrawal, but was selected because it produces maximal withdrawal behaviors that persist for up to 6 h allowing for the time course that was conducted (Rasmussen *et al.*, 1990) though such a high dose raises questions regarding possible off-target effects. Upregulation of adenylyl cyclase appears dependent upon CREB as increases in levels of immunoreactivity of AC VIII were reduced following bilateral treatment of CREB antisense oligonucleotides in LC as compared with the contralateral LC. Morphine withdrawal behaviors were reduced in rats receiving bilateral infusion of CREB antisense oligonucleotide and increases in firing rates of LC neurons were reduced (Lane-Ladd *et al.*, 1997). Nestler and colleagues have suggested that increases in firing of LC neurons is driven by PKA (Nestler, 2001a). Increases in cAMP-dependent PKA activity have been observed with chronic morphine in the LC (Nestler *et al.*, 1988) and an inhibitor of PKA, H-89, suppressed naloxone-precipitated morphine withdrawal induced hyperactivity of LC neurons (Ivanov *et al.*, 2001) and also reduced somatic signs of naloxone-precipitated morphine withdrawal in rats (Seyedi *et al.*, 2013). The role of PKA in morphine withdrawal has been suggested to be related to activation of an inward sodium current (Alreja *et al.*, 1993; Nestler, 2001b). Hyperexcitability of LC neurons causes an increased noradrenergic outflow (Crawley *et al.*, 1979; Van Bockstaele *et al.*, 2008) through efferents which connect widely throughout the brain to affect cognition, emotionality, anxiety, arousal and stress (Dunn *et al.*, 2004). Thus, the hyperexcitability of these neurons following withdrawal leading to increased sympathetic outflow is consistent with the wide range of consequential aversive effects of opioid withdrawal in humans.

The degree to which LC vs. periaqueductal grey (PAG) neurons contribute to opioid withdrawal has been questioned (Christie *et al.*, 1997). For example, microinjections of the mu

opioid antagonists methylnaloxonium (Maldonado *et al.*, 1992) or naloxone (Esposito *et al.*, 1987) into the LC or PAG were shown to elicit morphine withdrawal signs (teeth chattering, mastication, rearing, wet dog shakes and jumping) in rats, and electrolytic lesion of LC reduced withdrawal severity following intracranial ventricular (i.c.v.) administration of methylnaloxonium in morphine-pelleted rats (Maldonado *et al.*, 1993) suggesting that the LC is important for expression of somatic withdrawal signs. In contrast, others have shown that near-complete (94%) chemical lesion of noradrenergic neurons in the LC by 6-hydroxydopamine did not affect naloxone-precipitated (7.5-15 $\mu\text{g/kg}$) withdrawal-induced CPA in rats implanted with two subcutaneous 75 mg morphine pellets nor did it affect somatic signs of naloxone-precipitated (10-1000 $\mu\text{g/kg}$) morphine withdrawal (Caillé *et al.*, 1999). In addition to PAG and LC, noradrenergic neurons arising from the bed nucleus of the stria terminalis (BNST) have been implicated in morphine withdrawal aversion and somatic signs. Chemical lesion of ventral noradrenergic bundle which projects from the medulla to the BNST attenuated acquisition of morphine withdrawal CPA, but did not affect somatic signs. In contrast, lesion of the dorsal noradrenergic bundle projecting from the LC did not affect CPA or somatic withdrawal signs (Delfs *et al.*, 2000). These data suggest that multiple neural substrates contribute to the expression of different opioid withdrawal responses.

1.4. Treatment of opioid withdrawal

Currently, there are few available treatment options for opioid dependence. These medications either 1) act either directly upon mu opioid receptors (substitution therapy), or 2) target the increased noradrenergic tone that results from hyperexcitability of LC neurons. Methadone ($t_{1/2} = 28$ h), a full mu agonist, and buprenorphine ($t_{1/2} = 37$ h), a partial mu agonist,

are abused in the United States (Cicero *et al.*, 2005) and are not fully effective at combating withdrawal (Dyer *et al.*, 1999; Kuhlman *et al.*, 1998); thus these treatment strategies have limitations. Dyer *et al.* (1999) reported that opioid-dependent patients receiving once daily methadone maintenance treatment exhibited withdrawal severity that was inversely correlated with plasma methadone concentrations. Eight hours after subjects received their daily methadone dose, they reported significant increases in withdrawal severity. Kuhlman *et al.* reported that patients receiving buprenorphine (8 mg/day) had elevated withdrawal scores following the end of the study, peaking on day 5 then gradually returning to baseline by 14 days post-study. A review of clinical studies suggested that buprenorphine may be a more effective treatment for opioid dependence than methadone or clonidine as measured by patient retention, possibly because buprenorphine is associated with fewer adverse effects or because withdrawal symptoms may have resolved more quickly with buprenorphine (Gowing *et al.*, 2009). Treatment with alpha-2 adrenergic agonists (e.g. clonidine, lofexidine) attenuate the increased sympathetic activity during withdrawal, but these drugs can produce postural hypotension, sedation and cognitive impairment, and are not fully effective at attenuating the withdrawal symptoms in all patients (Gerra *et al.*, 2001; Gossop, 1988; Lobmaier *et al.*, 2010). While current treatment strategies can ward off some aspects of withdrawal and facilitate patient completion, better medications with fewer side effects could improve retention rates in detoxification programs and are warranted.

There is clinical evidence suggesting that cannabinoids may be effective at attenuating opioid withdrawal and improving patient retention in treatment programs. In a study of heroin abusers who underwent a 9 month drug-free treatment program, cannabis use was associated with greater retention (Ellner, 1977). Another study reported that intermittent cannabis use was associated with decreased percentage of heroin-positive urine during treatment and increased pill

compliance in patients undergoing naltrexone treatment for opiate-dependence (Church *et al.*, 2001). Similarly, cannabis use was also associated with greatly improved retention as well as pill-taking adherence in a naltrexone-treatment program for opioid dependence (Raby *et al.*, 2009). Additionally, based on pilot data, cannabis use during methadone maintenance treatment was reportedly associated with decreased withdrawal severity (Scavone *et al.*, 2013b). Cannabis has also been reportedly used as a substitute for prescription opioids (Lucas *et al.*, 2012). However, double-blind multi-center studies examining whether cannabinoids actually reduce opioid dependence are lacking.

1.5. The endogenous cannabinoid system

1.5.1. Cannabinoid receptors

The existence of cannabinoid receptors were first suggested by the ability of cannabinoids to inhibit adenylyl cyclase activity (Howlett *et al.*, 1984) and the demonstration of specific binding of [³H]CP55,940 in rat brain (Devane *et al.*, 1988). Subsequently, CB₁ (Matsuda *et al.*, 1990) and CB₂ (Munro *et al.*, 1993) receptors were cloned and determined to be G_{i/o}-protein coupled receptors which upon activation lead to 1) inhibition adenylyl cyclase and L-, N- and P/Q-type voltage-gated calcium channels (Caulfield *et al.*, 1992; Mackie *et al.*, 1992; Twitchell *et al.*, 1997), and 2) activation of inwardly-rectifying potassium channels (Guo *et al.*, 2004), and mitogen-activated protein kinase (Derkinderen *et al.*, 2001). In regard to tissue distribution, CB₁ is heterogeneously expressed at high levels in the central nervous system (CNS) and in peripheral tissue (e.g., liver and adipocytes), while CB₂ is localized mainly on immune cells and microglia and is associated with immune function but is also expressed on neurons (Galiègue *et al.*, 1995; Gong *et al.*, 2006).

1.5.2. Endocannabinoids

There are two main endogenous ligands that bind to CB₁ and CB₂ receptors, 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995) and *N*-arachidonylethanolamide (anandamide; AEA) (Devane *et al.*, 1992). Several differences exist between these two endocannabinoids. First, 2-AG levels in the central nervous system are approximately 100 fold higher than those of AEA (Ahn *et al.*, 2009), though the relative amount of each endogenous ligand that is actually signaling is likely much more similar. Long *et al.* demonstrated via *in vivo* microdialysis that interstitial levels of AEA and 2-AG are much more similar than whole brain levels of AEA and 2-AG. Specifically, baseline dialysate 2-AG levels were 4.6±0.7 nM while AEA levels were 0.54±0.1 nM in contrast to whole brain 2-AG levels in the nmol/g range in contrast to the pmol/g range for AEA (Long *et al.*, 2009a). Another reported difference between 2-AG and AEA is that AEA is a partial agonist of CB₁ while 2-AG is a full agonist as determined by increases in intracellular Ca²⁺ in NG108-15 cells (Sugiura *et al.*, 1999). Also, AEA is an agonist at TRPV1 (Ross, 2003) and PPARα receptors (Jhaveri *et al.*, 2008). Other endocannabinoids, which have not been extensively characterized, include noladin ether (Hanuš *et al.*, 2001), virodhamine (Porter *et al.*, 2002) and *N*-arachindonyl dopamine (Huang *et al.*, 2002). Endocannabinoids are not stored in vesicles, but rather are post-synaptically synthesized and released on demand via cleavage of phospholipids from the cellular membrane in response to Ca²⁺ (Ahn *et al.*, 2008).

1.5.2.1. 2-AG biosynthesis and enzymatic regulation

Biosynthesis of 2-AG is activity-dependent (Kano *et al.*, 2009) and is primarily produced via a two-step process involving phospholipase C (PLC) hydrolysis of membrane phospholipids resulting in the release of a arachidonic acid-containing diacylglycerol which is then converted

into 2-AG by diacylglycerol lipase (DAGL) (Gao *et al.*, 2010; Min *et al.*, 2010; Stella *et al.*, 1997; Tanimura *et al.*, 2010). Two isoforms, DAGL α and DAGL β , have been identified (Bisogno *et al.*, 2003). 2-AG is primarily degraded by the pre-synaptically localized (Gulyas *et al.*, 2004) enzyme monoacylglycerol lipase (MAGL) which breaks down 2-AG into arachidonic acid and glycerol (Dinh *et al.*, 2002). Minor enzymes that play a role in the hydrolysis of 2-AG include ABHD6 and ABHD12 (Blankman *et al.*, 2007), the former of which has been shown to be expressed post-synaptically and to be involved in 2-AG regulation as well as CB₁-dependent long-term depression (Marrs *et al.*, 2010). Additionally, cyclooxygenase-2 and 12- and 15-lipoxygenases are known to oxidize both 2-AG and AEA (Matias *et al.*, 2006).

Pharmacological inhibition or genetic deletion of MAGL elevates 2-AG (Long *et al.*, 2009a; Schlosburg *et al.*, 2010a). Upon repeated administration of JZL184 (40 mg/kg i.p., 6 days), mice became tolerant to its antinociceptive and anti-allodynic effects. MAGL knockout mice also exhibited tolerance to JZL184's antinociceptive and anti-allodynic effects. In either MAGL knockout mice or wild-type mice treated repeatedly with JZL184 (40 mg/kg i.p., 6 days), decreases in receptor binding by tritiated rimonabant and reductions in CP55,940-stimulated GTP γ S binding were also observed (Schlosburg *et al.*, 2010a). Furthermore, repeated JZL184 treatment also led to dependence as exhibited by marked increases in paw flutters following rimonabant (10 mg/kg i.p.) challenge.

1.5.2.2. AEA biosynthesis and enzymatic regulation

Biosynthesis of AEA from phospholipid precursors is also activity-dependent (Di Marzo *et al.*, 1994). AEA is biosynthesized by hydrolysis of N-acylphosphatidylethanolines (NAPEs), but the specific enzymes involved are less well characterized than those of 2-AG and remains an

open area of study. AEA biosynthesis was originally thought to be primarily mediated by NAPE-selective phospholipase D (NAPE-PLD) (Wang *et al.*, 2008). However, NAPE-PLD (-/-) mice possess wild type AEA brain levels, which suggests alternate biosynthetic pathways for this endocannabinoid (Leung *et al.*, 2006). Proposed pathways include the serine α/β hydrolase 4 (ABHD4) into lyso-NAPE that is converted into AEA either directly by lyso-phospholipase D or via a two-step process involving further hydrolysis of lyso-NAPE by ABHD4 into glycerophospho-NAE followed by glycerophosphodiesterase 1 (GDE1) cleavage of the glycerophosphate group (Simon *et al.*, 2006). However, GDE1 (-/-) mice displayed wild type levels of AEA, suggesting the involvement of additional enzymatic pathway(s) in AEA biosynthesis (Simon *et al.*, 2010).

Degradation of AEA is carried out primarily by the post-synaptically localized (Gulyas *et al.*, 2004) enzyme fatty-acid amide hydrolase (FAAH) resulting in the release of arachidonic acid and ethanolamine (Cravatt *et al.*, 2001b). Cellular reuptake of AEA may involve the FAAH-like AEA transporter (FLAT), a truncated and catalytically inactive variant of FAAH that binds AEA and facilitates its translocation into cells (Beltramo *et al.*, 1997; Fu *et al.*, 2011; Piomelli *et al.*, 1999). However, a recent study was unable to detect FLAT in mouse brain, spinal cord or L3-L5 dorsal root ganglia and FLAT generated from FAAH via polymerase chain reaction was characterized and shown to have residual catalytic activity (Leung *et al.*, 2013). Pharmacological inhibition or genetic deletion of FAAH has also been shown to elevate AEA (Cravatt *et al.*, 2001b; Fegley *et al.*, 2005; Lichtman *et al.*, 2004).

1.5.3. Depolarization-induced suppression of inhibition or excitation

Endocannabinoids are involved in a form of CB₁-dependent short-term synaptic plasticity known as depolarization-induced suppression of inhibition (DSI) or excitation (DSE) in which they are synthesized and released on demand in response to Ca²⁺ and travel in a retrograde manner to the presynaptic terminal where they inhibit release of either gamma-Aminobutyric acid (GABA) (DSI) or glutamate (DSE) (Diana *et al.*, 2004). Complete abolition of this retrograde inhibitory effect by genetic deletion of DAGL α suggests that this effect is mediated by 2-AG (Tanimura *et al.*, 2010). Further evidence to support this is that MAGL inhibitors, but not FAAH inhibitors, augment DSI (Makara *et al.*, 2005) or DSE (Straiker *et al.*, 2005) and that MAGL knockout mice exhibit prolonged DSI (Pan *et al.*, 2011).

1.6. Interactions between the endogenous cannabinoid and opioid systems

Cannabinoids and opioids have a number of similarities including the effects they produce (e.g. antinociception, sedation and reward) as well as similar distribution of their respective receptors and the types of receptors themselves (Vigano *et al.*, 2005). CB₁ and mu opioid receptors are both G-protein coupled receptors that couple with G_{i/o} proteins and thus have similar signal transduction pathways. Because of this similarity between these receptors, their interaction could involve downstream signaling events, including mutual inhibition of adenylyl cyclase (Childers *et al.*, 1992) via activation of G_{i/o} proteins (Robledo *et al.*, 2008), blockade of voltage-dependent Ca²⁺ channels or activation of GIRK channels. Since both cannabinoids and opioids can be localized pre-synaptically, they could also interact through mutual inhibition of neurotransmitter release such as norepinephrine, GABA, dopamine, and glutamate. Additionally, CB₁ and mu opioid receptors can interact through the formation of heterodimers as determined by fluorescence resonance energy transfer (Hojo *et al.*, 2008).

CB₁ and mu opioid receptors are both found in many areas consistent with the observed interactions of these two systems in processes related to reward, withdrawal, and antinociception. CB₁ and mu opioid receptors have been colocalized in lamina II interneurons of the dorsal horn of the spinal cord which may account for some of the interactions between cannabinoids and opioids in nociception (Salio *et al.*, 2001). In rat caudate putamen, CB₁ and mu opioid receptors are co-localized in somata and dendrites of spiny neurons (Rodríguez *et al.*, 2001). In the nucleus accumbens (NAc), of immunolabeled presynaptic CB₁ receptors localized to terminals, approximately 3% were coexpressed with mu opioid receptors. Of the presynaptic CB₁ receptors in junctions at dendrites and dendritic spines, 19% (NAc shell) and 13% (NAc core) were localized with targets containing mu opioid receptors. Presynaptically located mu opioid receptors, 20% (NAc shell) and 10% (NAc core) were at terminals synapsing onto CB₁ immunoreactive dendrites. In dendritic shafts, 21% (NAc shell) and 13% (NAc core) of those containing CB₁ receptors co-localized with mu opioid receptors (Pickel *et al.*, 2004). CB₁ and mu opioid receptors are also co-localized in the PAG with approximately 32% of stained cells exhibiting immunoreactivity for both receptors (Wilson-Poe *et al.*, 2012). In the locus coeruleus, electron microscopy of sections stained using immunogold and peroxidase for both CB₁ and mu opioid receptors (i.e. half immunogold-CB₁/peroxidase mu opioid receptor; half immunogold mu opioid receptor/peroxidase CB₁) revealed CB₁ and mu opioid colocalization in postsynaptic terminals as well as localization of CB₁ receptors presynaptically to postsynaptic mu opioid receptors (Scavone *et al.*, 2010). Thus, CB₁ and mu opioid receptors are localized to brain regions important for somatic (LC, PAG) as well as negative motivational aspects of opioid withdrawal (NAc).

In terms of translational implications, there is evidence to support interactions between endogenous opioid and endocannabinoid systems in multiple physiological processes. For example, the increased duration of time spent in the light side of the light/dark box by delta-9-tetrahydrocannabinol (THC; 0.3 mg/kg, i.p.), an effect interpreted as “anxiolytic”, was antagonized by the mu opioid receptor antagonist beta-funaltrexamine (5 mg/kg i.p.) (Berrendero *et al.*, 2002). Zarrindast et al. demonstrated that naloxone (0.1 mg/kg), which alone had no effect, reduced the increased time spent in the open-arms of the EPM by the CB₁ agonist arachidonylpropylamide (1.25 and 5 ng/infusion into amygdala) (Zarrindast *et al.*, 2008). In terms of interactions between cannabinoids and opioids in reward, pretreatment with the mu opioid antagonist naloxone (2 mg/kg i.p.) blocks conditioned place preference (CPP) produced by the CB₁ agonist CP55,940 (20 µg/kg i.p.) (Bairda *et al.*, 2001). Furthermore, naloxone (2 mg/kg), a dose that did not elicit CPP or CPA alone, blocked CPP to THC (0.75 mg/kg) (Bairda *et al.*, 2004). Likewise, CPP to morphine has a CB₁ component (Chaperon *et al.*, 1998; Martin *et al.*, 2000; Mas-Nieto *et al.*, 2001; Singh *et al.*, 2004). Chaperon et al. demonstrated that morphine (4 mg/kg) CPP was attenuated by pretreatment of the CB₁ antagonist rimonabant (Chaperon *et al.*, 1998) and Martin et al. demonstrated that morphine CPP (5 mg/kg s.c.) was absent in CB₁ knockout mice (Martin *et al.*, 2000). Mas-Nieto et al. also demonstrated that rimonabant (5 mg/kg) reduced morphine CPP (3 mg/kg) in mice while rimonabant did not affect place conditioning on its own (Mas-Nieto *et al.*, 2001). Singh et al. demonstrated in rats that rimonabant (0.1 mg/kg i.p.) blocked the acquisition of morphine CPP (4 mg/kg s.c.) in rats (Singh *et al.*, 2004). CB₁ antagonists attenuate morphine and heroin self-administration (Caille *et al.*, 2003; Navarro *et al.*, 2001) and prevent its reinstatement (De Vries *et al.*, 2003; Fattore *et al.*, 2003). In rats trained to self-administer heroin (0.02 mg/infusion i.v.), the CB₁ antagonist/inverse

agonist rimonabant (0.3 – 3 mg/kg i.p.) pretreatment reduced the number of infusions during a 3 h session under a fixed-ratio 1 schedule of reinforcement and lowered breakpoints for heroin (0.003 mg/kg/infusion i.v.) reinforcement (Caille *et al.*, 2003). In another self-administration study, rimonabant (0.25 mg/kg i.p.) reduced the number of morphine (0.002 mg/kg/infusion i.v.) infusions during a 4 h session in a mouse self-administration paradigm (Navarro *et al.*, 2001). Additionally, morphine intravenous self-administration (0.001 – 0.004 mg/kg/infusion i.v.) was reduced in CB₁ (-/-) mice as compared to (+/+) controls (Ledent *et al.*, 1999) suggesting this system is important for the rewarding effects of opioids. Together, these data suggest a strong relationship in the rewarding and reinforcing aspects of cannabinoids and opioids.

1.7. Cannabinoids and Opioid Withdrawal

Anecdotal evidence for the potential of cannabinoids to be used in treatment of opioid withdrawal extends to the 19th century when a cannabis tincture was reported as effective at reducing opium withdrawal in a case study. A man who had been using approximately two ounces of laudanum (10% powdered opium) daily was suffering from “insomnia, anorexia, disordered bowels and conscious delusions” and upon being placed on a cannabis tincture the physician found that it had restored his appetite and improved his sleep (Birch, 1889). Since then, experimental evidence for the potential of cannabinoids to attenuate opioid withdrawal has been demonstrated in laboratory animals undergoing morphine withdrawal (see Table 1). It was demonstrated in the 1970s that THC reduces these somatic withdrawal signs in rats and mice (Bhargava, 1976; Hine *et al.*, 1975). Specifically, THC (2.5 – 10 mg/kg) administered i.p. 30 min prior to naloxone-precipitated morphine withdrawal in morphine-pelleted mice significantly reduced the number of withdrawal jumps (Bhargava, 1976). In rats implanted with a 75 mg

subcutaneous morphine pellet, THC (5 mg/kg i.p.) reduced naloxone-precipitated (4 mg/kg i.p.; 72 h following pellet implantation) withdrawal induced wet shakes and diarrhea (Hine *et al.*, 1975). Endocannabinoids have also been shown to possess similar potential. AEA (5 mg/kg) administered intravenously decreased jumps and body weight loss following naloxone (1 mg/kg) challenge in morphine (75 mg) pelleted mice 72 h after implantation (Vela *et al.*, 1995). As well, 2-AG (10 μ g i.c.v.) reduced naloxone (3.2 mg/kg i.p.) precipitated withdrawal signs in mice following 5 days of morphine (8 – 45 mg/kg s.c. twice daily) injections (Yamaguchi *et al.*, 2001) though CB₁ receptor mediation was not tested in either the Vela *et al.* nor the Yamaguchi *et al.* studies. Furthermore, rapid hydrolysis of endocannabinoids precludes their potential therapeutic use.

Inhibition of endocannabinoid catabolic enzymes reduces somatic signs of opioid withdrawal via a CB₁ receptor mechanism of action, which supports the notion of endocannabinoid enzyme inhibition as a strategy to treat opioid withdrawal (Ramesh *et al.*, 2011). Mice implanted with morphine (75 mg) pellets were administered either the MAGL inhibitor JZL184 or the FAAH inhibitor PF-3845 and then 2 h later given a naloxone (1 mg/kg s.c.) challenge immediately prior to a 30 min observation period during which withdrawal signs were scored. JZL184 (4-40 mg/kg i.p.) significantly reduced the number of jumps while JZL184 (16-40 mg/kg i.p.) reduced the number of paw flutters, amount of weight loss, and percentage of mice that presented with diarrhea. JZL184's (40 mg/kg i.p.) effects were all blocked by pretreatment with the CB₁ receptor antagonist rimonabant (3 mg/kg i.p.), but not the CB₂ antagonist SR144528 (3 mg/kg i.p.). The FAAH inhibitor PF-3845 (10 mg/kg i.p.) reduced jumping and paw flutters, an effect that was blocked with rimonabant pretreatment, however there was no observed effect on weight loss or the presence of diarrhea. These data suggest that

MAGL inhibition reduced all somatic withdrawal signs while inhibition of FAAH reduced only a subset, i.e. jumping and paw flutters. Interestingly, both JZL184 and PF-3845 were shown to reduce the increased contractility of morphine treated ilea following challenge with naloxone (30 μ M), however while PF-3845's effects were blocked by the CB₁ antagonist rimonabant, JZL184's effects were not. Nonetheless, all of the *in vivo* effects of JZL184 and PF-3845 were blocked by rimonabant suggesting CB₁ receptors are necessary for these effects. Aside from rodent models, a brief report in rhesus monkeys indicated that THC (0.1 – 3 mg/kg s.c.) did not affect morphine withdrawal signs (Woods *et al.*, 1982) but another study in dogs reported that THC (2 mg/kg) did reduce morphine withdrawal signs (125 mg/day) (Gilbert, 1981).

In humans, there have been some reports regarding the effects of cannabis use during treatment for opioid dependence but the results are mixed. A study of opioid-dependent patients found that concurrent marijuana use did not affect retention in the methadone treatment program (65% vs. 60%) or the number of continuous weeks of opiate abstinence (8.4 ± 6.5 vs. 8.5 ± 7.2) (Budney *et al.*, 1998). A retrospective analysis of three clinical trials with heroin-dependent participants receiving methadone maintenance treatment found no association of cannabis use with patient retention or heroin use during or after the methadone treatment period (Epstein *et al.*, 2003). Similar findings reported that cannabis use was not associated with retention rate nor did it affect any of the psychological measures assessed such as anxiety or mood disorders (Weizman *et al.*, 2004). Chronic marijuana also has been reported to have no effect on heroin use prior to treatment and during treatment did not affect the increased HPA axis activity as measured by increased plasma ACTH. Additionally, during treatment there was no effect of concurrent marijuana use on withdrawal signs (gooseflesh, mydriasis, vomiting, tremor, profuse sweating, restlessness, lacrimation, and nasal congestion, uncontrollable yawning, feeling to

change in temperature, stomach pain, and muscle aching) and no effect on heroin craving (Nava *et al.*, 2007). In contrast, some studies have reported that cannabis use may have beneficial effects in opioid-dependent patients undergoing treatment. Intermittent marijuana use was associated with a decrease in percent of heroin-positive urine samples and an increase in pill compliance during naltrexone treatment (Church *et al.*, 2001). An association between marijuana use, but not alcohol use, was associated with improved retention in heroin abusers during a 9 month treatment program and marijuana was also reportedly used as a heroin substitute (Ellner, 1977). Another study similarly reported that intermittent marijuana use was associated with improved retention in a naltrexone-treatment program and also associated with greater adherence to pill-taking (Raby *et al.*, 2009). Recently it was reported that when methadone-maintenance therapy patients were grouped by either low- or moderate-withdrawal severity, cannabis users were significantly more likely to fall into the low-withdrawal severity group suggesting that cannabis use may have reduced withdrawal in these patients, although these were preliminary pilot data (Scavone *et al.*, 2013b).

While the rodent literature suggests that cannabinoids may be effective at reducing opioid withdrawal, the data available in humans is less convincing. The results from human studies are based upon reported concurrent cannabis use during treatment rather than controlled studies looking at cannabinoids directly for their ability to attenuate opioid withdrawal and while some studies report no effect of cannabis use others do. It will be important to continue studying the effects of cannabinoids on opioid withdrawal to determine whether they may offer the potential to reduce withdrawal in humans.

Table 1. Effects of cannabinoids on opioid withdrawal signs in rodents

Species	Dependence Induction	Withdrawal type	Cannabinoid agent	Dependent measures/outcome	Reference
Rats	75 mg morphine pellet (72 h), s.c.	Naloxone, 4 mg/kg, i.p.	THC, 5-10 mg/kg, i.p.	Reduced wet shakes and diarrhea	(Hine <i>et al.</i> , 1975)
Mice	75 mg morphine pellet (72 h), s.c.	Naloxone, i.p. (specific doses not reported)	THC, 2.5-10 mg/kg, i.p.	Reduced jumping, defecation, and rearing; shifted naloxone ED50	(Bhargava, 1976)
Guinea pigs	Slow release suspension of morphine, 300 mg/kg (48 h)	Naloxone, 0.1 mg/kg, s.c.	THC, 2 mg/kg, p.o.	Reduced total withdrawal score (jumping, body shakes, digging, yawning, ptosis, diarrhea among)	(Frederickson <i>et al.</i> , 1976)
Mice	75 mg morphine pellet (72 h), s.c.	Naloxone, 1 mg/kg, i.p.	THC, 10 mg/kg, i.v. or AEA, 1-5 mg/kg i.v.	AEA (5 mg/kg) and THC (10 mg/kg) reduced no. jumps and weight loss	(Vela <i>et al.</i> , 1995)
Mice	Morphine BID, 8-45 mg/kg (5 days), s.c.	Naloxone, 5 mg/kg, i.p.	AEA, 0.1-5 mg/kg, i.v.	AEA (1-5 mg/kg) reduced no. jumps and weight loss	(Vela <i>et al.</i> , 1995)
Mice	Morphine BID, 8-45 mg/kg (5 days), s.c.	Naloxone, 3.2 mg/kg, i.p.	2-AG, 1-10µg, i.c.v.	2-AG (10 µg) reduced no. jumps, paw tremors	(Yamaguchi <i>et al.</i> , 2001)
Mice	Morphine BID, 20-100 mg/kg (5 days), s.c.	Spontaneous withdrawal; naloxone, 1 mg/kg, i.p.	AM404 (AEA transport inhibitor), 2-10 mg/kg, i.p.	AM404 (2 and 10 mg/kg) reduced no. jumps in spontaneous withdrawal; no effect	(Del Arco <i>et al.</i> , 2002)
Mice	Morphine BID, 200-300 mg/kg (7 days), p.o.	Naloxone, 1 mg/kg, s.c.	THC, 1-50 mg/kg, p.o.	THC (1, 20, and 50 mg/kg) reduced the no. mice exhibiting platform jumping	(Cichewicz <i>et al.</i> , 2003)
Mice	75 mg morphine pellet (72 h), s.c.	Naloxone, 1 mg/kg, s.c.	THC, 0.1-10 mg/kg, p.o.	THC (0.3-10 mg/kg) reduced paw tremors and head shakes	(Lichtman <i>et al.</i> , 2001a)
Mice	75 mg morphine pellet (72 h), s.c.	Naloxone, 1 mg/kg, s.c.	JZL184 (MAGL inhibitor), 4-40 mg/kg; PF-3845 (FAAH inhibitor), 10 mg/kg; i.p.	JZL184 (4-40 mg/kg) reduced jumps, (16-40 mg/kg) reduced paw flutters, weight loss and diarrhea; PF-3845 (10 mg/kg) reduced jumps and paw flutters*	(Ramesh <i>et al.</i> , 2011)

*CB₁ mediation demonstrated

1.8. Rationale and Hypothesis

Elevation of endocannabinoids may reduce opioid withdrawal via activation of CB₁ receptors on neurons through interactions with mutual second messenger systems shared by both cannabinoid and opioid receptors or by modulating other systems such as the increased efflux of norepinephrine during opioid withdrawal. As previously described (see section 1.3), naloxone-precipitated opioid withdrawal leads to a superactivation of adenylyl cyclase which results in an overshoot of cAMP production (Avidor-Reiss *et al.*, 1995). Since CB₁ receptor activation of G_{i/o} proteins leads to inhibition of AC (Matsuda *et al.*, 1990; Vogel *et al.*, 1993) and these receptors are colocalized with mu opioid receptors in LC, a region important for opioid withdrawal (Scavone *et al.*, 2010), it is possible CB₁ receptor activation could resume inhibition of AC following opioid withdrawal which would thereby dampen withdrawal effects.

Given the colocalization of CB₁ and mu opioid receptors in the LC (Scavone *et al.*, 2010), PAG (Wilson-Poe *et al.*, 2012) and NAc (Pickel *et al.*, 2004), cannabinoid receptors are advantageously positioned to compensate for the increased signaling and subsequent hyperactivity in neurons that are key to the expression of both somatic and motivational aspects of opioid withdrawal. In LC neurons, CB₁ receptors located presynaptically on glutamatergic afferents could inhibit glutamate release onto noradrenergic neurons, resulting in reduction in activity and release of norepinephrine (Scavone *et al.*, 2010). Additionally, CB₁ receptors located postsynaptically with mu opioid receptors (Scavone *et al.*, 2010) could inhibit AC and reduce the cAMP overshoot that occurs during opioid withdrawal. Clonidine has been shown to reduce both cannabinoid (Lichtman *et al.*, 2001b) and opioid withdrawal (Dehpour *et al.*, 2001; Gold *et al.*, 1978) signs suggesting that both systems are involved in sympathetic outflow and thus effects of cannabinoids on opioid withdrawal may involve direct actions on the noradrenergic system. See

Figure 1 for a diagram of how CB₁ receptors may modulate hyperactivity of LC neurons and how endocannabinoid catabolic inhibitors could indirectly activate CB₁ receptors through elevation of endocannabinoids.

As discussed in the previous section, cannabinoids such as THC as well as inhibitors of endocannabinoid catabolism reduce somatic withdrawal signs in rodents. Previous work (Ramesh *et al.*, 2011) examining inhibitors of MAGL and FAAH, JZL184 and PF-3845 respectively, demonstrated that indirect activation of CB₁ receptors through elevation of endocannabinoids reduced somatic withdrawal signs in mice. JZL184 attenuated somatic withdrawal signs (Ramesh *et al.*, 2011) but also produced cannabimimetic effects (hypomotility, hypothermia) (Long *et al.*, 2009a), dependence liability and functional CB₁ receptor tolerance after repeated dosing (Schlosburg *et al.*, 2010a). While full FAAH inhibition did not produce cannabimimetic effects and dependence liability, it only reduced a subset of withdrawal signs in mice (jumps and paw flutters). There is some evidence to support the possibility of differential roles of elevating either 2-AG or AEA on opioid withdrawal. While MAGL is localized pre-synaptically, FAAH is localized post-synaptically (Gulyas *et al.*, 2004) thus inhibition of MAGL may preferentially elevate levels of 2-AG presynaptically and inhibition of FAAH may preferentially elevate levels of AEA post-synaptically. As discussed previously, CB₁ receptors have been localized in LC neurons both presynaptically on glutamatergic afferents and post-synaptically alongside mu opioid receptors on LC neurons as well as in the cytoplasm of somatodendritic profiles (Scavone *et al.*, 2010). It is possible then that 2-AG and AEA are producing their effects on noradrenergic circuitry via different mechanisms, i.e. 2-AG inhibiting glutamate release onto LC neurons via pre-synaptic CB₁ receptors and AEA inhibiting activation of LC neurons via post-synaptic CB₁ receptors. Considering that full FAAH inhibition reduces a

subset of opioid withdrawal signs and does not produce cannabimimetic effects and that partial MAGL inhibition also reduces somatic withdrawal signs without producing cannabimimetic effects, it's possible that combined full inhibition of FAAH with partial MAGL inhibition may reduce somatic signs (jumps, paw flutters, diarrhea) of morphine withdrawal without the adverse consequences of maximal MAGL inhibition.

While reductions in somatic withdrawal signs by inhibition of endocannabinoid catabolism signs suggest that these drugs may be attenuating opioid withdrawal, it is important to examine other aspects of opioid withdrawal. As discussed in section 1.3, motivational components and somatic signs of withdrawal are dissociable by brain region and naloxone dose (Frenois *et al.*, 2002; Hand *et al.*, 1988; Stinus *et al.*, 1990) suggesting that distinct regions mediate the expression of somatic withdrawal signs and aversive aspects of withdrawal. While endocannabinoid catabolic inhibitors may reduce somatic signs of morphine withdrawal, it is not known whether they will also reduce the aversive aspects of withdrawal. Therefore, it is important to establish other behavioral assays sensitive to morphine withdrawal and determine if endocannabinoid catabolic inhibitors are able to reduce other signs of morphine withdrawal (e.g. CPA).

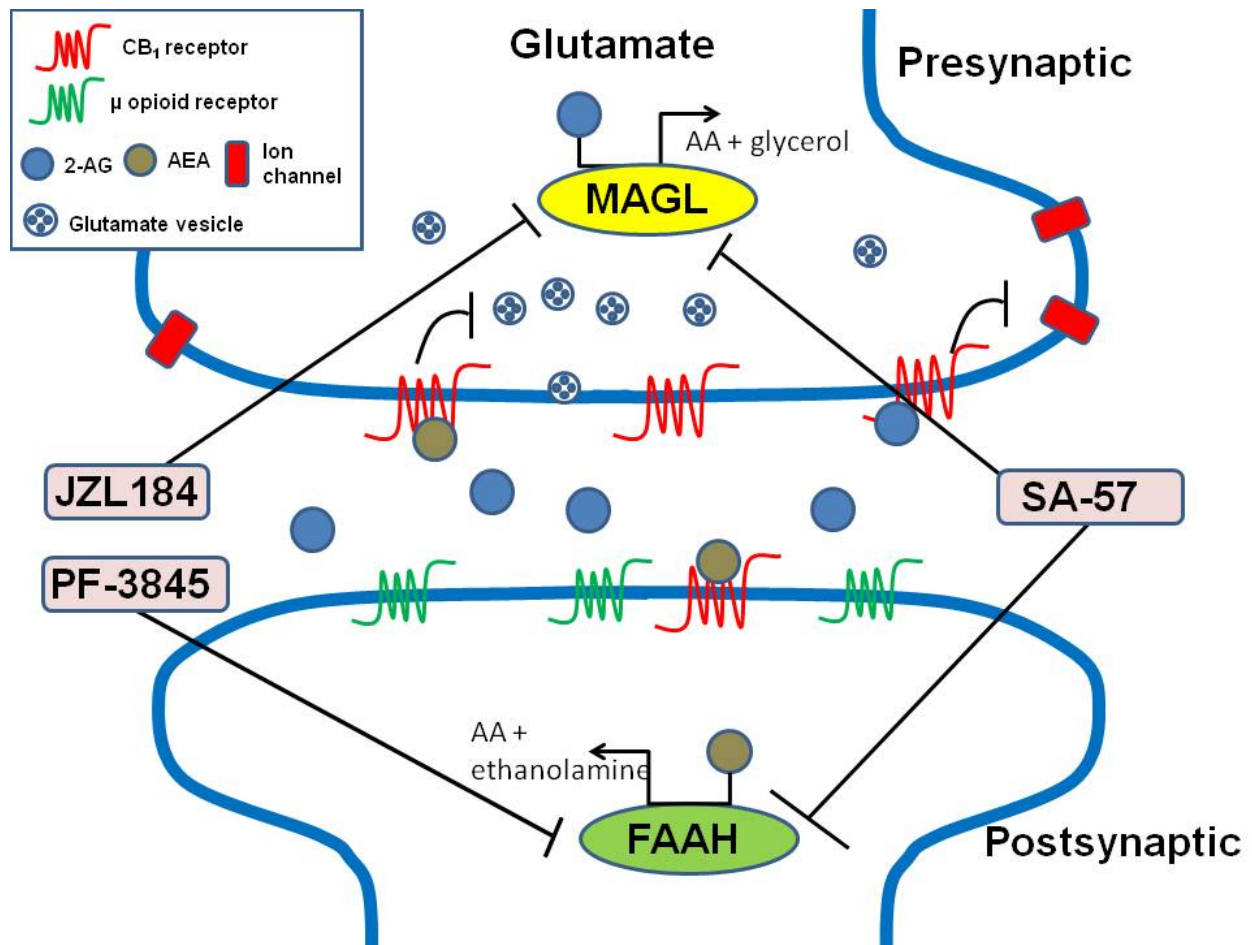


Figure 1. Localization of CB1 and mu opioid receptors, endocannabinoids and their catabolic enzymes, and targets of endocannabinoid catabolic inhibitors in LC neurons. CB₁ receptors have been colocalized with mu opioid receptors on the same post-synaptic terminals as well as located presynaptically on glutamatergic afferents of postsynaptic mu opioid receptors in LC neurons (Scavone *et al.*, 2010). Cannabinoids are hypothesized to reduce hyperactivity of noradrenergic neurons during opioid withdrawal in LC by either inhibiting glutamate release onto noradrenergic neurons or compensatory inhibition of cAMP overshoot following opioid withdrawal in noradrenergic neurons. Catabolic inhibitors elevate levels of 2-AG or AEA by inhibiting either MAGL or FAAH respectively, thereby indirectly activating CB₁ receptors. AA = Arachidonic acid; 2-AG = 2-arachidonoylglycerol; AEA = arachidonylethanolamide;

MAGL = monoacylglycerol lipase, primary catabolic enzyme for 2-AG; FAAH = fatty acid amide hydrolase, primary catabolic enzyme for AEA; JZL184 = selective MAGL inhibitor; PF-3845 = selective FAAH inhibitor; SA-57 = dual FAAH/MAGL inhibitor, more selective for FAAH than MAGL. [Adapted from (Ahn *et al.*, 2008; Scavone *et al.*, 2013a; Scavone *et al.*, 2010)]

1.8.1. Overall Hypothesis

Inhibition of endocannabinoid catabolic enzymes FAAH and/or MAGL will reduce somatic and negative motivational signs of opioid withdrawal in mice via CB₁ mechanism of action.

1.8.2. Objectives

Our aims for these studies were to 1) assess the efficacy of dual inhibition of FAAH and MAGL on somatic signs of opioid withdrawal and 2) determine whether these treatments would produce cannabimimetic effects (hypomotility, catalepsy, antinociception and hypothermia) ; 3) develop other behavioral assays of opioid withdrawal; and 4) determine if endocannabinoid catabolic inhibitors would reduce the acquisition of opioid withdrawal induced CPA as a measure of the negative motivational consequences of opioid withdrawal.

1.8.3. Selection of endocannabinoid catabolic inhibitors

1.8.3.1. MAGL inhibitors

There are several MAGL inhibitors currently available but among them only a few have sufficient selectivity and potency *in vivo* for behavioral studies. The MAGL inhibitor URB602 lacks potency (IC₅₀ = 25 μM) and selectivity for MAGL as it was shown to also inhibit AEA hydrolysis (IC₅₀ = 17μM) (Vandevorde *et al.*, 2007) whereas the newer inhibitor, JZL184 selectively inhibits MAGL (IC₅₀ = 8 nM), though it can also inhibit FAAH (IC₅₀ = 4,000 nM). However, JZL184 does not alter AEA acutely and only after repeated administration of JZL184 are elevations of AEA apparent (Long *et al.*, 2009a; Long *et al.*, 2009b). KML29, a new MAGL inhibitor with greater selectivity for MAGL (IC₅₀ = 15 nM) over FAAH (IC₅₀ > 50,000 nM) elevates 2-AG without elevating AEA upon repeated treatment, an effect which has been

observed with JZL184 (Chang *et al.*, 2012). KML29 was reported to produce anti-allodynic and anti-edematous effects in mice demonstrating that this compound is effective *in vivo* (Ignatowska-Jankowska *et al.*, 2013). MJN110 is another new MAGL inhibitor with greater selectivity for MAGL ($IC_{50} = 2.1$ nM) over FAAH ($IC_{50} > 50,000$ nM) and approximately 100 fold more selective for MAGL than ABHD6 (Niphakis *et al.*, 2013). Since JZL184 acutely elevates 2-AG and not AEA and has previously been used to assess the effects of MAGL inhibition of morphine withdrawal in mice (Ramesh *et al.*, 2011) we selected this compound for these studies.

1.8.3.2. FAAH inhibitors

The irreversible FAAH inhibitor AM374 has marked potency over the non-selective serine protease inhibitor phenylmethylsulfonyl fluoride at inhibiting AEA hydrolysis, but AM374 also binds to CB₁ receptors ($IC_{50} = 520$ nM) (Deutsch *et al.*, 1997). Derivatives of the sulfonyl fluoride inhibitors have recently been characterized which are approximately 200 fold more selective for rat FAAH over human MAGL, though these compounds also bind to CB₁ receptors in the 100 nM range (Alapafuja *et al.*, 2012). URB597 has been well characterized though it inhibits other serine hydrolases and carboxylesterases (Lichtman *et al.*, 2004; Zhang *et al.*, 2007). OL-195, a reversible alpha-ketoheterocycle FAAH inhibitor, has greater selectivity for FAAH compared to URB597, (Boger *et al.*, 2005). PF-3845 selectively inhibits FAAH (Ahn *et al.*, 2009) and is more selective than URB597 (Lichtman *et al.*, 2004), and thus is one of the most potent and selective FAAH inhibitors available. PF-3845 has also previously been used to investigate the effects of FAAH inhibition on opioid withdrawal (Ramesh *et al.*, 2011) and is therefore an ideal FAAH inhibitor for these studies.

1.8.3.3. Dual FAAH/MAGL inhibitors

In addition to the selective FAAH and MAGL inhibitors there are dual inhibitors that can elevate both AEA and 2-AG. JZL195 was shown to equipotently ($IC_{50} = 2-4$ nM) inhibit FAAH and MAGL and at doses that fully inhibited both enzymes it produced pronounced cannabimimetic effects (hypomotility, catalepsy, antinociception and hypothermia), THC-like discriminative stimulus effects (Long *et al.*, 2009c), and spatial learning deficits in the Morris water maze as measured by path length and escape latency (Wise *et al.*, 2012). AM6701, a non-selective dual FAAH/MAGL inhibitor, provided greater neuroprotective effects than AM6702 which has 44-fold more selectivity for FAAH than MAGL. Excitotoxic damage by kainic acid in rat hippocampal slice cultures was reduced by AM6701 to a greater degree than AM6702. Furthermore, AM6701 reduced seizure activity in rats following kainic acid more effectively than AM6702 and also improved balance and coordination on the rotarod test. The authors suggested from these data that dual inhibition of FAAH and MAGL has enhanced neuroprotective effects (Naidoo *et al.*, 2012). Another new dual inhibitor, SA-57, is more selective for FAAH ($IC_{50} = 10$ nM) than MAGL ($IC_{50} = 410$ nM) allowing for the partial inhibition of MAGL while fully inhibiting FAAH (Niphakis *et al.*, 2011).

The mixed FAAH/MAGL inhibitor SA-57 was employed to investigate the effects of full FAAH inhibition and partial MAGL inhibition as it has significantly greater potency at inhibiting FAAH over MAGL, allowing for full inhibition of FAAH while only partially inhibiting MAGL (Niphakis *et al.*, 2011).

1.8.4. Chapter 2: Effects of dual FAAH/MAGL inhibition on somatic signs of opioid withdrawal

In Chapter 2 we investigated the effects of dual inhibition of FAAH and MAGL on somatic signs of opioid withdrawal in mice. While inhibition of either FAAH or MAGL has been

shown to reduce somatic signs of opioid withdrawal in mice, FAAH inhibition only attenuated a subset of withdrawal signs (Ramesh *et al.*, 2011). While MAGL inhibition attenuated all observed signs of opioid withdrawal, it has also been shown to produce a subset of cannabimimetic effects (hypomotility, hypothermia, and hyperreflexia) (Long *et al.*, 2009a) and produce dependence upon repeated treatment (Schlosburg *et al.*, 2010a). We tested the hypothesis that full inhibition of FAAH and partial inhibition of MAGL would attenuate morphine withdrawal at doses that do not produce significant cannabimimetic effects.

1.8.5. Chapter 3: Development of behavioral assays of opioid withdrawal

In Chapter 3, we described the development of additional assays for opioid withdrawal in mice. In order to investigate the ability of endocannabinoid catabolic inhibitors to reduce other behaviors associated with opioid withdrawal, we examined the effects of opioid withdrawal in marble burying, novelty-induced hypophagia, the light/dark box, a novel procedure developed to assess “escape behavior” and the CPA procedure. The light/dark box, novelty-induced hypophagia, and marble burying assays have been used to assess the effects of anxiolytic drugs and have been used to infer treatments as “anxiolytic” or “anxiogenic” while CPA has been used to infer aversive aspects of withdrawal.

1.8.6. Chapter 4: Effects of endocannabinoid catabolic inhibitors on negative motivational aspects of opioid withdrawal

In Chapter 4, we investigated the effects of endocannabinoid catabolic inhibitors on the acquisition of CPA to opioid withdrawal in mice. While endocannabinoid catabolic inhibitors have been shown to reduce somatic signs of morphine withdrawal via CB₁ mechanism of action in mice, this thesis represents the first study examining the effects of cannabinoids on acquisition

of opioid withdrawal induced CPA. We examined whether inhibition of endocannabinoid catabolism would block the acquisition of naloxone-precipitated morphine withdrawal CPA and reduce the percentage of mice that exhibited jumping behavior during the conditioning sessions.

Chapter 2: Effects of dual FAAH/MAGL inhibition on somatic signs of morphine withdrawal

2.1. Introduction: Maximal inhibition of MAGL reduces somatic withdrawal signs in mice but produces cannabimimetic effects

Cannabinoids have been known for almost four decades to reduce somatic signs of opioid withdrawal (Bhargava, 1976; Hine *et al.*, 1975). Recently it has been shown that a high dose of the MAGL inhibitor JZL184 reduces somatic measures of morphine withdrawal, including jumps, paw flutters, weight loss, and diarrhea (Ramesh *et al.*, 2011). However, a problem with JZL184 remains in that it also elicits cannabimimetic (i.e. THC-like) effects, including hypomotility, hypothermia and hyperreflexia (Long *et al.*, 2009a) and repeated administration of high dose JZL184 results in cannabinoid dependence and functional CB₁ receptor tolerance (Schlosburg *et al.*, 2010b). Although the antinociceptive and anxiolytic-like effects of JZL184 do not undergo tolerance following repeated administration of low doses (Busquets-Garcia *et al.*, 2011; Kinsey *et al.*, 2013; Sciolino *et al.*, 2011), these same low doses of this compound lack effectiveness in reducing opiate withdrawal responses (Ramesh *et al.*, 2011). Whereas high doses of FAAH inhibitors do not elicit cannabimimetic side effects and sustained pharmacological inhibition of FAAH does not lead to CB₁ receptor functional tolerance or cannabinoid dependence (Falenski *et al.*, 2010; Schlosburg *et al.*, 2010b), inhibition of this enzyme prevents only a subset of withdrawal responses in morphine-dependent mice (Ramesh *et al.*, 2011). Therefore, the primary objective of these experiments was to determine whether dual inhibition of FAAH and MAGL would reduce withdrawal signs in morphine-dependent mice, with minimal cannabimimetic side effects.

2.1.1. Rationale and hypothesis

Inhibition of endocannabinoid catabolism presents a novel treatment strategy for opioid withdrawal, but high doses of JZL184 which maximally attenuate somatic withdrawal signs also produce cannabimimetic effects (Long *et al.*, 2009a), dependence liability and functional CB₁ receptor tolerance after repeated dosing (Schlosburg *et al.*, 2010b). Full FAAH inhibition attenuates a subset of withdrawal signs but is without the cannabimimetic effects and dependence liability that are observed with high dose JZL184. Thus, combined full inhibition of FAAH with partial MAGL inhibition may reduce somatic withdrawal signs without the adverse consequences of maximal MAGL inhibition.

Therefore, we hypothesized that full FAAH inhibition combined with partial MAGL inhibition would reduce the somatic signs of spontaneous and precipitated morphine withdrawal and that this treatment would not produce cannabimimetic effects. We tested the efficacy of a novel combination of low dose of the MAGL inhibitor JZL184 and high dose of the FAAH inhibitor PF-3845 as well as the novel dual inhibitor SA-57, which is more potent in inhibiting FAAH ($IC_{50} < 10$ nM) than MAGL ($IC_{50} = 410$ nM) (Niphakis *et al.*, 2012), in reducing precipitated and spontaneous morphine withdrawal signs. In addition, endocannabinoid levels were quantified in whole brain following SA-57 treatment. In these studies, we employed a spontaneous withdrawal model because it possesses greater face validity than naloxone-precipitated withdrawal and because somatic signs of spontaneous withdrawal persist for an extended duration of time (8 h), allowing for time course assessment. Specific withdrawal signs examined were jumping (platform jumping in the case of spontaneous withdrawal), paw flutters,

head shakes, diarrhea, and weight loss. To assess cannabimimetic effects, we tested SA-57 in the cannabinoid tetrad which is a battery of tests that measure locomotor activity, catalepsy, warm-water tail withdrawal, and rectal temperature.

2.2. Methods

2.2.1. Subjects

Male ICR mice (Harlan laboratories; Indianapolis, IN) weighing between 26 and 30 g served as subjects. The mice were housed 4-5 per cage in a temperature controlled (20-22°C) environment, in a facility approved by the American Association for the Accreditation of Laboratory Animal Care. The mice were kept on a 12 h light/dark cycle, with all experiments performed during the light cycle. Food and water were available *ad libitum* except while mice were observed during the withdrawal procedure. The study was performed with the approval of the Institutional Animal Care and Use Committee at Virginia Commonwealth University in accordance with the Guide for the Care and Use of Laboratory Animals.

2.2.2. Drugs

Morphine pellets (75 mg), placebo pellets, morphine sulfate, THC, and rimonabant were obtained from the National Institute on Drug Abuse (Bethesda, MD). Naloxone hydrochloride was purchased from Sigma Aldrich (St. Louis, MO). JZL184 and PF-3845 were synthesized as described previously and supplied by Organix Inc. (Woburn, MA) (Ahn *et al.*, 2009; Long *et al.*, 2009a). SA-57 was synthesized as previously described at The Scripps Research Institute (Niphakis *et al.*, 2012). THC, PF-3845, JZL184, SA-57 and rimonabant were dissolved in ethanol, followed by addition of Emulphor-620 (Rhone-Poulenc, Princeton, NJ), and diluted with 0.9% saline to form a vehicle mixture of ethanol, emulphor, and saline in a ratio of 1:1:18.

Naloxone was dissolved in 0.9% saline and doses calculated by weight of the salt. All injections were administered in volume of 0.01 ml per 1 g body weight. THC and naloxone were administered via subcutaneous (s.c.) injection, whereas PF-3845, JZL184, SA-57 and rimonabant were given via intraperitoneal (i.p.) injection. In spontaneous withdrawal studies, all injections were given 1 h after pellet removal. For experiments quantifying brain endocannabinoid levels, SA-57 was administered 2 h before decapitation.

2.2.3. Scoring of somatic signs of morphine withdrawal

For the spontaneous withdrawal experiments, two trained observers (Thomas Gamage and Divya Ramesh) blinded to the treatment condition live-scored two mice each simultaneously so that a total of 4 mice were scored per observation session. For the precipitated-withdrawal studies, one blinded observer (Thomas Gamage) scored the recorded videos. The primary behavioral signs that were scored were frequency of jumps (either off the platform or within the observation chamber) and front paw flutters (including single and double paw flutters and twitches, which are not commonly displayed by naïve mice). The occurrence of diarrhea during the testing period was noted if liquid feces were present or if fecal boli were soft enough to “smear” with gentle pressure. All behaviors were recorded as new incidences when separated by at least 1 s or interrupted by any other normal behavior. In addition, mice were weighed before and immediately after the 30 min test session to assess body weight loss.

2.2.4. Spontaneous withdrawal

Approximately 72 h after morphine pellet implantation, mice were weighed and assessed for baseline withdrawal behavior. The mice were then given light isoflurane anesthesia, the pellets were surgically extracted, and the mice were housed individually in cages that were

placed on heating pads for 2 h. At 1 h after pellet removal, groups received an injection of drug or vehicle. The animals were observed for spontaneous withdrawal signs for 15 min intervals at 2, 4, 6 and 8 h post-pellet removal. Spontaneous withdrawal signs were quantified using a procedure adapted from Way et al. (1969) that included the percentage of mice jumping off a circular platform (12 cm diameter x 70 cm height), the total number of paw flutter and head shake incidences, and body weights recorded at each time point. Paw flutters and head shakes were each pooled across all time points to represent the total number of incidences of the behaviors observed. The percentage of mice presenting with diarrhea across the 8 h test session was also recorded. The mice remained singly housed throughout the testing period, and food and water were available *ad libitum*, except during the 15 min observation periods.

2.2.5. Pellet implantation surgery

In order to induce opioid dependence, mice were implanted with morphine pellets as previously described. In brief, anesthesia was induced with 2.5% isoflurane, the fur was shaved, the skin was disinfected with a sterile betadine swab (Purdue products, Stamford, CT), and a 1 cm horizontal incision was made in the midscapular region, using sterile surgical scissors. A 75 mg morphine sulfate pellet was inserted subcutaneously, and the incision was closed with a sterile staple. The mice were given a minimum 1 h recovery period in heated home cages for 1 h after surgery and then returned to the vivarium until testing.

2.2.6. Naloxone precipitated withdrawal

Somatic withdrawal signs were scored, as previously described (Schlosburg *et al.*, 2009). In brief, mice were placed in white acrylic chambers (20x20 cm), with a clear acrylic front panel and a mirrored back panel for a 30 min acclimation period. The chambers were enclosed in

sound-attenuating cabinets that contained an indirect filtered LED light source and fans for air circulation and white noise. The mice were briefly removed from the chambers for naloxone administration and immediately returned to the chambers for a 30 min observation period. Behavior was recorded using a series of Fire-iTM digital cameras (Unibrain, San Ramon, CA), and the videos were saved using the ANY-mazeTM video tracking software (Stoelting Co., Wood Dale, IL). Chambers were changed between tests and cleaned at the end of testing with an ammonia based cleanser and left to dry for two days, to allow for odors to dissipate. The recorded videos were randomized and scored by a trained observer, who was blinded with respect to treatment condition. The primary behavioral signs of interest were frequency of jumps and front paw tremors (including single and double paw flutters and twitches, which are not commonly displayed by naïve mice). The occurrence of diarrhea during the testing period was noted. All behaviors were recorded as new incidences when separated by at least 1 s or interrupted by any other normal behavior. In addition, mice were weighed before and immediately after the 30 min test session to assess body weight loss.

2.2.7. Cannabinoid tetrad

For this study mice were injected with either vehicle or a dose of SA-57 i.p. (1.25, 2.5, 5, and 12.5 mg/kg) and tested 2 h later. Locomotor activity was recorded by ANY-maze Software (Stoelting, Wood Dale, IL) during a 5 min test in which mice were placed in a Plexiglas cage with black flooring located within a lit sound attenuating chamber equipped with a ventilation fan. Catalepsy was evaluated using the bar test, in which the front paws of each subject were placed on a rod (0.75 cm diameter) that was elevated 4.5 cm above the surface. Mice were timed if they remained motionless with their paws on the bar (with the exception of respiratory

movements), and the time motionless from 3 attempts to place on the bar were totaled with a cutoff of 60 s. Hyper-reflexive popping and jumping away from the bar was also scored. In the tail immersion test, each mouse was placed head first into a small bag fabricated from absorbent under pads (VWR Scientific Products; 4 cm diameter, 11 cm length) with the tail out of the bag. The experimenter gently held the mouse and immersed approximately 1 cm of the tip of the tail into a water bath maintained at 52.0°C. The latency for the animal to withdraw its tail from the water within a 10 s cutoff time was scored and calculated as percent of maximum possible effect (%MPE = [(test - control latency) / (100 - control)] x 100). Rectal temperature was determined by inserting a thermocouple probe 2.0 cm into the rectum and temperature was obtained from a telethermometer. Before any injections, baseline tail nociceptive latencies and rectal temperatures were assessed for all tests.

2.2.8. Quantification of endocannabinoids

Mice were treated with vehicle or a dose of SA-57 i.p. (1.25, 2.5, 5, and 12.5 mg/kg). Two hours after last injection, the mice were decapitated, and brains were harvested and split in half. Twenty-four h after the last injection, the mice were decapitated, and brains were snap frozen in liquid nitrogen and stored at -80°C until lipid extraction.

On the day of processing, tissues were weighed and homogenized with 1.4 ml chloroform/methanol (2:1 v/v containing 0.0348 mg PMSF/ml) after the addition of internal standards to each sample (2 pmol AEA-d8 and 1 nmol 2-AG-d8). Homogenates were then mixed with 0.3 ml of 0.73% w/v NaCl, vortexed, and then centrifuged for 10 min at 3220 g (4° C). The aqueous phase plus debris were collected and extracted two more times with 0.8 ml chloroform. The organic phases from the three extractions were pooled and the organic solvents were

evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 ml chloroform and mixed with 1 ml ice-cold acetone. The mixtures were then centrifuged for 5 min at 1811 g and 4° C to precipitate the proteins. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 ml methanol and placed in autosample vials for analysis.

LC/MS/MS was used to quantify AEA, 2-AG, arachidonic acid. The mobile phase consisted of (10:90) water/methanol with 0.1% ammonium acetate and 0.1% formic acid. The column used was a Discovery HS C18, 4.6×15 cm, 3 µm (Supelco, PA). The mass spectrometer was run in Electrospray Ionization, in positive mode. Ions were analyzed in multiple-reaction monitoring mode, and the following transitions were monitored: (348>62) and (348>91) for AEA; (356>62) for AEAd8; (379>287) and (279>269) for 2-AG; and (387>96) for 2-AG-d8. A calibration curve was constructed for each assay based on linear regression using the peak area ratios of the calibrators. The extracted standard curves ranged from 0.03 to 40 pmol for AEA and from 0.05 to 64 nmol for 2-AG.

2.2.9. Statistical Analysis

All data are reported as mean±SEM. In the precipitated withdrawal experiments, the numbers of jump, head shake and paw tremor incidences were tallied. In the spontaneous withdrawal experiments, the occurrence of jumps was scored as a binary event for the entire 15-min period at each time point. Diarrhea was also scored as a binary event for both spontaneous and precipitated withdrawal. Weight loss (g) was calculated by subtracting the body weight at the conclusion of each 15-min observation period from the pre-pellet removal weight. Data were analyzed using one-way or two-way between measures analysis of variance. Dunnett's test was

used to compare drug treatments with vehicle and Newman-Keuls post-hoc test was employed for comparisons between various treatments. The percentage of mice between groups presenting with diarrhea and percentage of mice that jumped off platforms were analyzed by the z-test of two proportions. Differences were considered statistically significant at $p < 0.05$.

2.3. Results

2.3.1. Withdrawal scoring between observers

Two observers scored the spontaneous withdrawal experiments simultaneously. Both observers scored pre-recorded withdrawal videos to determine similarities between scoring criteria and there were positive correlations between both observers for paw flutters [$r=0.94$, $n=54$, $p<0.0001$], head shakes [$r=0.90$, $n=54$, $p<0.0001$], and jumps [$r=0.99$, $n=54$, $p<0.0001$].

2.3.2. Dual inhibition by combination low dose JZL184 and high dose PF-3845 reduces naloxone-precipitated somatic withdrawal signs in mice

To evaluate whether combined inhibition of MAGL and FAAH reduces precipitated opioid withdrawal signs, mice were co-administered a low dose of JZL184 (4 mg/kg, i.p.) or vehicle and a high dose of PF-3845 (10 mg/kg, i.p.) or vehicle. The combination treatment blocked the occurrence of withdrawal signs including the frequencies of jumping [$F(3, 26) = 4.6$; $p < 0.01$; Figure 2A] and paw flutter incidents [$F(3,26) = 5.6$; $p < 0.01$; Figure 2B], as well as weight loss [$F(3,26) = 8.9$; $p < 0.001$; Figure 2C], and diarrhea [Figure 2D].

2.3.3. Dual inhibition by combination low dose JZL184 and high dose PF-3845 reduces spontaneous somatic withdrawal signs in mice

JZL184 (4 mg/kg) and PF-3845 (10 mg/kg) given in combination reduced the full spectrum of spontaneous withdrawal signs, while single administration of these drugs reduced only a subset of withdrawal signs. The combination of JZL184 and PF-3845, but neither drug alone, significantly reduced jumping at 4, 6, and 8 h after morphine pellet removal [Figure 3A]. The combination as well as each drug given alone significantly reduced the intensity of total head shakes [$F(3,26) = 4.4$; $p<0.01$; Figure 3B] and paw flutters [$F(3,26) = 12.3$; $p<0.001$; Figure 3C]. However, only the combination of JZL184 and PF-3845 significantly ameliorated

the intensity of weight loss [$F(3,26) = 4.8$; $p < 0.01$; Figure 3D] and blocked the occurrence of diarrhea [Figure 3E].

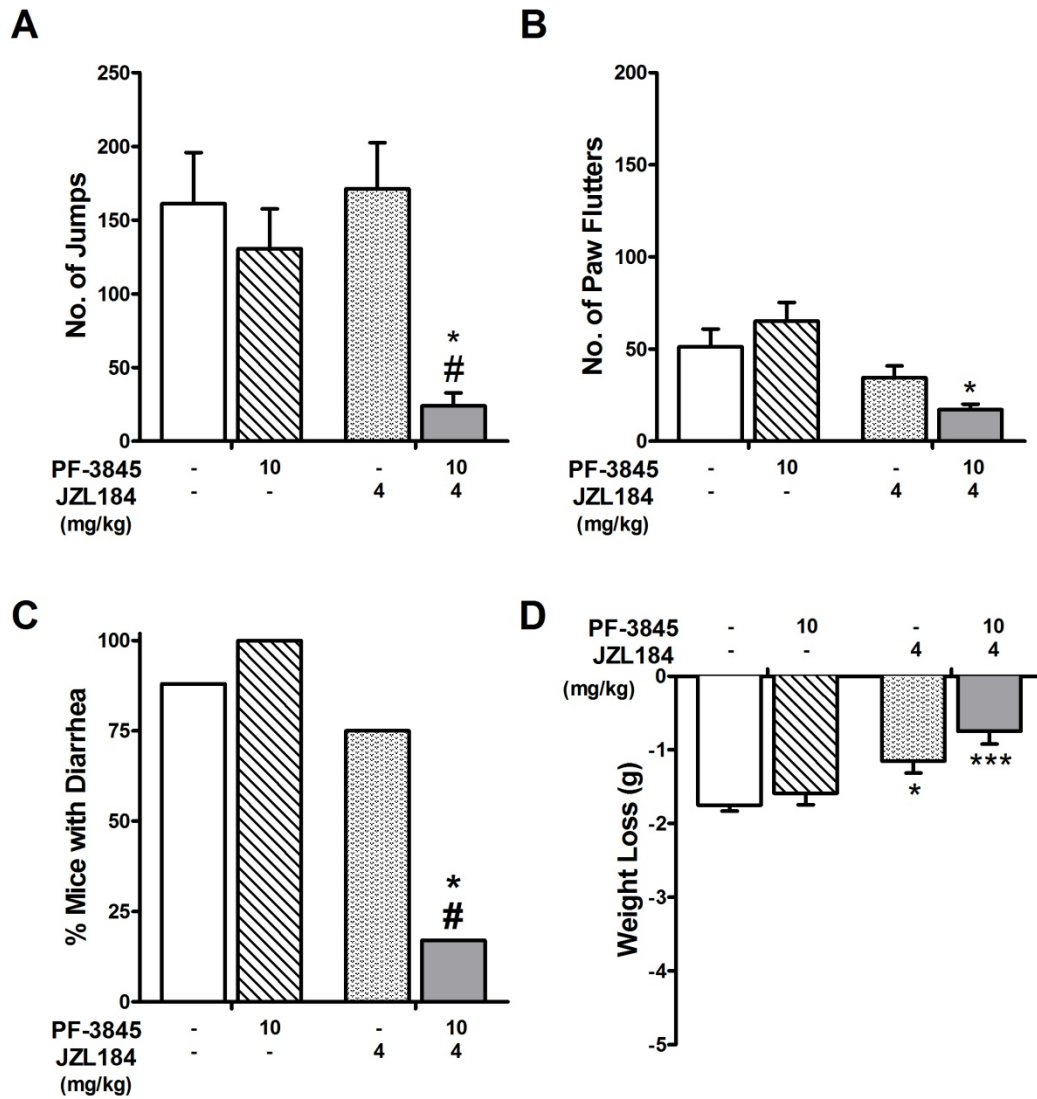


Figure 2. Combined administration of a low dose of JZL184 (4 mg/kg) and high dose of PF-3845 (10 mg/kg) attenuated the occurrence of the naloxone-precipitated withdrawal jumping and diarrhea to a greater extent than either inhibitor alone. $n=6-8$ per group. Data expressed as mean \pm SEM for panels A-C. * $p<0.05$, ** $p<0.01$. *** $p<0.001$ vs. vehicle, # $p<0.05$ compared to PF-3845 and JZL184 alone.

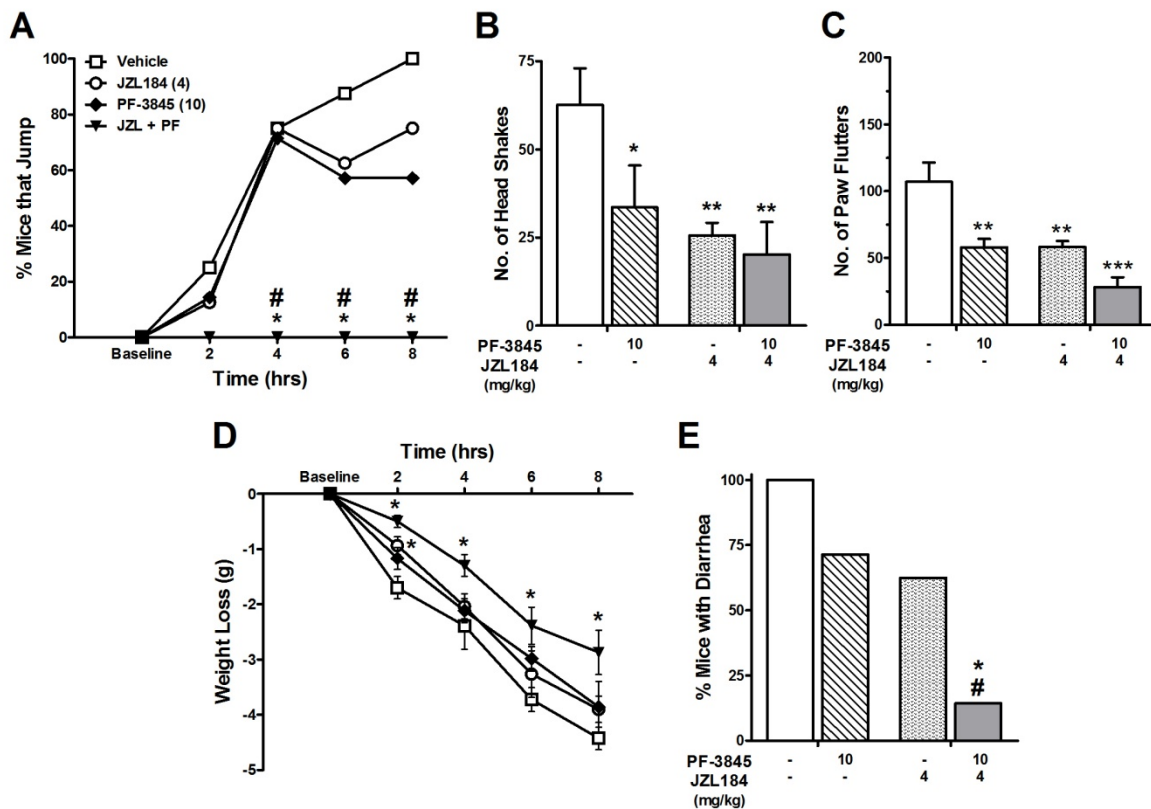


Figure 3. Combined administration of a threshold dose of JZL184 (4mg/kg) and high dose of PF-3845 (10 mg/kg) attenuated the occurrence of the spontaneous withdrawal jumping and diarrhea to a greater extent than either inhibitor alone. $n=7-8$ per group. The withdrawal signs measured include: (A) jumps, (B) headshakes, (C) paw flutters, (D) weight loss, and (E) diarrhea. $n=7-8$ per group. Data expressed as mean \pm SEM for panels B-D. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. vehicle, # $p<0.05$ compared to PF-3845 and JZL184.

2.3.4. Dual FAAH/MAGL inhibition reduces somatic signs of spontaneous morphine withdrawal via CB1 receptor mechanism of action

Rimonabant completely prevented all reductions in somatic withdrawal signs by combined administration of JZL184 (4 mg/kg) and PF-3845 (10 mg/kg) in morphine-dependent mice following pellet removal, including the occurrence of jumps [Figure 4A], head shakes [$F(1,37) = 4.4$; $p < 0.05$; Figure 4B], paw flutters [$F(1,37) = 9.9$; $p < 0.01$; Figure 4C], weight loss [$F(12,108) = 2.8$; $p < 0.01$; Figure 4D], and diarrhea [Figure 4E],

2.3.5. Dual inhibition by the mixed FAAH/MAGL inhibitor SA-57 reduces somatic signs of naloxone-precipitated morphine withdrawal in mice

To determine the efficacy of a dual FAAH and MAGL inhibitor on morphine-dependent mice undergoing spontaneous withdrawal, we employed SA-57, which is greater than 100-fold more potent as a FAAH inhibitor than as a MAGL inhibitor (Niphakis *et al.*, 2012). SA-57 dose-dependently reduced the occurrence of platform jumping [Figure 5A], the total numbers of paw flutters [$F(3,32) = 18.0$; $p < 0.0001$; Figure 5B] and head shakes [$F(3,32) = 4.34$; $p < 0.05$; Figure 5C], attenuated weight loss at all time points at the 5 mg/kg dose [$F(3,32) = 5.99$; $p < 0.01$; Figure 5D] and fully blocked the presence of diarrhea at 5 mg/kg [Figure 5E].

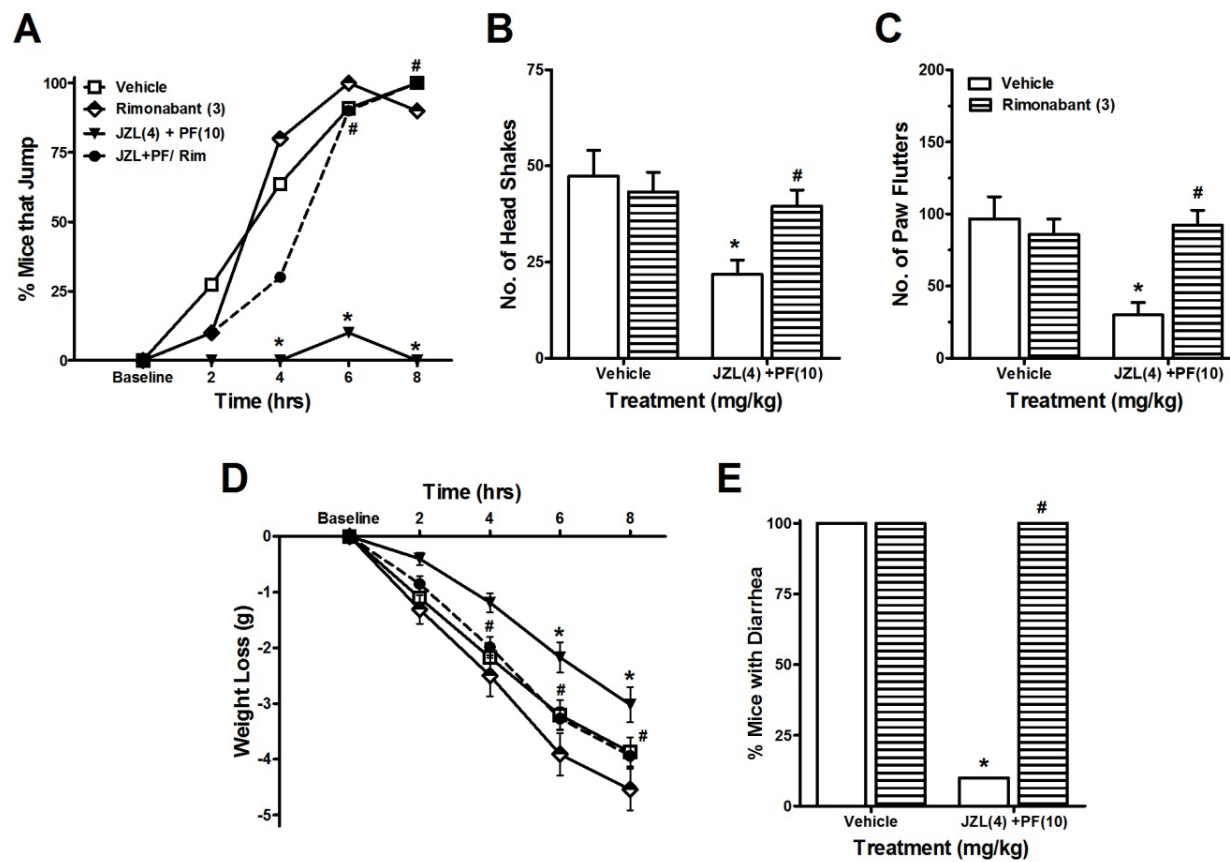


Figure 4. CB₁ receptors mediate the effects of combined administration of a threshold dose of JZL184 (4 mg/kg) and a high dose of PF-3845 (10 mg/kg) in on spontaneous morphine withdrawal signs. n=10-11 per group. The withdrawal signs measured include: (A) jumps, (B) head shakes, (C) paw flutters, (D) weight loss, and (E) diarrhea. n=10-11 per group. Data are presented as mean±SEM for panels B-D. *p<0.05 vs. vehicle, #p<0.05 vs JZL+PF condition

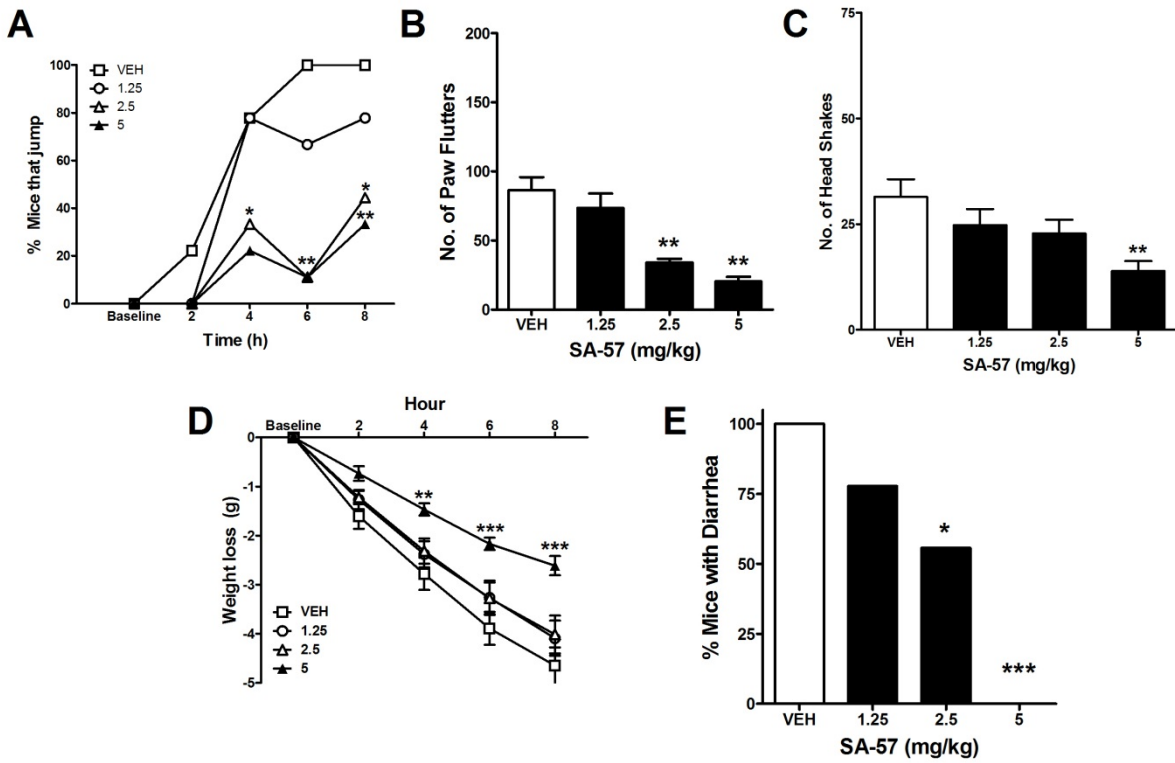


Figure 5. The dual FAAH/MAGL inhibitor SA-57 reduced all measured signs of spontaneous morphine withdrawal. The withdrawal signs measured include (A) jumps, (B) paw flutters, (C) head shakes, (D) weight loss and (E) diarrhea. $n=9$ per group. Data expressed as mean \pm SEM for panels B-D. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. vehicle.

2.3.6. SA-57 is more potent at elevating AEA than 2-AG in mouse brain

As shown in Figure 6A, 0.125 and 1.25 mg/kg SA-57 did not alter brain 2-AG levels, but 2.5, 5, and 12.5 mg/kg SA-57 elevated brain 2-AG levels respectively by 3-4 fold, 7 fold, and greater than 10-fold [$F(5,24)=305.5$; $p<0.0001$]. SA-57 was considerably more potent in elevating brain AEA levels ($ED_{50} = 0.4$ mg/kg) than 2-AG levels ($ED_{50} = 5.0$ mg/kg), with 0.125 mg/kg increasing AEA approximately 3-fold and each of the higher doses producing maximally elevated brain AEA levels [$F(5,24)=49.02$; $p<0.0001$; Figure 6C]. As shown in Figure 6B, 5 and 12.5 mg/kg SA-57 significantly reduced brain AA levels [$F(5,24)=10.93$; $p<0.0001$] by 33% and 60%, respectively.

2.3.7. The dual FAAH/MAGL inhibitor SA-57 produces minimal cannabinimimetic effects

To evaluate whether dual blockade of MAGL and FAAH would elicit cannabinimimetic effects, we tested SA-57 (1.25-12.5 mg/kg) in the cannabinoid tetrad assay. As shown in Figure 7, SA-57 produced immobility, catalepsy and hypothermia at only the highest dose tested (12.5 mg/kg) which maximally elevates both AEA and 2-AG [Figure 6]. Specifically, SA-57 significantly increased [Figure 7A] immobility [main effect of treatment, $F(4,30)=4.56$, $p<0.01$] at the highest dose tested with no main effect for time [$p=0.37$] or interaction effect [$p=0.90$]. SA-57 also produced [Figure 7B] catalepsy at 4, 6, and 8 h post-treatment [main effect of treatment, $F(4,124)=6.0$, $p<0.01$; main effect of time, $F(4,124)=8.6$, $p<0.0001$; interaction effect, $F(16,124)=3.1$, $p<0.001$]. There was no effect of SA-57 on antinociception at any dose at any time point [Figure 7C]. Finally, SA-57 produced a significant reduction in rectal temperature at 1, 2 and 4 h [main effect of treatment, $F(4,124)=9.5$, $p<0.0001$; main effect of time, $F(4,124)=57.6$, $p<0.0001$; interaction effect, $F(16,124)=16.0$, $p<0.0001$; Figure 7D].

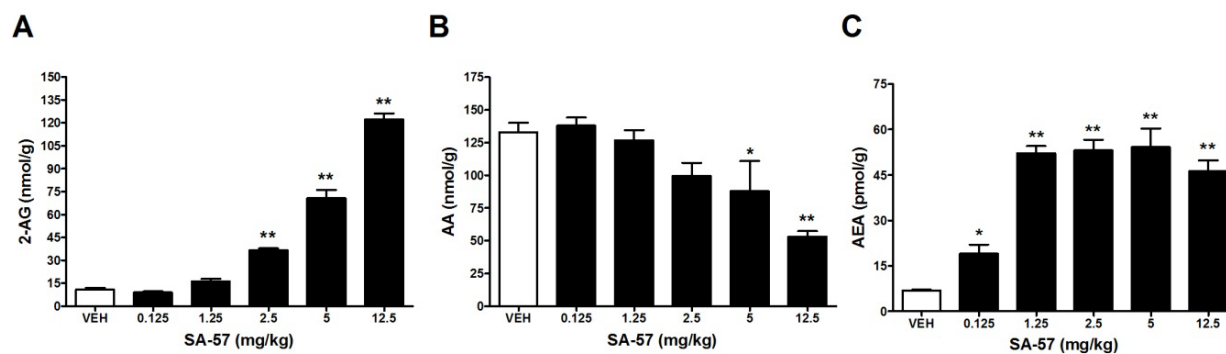


Figure 6. Effects of dual FAAH/MAGL inhibitor SA-57 on whole brain levels of 2-AG, arachidonic acid, and AEA. The dual FAAH/MAGL inhibitor SA-57 dose-dependently increased whole-brain levels of (A) 2-AG, and concomitantly decreased (B) arachidonic acid, but was most potent at elevating (C) brain AEA levels. $n=5-7$ per group. Data are expressed as mean \pm SEM. * $p<0.05$, ** $p<0.05$, compared to vehicle.

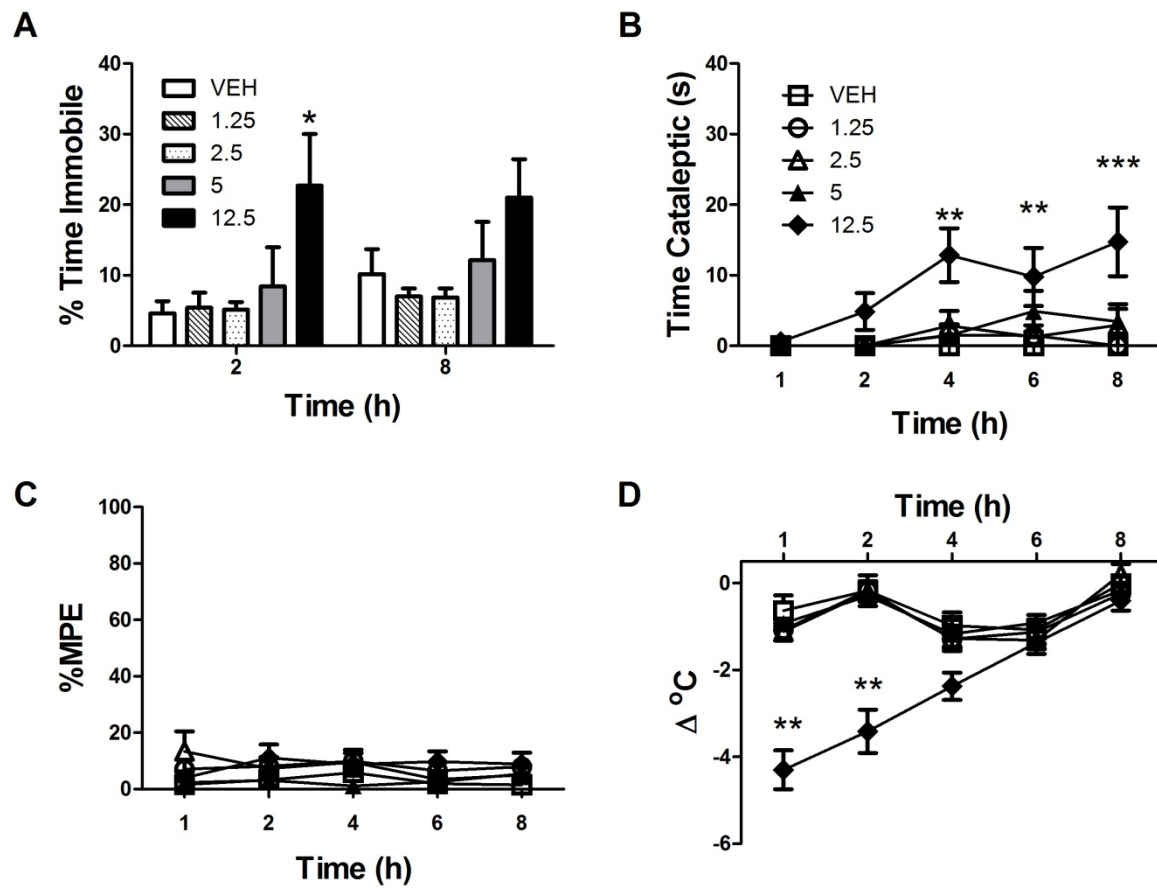


Figure 7. Effects of dual FAAH/MAGL inhibitor SA-57 in the cannabinoid tetrad. The dual FAAH/MAGL inhibitor SA-57 produced (A) immobility, (B) catalepsy and (D) hypothermia only at the highest dose tested (12.5 mg/kg), but did not produce (C) antinociception. $n=5$ per group. Data are expressed as mean \pm SEM. * $p<0.05$, ** $p<0.05$ compared to vehicle.

2.4. Summary

The major finding of the preceding experiments is that partially blocking MAGL in combination with full FAAH blockade reduced somatic withdrawal signs at doses that did not produce cannabimimetic effects. Specifically, combined administration of low dose of the MAGL inhibitor JZL184 (i.e., 4 mg/kg) and high dose of the FAAH inhibitor PF-3845 (i.e., 10 mg/kg) reduced precipitated [Figure 2] and spontaneous [Figure 3] morphine withdrawal and these effects were CB₁ mediated [Figure 4]. Additionally, dual inhibition with PF-3845 (10 mg/kg) and JZL184 (4 mg/kg) produced greater effects on jumps and diarrhea than either treatment alone. The dual FAAH/MAGL inhibitor SA-57 [Figure 5] significantly reduced paw flutters, head shakes, jumps, diarrhea, and weight loss in morphine-dependent mice undergoing spontaneous withdrawal. Importantly, these studies have shown that dual inhibition of FAAH/MAGL at doses of SA-57 that only partially elevate 2-AG [Figure 6] do not produce detectable changes in measures of cannabimimetic activity [Figure 7].

Chapter 3: Development of behavioral assays of morphine withdrawal

3.1. Introduction

Opioid withdrawal in humans is characterized by a number of physical and affective components including physical aspects such as diarrhea, emesis, dehydration, hypertension, hyperthermia, tachycardia and body aches as well as affective aspects such as anxiety, dysphoria, and panic attacks (Farrell, 1994; Gossop, 1988; Jasinski, 1981; Wesson *et al.*, 2003). As described in the previous chapters, rodents exhibit a number of withdrawal signs following either precipitated or spontaneous opioid withdrawal which include jumping, paw fluttering, head/body shaking/twitching, ptosis, teeth chattering, weight loss and diarrhea (Way *et al.*, 1969). One concern regarding a drug's ability to attenuate these withdrawal signs is whether the drug is having a direct effect on withdrawal or if it is simply suppressing behavior in general, though this would unlikely be the case for diarrhea. Additionally, it's been suggested that different aspects of withdrawal are mediated by different brain regions and while a drug treatment may be effective at attenuating one subset of withdrawal signs it may be ineffective at reducing others (Schulteis *et al.*, 1996). A critical component of treating opioid dependence is reducing withdrawal states which are associated with the maintenance of abuse as continued drug use serves to avoid the withdrawal state thus serving as a negative reinforcer (Bart, 2012; Koob *et al.*, 2005). It's important then to have behavioral models which can effectively assess other aspects which are related to the aversive internal/affective states of opioid withdrawal. A drug treatment that only blocks one subset of withdrawal signs would have limited clinical efficacy. Thus, in order to determine if a drug treatment is differentially affecting aspects of withdrawal, additional behavioral assays are necessary.

The purpose of the research presented in this chapter was to examine established murine behavioral assays thought to be reflective of aversive aspects of opioid withdrawal. We examined the effects of morphine withdrawal in the marble burying, novelty-induced hypophagia, and light/dark box tests which have all been suggested to model anxiety. Specifically, anxiolytic drugs will reduce marble burying, inhibit the suppression of feeding by a novel environment, and increase the amount of time spent in the light side of the light/dark box. Additionally, we developed a novel procedure to examine escape behavior in mice to further investigate the behaviors that we observed following opioid withdrawal in the light/dark box. Finally, we examined the effects of naloxone-precipitated withdrawal in the conditioned place avoidance (CPA) procedure which has been used to study the negative motivational aspects of withdrawal. The objective of this work was to identify a procedure that could be used to infer an aversive state of withdrawal in order to test endocannabinoid catabolic enzyme inhibitors for their efficacy to reduce opioid withdrawal.

3.1.1. Elevated plus maze and light/dark box

Several behavioral assays are employed as screening tests for anxiolytic compounds and among them are the elevated plus maze (EPM) and the light/dark box. Both of these behavioral procedures utilize a rodent's innate avoidance of bright/exposed areas to assess the effects of a treatment on the rodent's behavior in the assay including measures such as the number of entries and the amount of time spent in areas of the apparatus. The EPM contains four arms, two of which are closed off by walls flanking either side creating an "alley" while the other two arms have no walls leaving them more exposed. The innate avoidance by rodents of the open-arms was first observed by Montgomery in the 1950s (Montgomery, 1955), who suggested the

preference of rats for the closed-arms indicated a difference in the relative amount of “fear” elicited as compared with the open-arms. He also suggested that both the open and closed-arms elicit an “exploratory drive” and there is a conflict between the rodent’s motivation to explore and its avoidance due to the “fear” of the open-arms. Handley and Mithani (1984) used this as the basis for the development of the elevated-plus maze as a screen for anxiolytic drugs and found that diazepam (0.5 – 5 mg/kg i.p.) increased the ratio of entries into the open-arms vs. closed arms but not total entries. Subsequently, Lister (1987) adapted the EPM to be used with mice and demonstrated that anxiolytic agents chlordiazepoxide (10 mg/kg) and ethanol (1.6 g/kg) increased the ratio of open-arm entries to total entries, and suggested that the EPM may work to screen anxiolytic compounds in mice. Furthermore, Lister demonstrated that drugs which are anxiogenic in humans, caffeine (60 mg/kg) and picrotoxin (2 mg/kg) reduced the ratio of open-arm to total entries, suggesting that the procedure has bidirectional drug sensitivity (Carobrez *et al.*, 2005). The EPM has since been used to assess the effect of various experimental manipulations (e.g. drug treatments, genetics) on behaviors in this apparatus (e.g. number of entries to open-arms, time spent in open-arms) and agents which are anxiolytic or anxiogenic in humans have been shown to increase or decrease the amount of time spent in the open-arms in this assay respectively (Carobrez *et al.*, 2005; Hogg, 1996).

The light-dark box, first described by Crawley (1981), is constructed of two compartments, a dark side that is typically one third of the entire apparatus and the light side which comprises two thirds (though some studies use 1/2 for each side). These two sides are separated by a wall containing an opening which allows the rodent to ambulate between compartments. Similar to the EPM, rodents will typically spend much less time in the light side than in the dark side, an avoidance behavior that is reduced by anxiolytic agents such as

benzodiazepines (Bourin *et al.*, 2003; Crawley, 1981). Crawley reported that mice treated with benzodiazepines clonazepam (0.1 – 0.5 mg/kg i.p.), diazepam (0.5 – 5 mg/kg i.p.), flurazepam (1 – 10 mg/kg i.p.), and chlordiazepoxide (5 – 10 mg/kg i.p.) exhibited increased number of transitions between sides, suggesting an increased exploratory behavior. This behavior was not observed with other drugs that do not possess anxiolytic effects in humans suggesting a degree of pharmacological specificity in this assay. Costall et al. (1989) further characterized the light/dark box, manipulating light intensity and testing several mouse strains and drug treatments. White lighting (15 – 40 watts) did not significantly reduce the amount of time on the light side compared to the dark side in which a red light (60 watt) was placed, however when the white light was increased to 60 or 100 watts, mice spent significantly less time on the light side and exhibited shorter latencies to exit to the black side, suggesting that greater light intensity produces an avoidance response in mice.

The use of behavioral assays typically used to screen for anxiolytic drug effects has also been extended into studies of drug dependence where rodents undergoing withdrawal exhibit shifts in behaviors in these assays. For example, in rats, withdrawal from morphine has been shown to reduce the amount of time spent in the open-arms which is inferred by investigators to suggest an anxiogenic state (Bhattacharya *et al.*, 1995; Castilho *et al.*, 2008; Harris *et al.*, 1993; Schulteis *et al.*, 1998b; Zhang *et al.*, 2008). Bhattacharya was among the first to demonstrate that morphine-dependent (10 mg/kg i.p. b.i.d. for 14 days) rats undergoing spontaneous withdrawal (24 h following last injection) spent significantly less time in the open-arms of the EPM than did vehicle-treated rats ($9.2 \pm 0.9\%$ vs. $24.3 \pm 3.6\%$, morphine vs. control) (Bhattacharya *et al.*, 1995). Subsequently, Schulteis et al. (1998b) demonstrated that rats undergoing both precipitated and spontaneous morphine withdrawal exhibited decreases in the time spent in the open-arms of the

EPM. Rats implanted with either morphine (2x75 mg) or placebo pellets for 64 h were tested 8 h later (72 h post pellet implantation) and morphine-pelleted rats undergoing spontaneous withdrawal exhibited significant decreases in the percent of time spent in the open-arms. For precipitated withdrawal, morphine-pelleted rats (2x75 mg; 72 h) received naloxone (0.03, 0.1 mg/kg s.c.) and subsequently exhibited significant reductions in the percent of time spent in the open-arms (Schulteis *et al.*, 1998b).

Examination of morphine withdrawal effects in the light/dark box is not as well characterized as morphine withdrawal in the EPM, however Castilho reported that naloxone (0.1 mg/kg i.p.) significantly reduced the time spent in the light side and the number of entries into the light side in morphine-dependent, but not non-dependent, rats (Castilho *et al.*, 2008). These decreases in time spent in the open-arms of the EPM and light side of the light/dark box are opposite to effects observed with drugs that are known to be anxiety-reducing and mimic effects of drugs that are known to be anxiety-provoking in humans and experimenters have therefore inferred that these behaviors are reflecting an anxiogenic state in the animal.

However, opioid-dependent mice undergoing either precipitated or spontaneous withdrawal display paradoxical behavioral effects in the EPM. Specifically, they exhibit increases in open-arm time, an effect which would typically be interpreted as anxiolytic-like (Buckman *et al.*, 2009; Hodgson *et al.*, 2008; Hodgson *et al.*, 2009). The first study to report increased open-arm time following opioid withdrawal in mice was reported by Hodgson *et al.* wherein morphine dependent mice (morphine 10-40 mg/kg s.c. b.i.d., 3 days) were challenged with naloxone (0.1-0.4 mg/kg) and at each dose mice treated repeatedly with morphine exhibited a significant increase in open-arm time whereas mice treated with vehicle did not (Hodgson *et al.*, 2008). Subsequently they demonstrated similar increases in open-arm time following

spontaneous withdrawal using a slightly longer dependence procedure (morphine 10-40 mg/kg s.c. b.i.d., 6 days) and mice were tested 2, 4, 8 and 24 h after the last morphine or vehicle injection. Morphine treated mice exhibited increases in time spent in the open-arms and increased number of entries into the open arms at the 8 h time point (Buckman *et al.*, 2009). Additionally, precipitated withdrawal by naloxone (0.2 mg/kg) in opioid-dependent mice resulted in significantly elevated levels of the stress hormone corticosterone in blood collected 10 min post naloxone treatment (Hodgson *et al.*, 2008). This effect has previously been reported in mice and rats following morphine withdrawal (Budziszewska *et al.*, 1996; Gonzalvez *et al.*, 1994). The effects of morphine withdrawal in mice in the light/dark box have not yet been reported. Here, we examined whether naloxone challenge in morphine-pelleted mice would cause a decrease in the duration of time in the light side, which would be consistent with the interpretation that opioid withdrawal produces anxiogenic-like effects.

3.1.2. *Escape behavior*

Unconditioned defensive behaviors exhibited by rodents have been suggested to include flight, startle, hiding, freezing, defensive threat, attack and risk assessment (Blanchard *et al.*, 2003; Rodgers *et al.*, 1997). These defensive behaviors have also been suggested to be controlled by environmental features such as the availability of escape and the proximity of the threat (Rodgers *et al.*, 1997). In the mouse defense test battery jumping behavior is scored as attempted escape. Jumping behavior is also observed during naloxone-precipitated morphine withdrawal in mice (Way *et al.*, 1969) and this has been termed by some as “escape jumping” (Frederickson *et al.*, 1976; Noda *et al.*, 2004; Stinus *et al.*, 1990). Observations that mice exhibit an increase in the amount of time spent in the open-arms of the elevated plus maze during morphine

withdrawal has been suggested to reflect a shift in defensive behaviors towards escape (Hodgson *et al.*, 2008). It seems likely that the jumping behavior and the increases in time spent in the open-arms of the EPM exhibited by mice are closely related. However, it is not clear whether the jumping or increased time spent in the open-arms of the EPM are directed towards escape. In order to determine whether morphine withdrawal is increasing escape behavior a procedure is necessary that allows for rodents to actually escape. Thus, we developed a procedure in which mice are placed in a novel environment containing an opening through which they can escape. We hypothesized that naloxone would significantly reduce the latency to escape in morphine-pelleted, but not placebo-pelleted, mice.

3.1.3. Novelty-induced hypophagia

Novelty-induced hypophagia is a decrease in food consumption and/or a reduced latency to begin feeding in a novel environment. Rodents will quickly approach and consume a food in their home cage if it is 1) familiar and 2) they are food deprived or if the food is highly palatable. However, if the same food is presented to the animal in a novel environment the latency to approach and consume the food is increased, the amount consumed is decreased, and this behavior has been termed “novelty-induced hypophagia” or “hyponeophagia”.

Stephens (1973) and Soubrie (1975) were among the first to demonstrate that rats and mice treated with benzodiazepines would consume larger quantities of food in a novel environment than control animals. Stephens reported that food deprived mice receiving subcutaneous injections of diazepam (0.1 – 62.5 mg/kg), chlordiazepoxide (0.39 – 100 mg/kg) and nitrazepam (0.1 – 62.5 mg/kg), elicited a dose-dependent increase in wet mash consumption in a novel environment.

Variations of the novelty-induced hypophagia task have been developed recently by Merali et al. (2003) in which animals are not food deprived, but instead are acclimated to a palatable food and subsequently tested in the home cage and a novel environment. The latency to consume is recorded in both the home cage and the novel environment and mice exhibit a very short latency to approach and consume the food in their home cage while taking a significantly longer amount of time to begin eating the food in a novel environment. Merali et al. evaluated pharmacological and environmental manipulations in this procedure. Among their findings, the addition of a predator scent (rat fecal bolus) significantly increased the latency over no scent in the novel condition. Treatment with diazepam (0.25 mg/kg i.p.) significantly reduced the latency to approach and consume the palatable food in the novel environment, but did not affect latency in the home cage suggesting context-specificity of diazepam's effects. Additionally, chlordiazepoxide (3 mg/kg i.p.) and propranolol (2 mg/kg i.p.) also reduced the latency to consume food in the novel condition but not in the home cage.

The novelty-induced hypophagia task has recently been used to examine the effects of nicotine withdrawal in mice. Turner et al. (2013) examined withdrawal in mice implanted with osmotic minipumps delivering nicotine (18 mg/kg/day). Fourteen days later, the pumps were removed to elicit spontaneous withdrawal. Mice were tested 24 h after minipump removal in the novelty-induced hypophagia test and the withdrawal group exhibited significantly increased elevations in latency to feed in the novel environment as compared to saline control animals, but withdrawal did not affect latency in the home cage, suggesting that the nicotine withdrawal state specifically increases novelty-induced hypophagia. There are presently no published reports of opioid withdrawal in this model of which we are aware.

3.1.4. Marble burying

The marble burying test was initially developed as a modified version of the defensive burying model, but as discussed below this explanation has been challenged. In the defensive burying model, a nociceptive or predator-related stimulus is presented to the rodent in an apparatus containing a substrate material (e.g. sand, bedding) which rodents push/kick up towards the stimulus. This effect was first demonstrated in rats by Pinel and Treit (1978) who reported that an electrified prod caused rats to bury it, but that rats would not bury another prod that did not produce a shock. This pattern of findings suggested that the rats were specifically burying the prod which produced a noxious stimulus and thus the behavior was described as defensive burying. Ethologically, rodents have been observed emitting this behavior from their burrows towards snakes (Deacon, 2006). Marble burying is a modified version of the defensive burying task in which marbles (typically 20-25) are placed in a grid pattern atop the substrate material and the number of marbles that are buried during the test is taken as an indicator of this burying behavior, though marble burying as a measure of defensive burying has been challenged and is now considered an indirect measure of digging behavior rather than defensive burying (Deacon, 2006). Anxiolytic drugs will reduce the number of marbles buried in the marble burying procedure (Broekkamp *et al.*, 1986; Njung'e *et al.*, 1991) as will endocannabinoid catabolic inhibitors (Kinsey *et al.*, 2011).

The effects of opioid withdrawal have been examined in the defensive burying task in rats. Morphine-dependent rats (10-80 mg/kg i.p. once daily, 11 days) or saline treated controls were tested 48 h after their last repeated injection and placed in a chamber containing a 3 cm diameter hole which contained an electric probe with a constant electrical current. Rats that had received repeated morphine (10 - 80 mg/kg i.p., q.i.d.; 11 days) were tested 48 h after their last

morphine injection and exhibited significant increases in the latency to begin burying as well as increased burying duration, suggesting that morphine withdrawal increased the defensive burying behavior (Harris *et al.*, 1993).

Additionally, morphine withdrawal in mice leads to increases in the number of marbles buried in the marble burying task. Mice treated repeatedly with morphine (5-20 mg/kg s.c. twice daily, 10 days) were tested 2, 8, 24, 48 and 96 h post withdrawal in the marble burying task. Mice were placed in the Plexiglas cage with 5 cm of bedding and 20 marbles placed in a 4x5 grid pattern and the number of marbles buried at the end of the 30 min session was counted. Mice at 8, 24, and 48 h post withdrawal buried more marbles as compared with saline treated controls (Umathe *et al.*, 2012). Thus, morphine withdrawal increases both defensive burying in rats and digging behavior in mice as assessed by the marble burying task.

3.1.5. Conditioned place avoidance

Place conditioning is a Pavlovian paradigm in which the primary motivational properties of a treatment (e.g. acute drug treatment, drug withdrawal) serve as an unconditioned stimulus which is paired with specific context that serves as a conditioned stimulus. The conditioned stimulus (CS) becomes predictive of the unconditioned stimulus (US) such that the CS elicits either approach (i.e. conditioned place preference) or avoidance (i.e. conditioned place avoidance). It is then inferred from the animal's behavior that the treatment is either rewarding or aversive depending upon the expressed behavior during free access to the entire apparatus after the conditioning procedure. Test compounds can then be administered prior to the conditioning sessions to determine their effect on the acquisition of CPA, wherein a reduction of CPA would suggest the test compound blocked the aversive unconditioned stimulus (Bardo *et al.*, 2000).

The first published study to examine place conditioning was by Horace Beach (1957) who developed a procedure in rats based upon early findings by Spragg (1940). Spragg demonstrated that monkeys would seek a certain colored box that contained a morphine syringe after the monkeys had been repeatedly treated with morphine from that specific box. Beach surmised that rats could also learn to associate the effects of morphine with a particular conditioned stimulus. Using a Y-maze, Beach exposed the rats to the apparatus, allowing them on repeated trials to enter one arm of the maze or the other but would not allow them to move backward, thus forcing them to make a “choice”. Rats were then made dependent for 8 weeks receiving daily morphine injections (5 mg/kg or 1 – 20 mg/kg s.c.) and then conditioned in what is now termed a “biased” procedure. Following the 8 weeks dependence procedure, rats were then conditioned for 1 h with saline on their preferred side and then 20 min after this conditioning session were administered morphine (i.e. the same dose administered daily) and placed in their non-preferred side. This procedure continued for 12 days until they were tested in 4 trial test sessions where the rats could choose to enter either the morphine paired or saline paired arms of the Y-maze. The arm selected in each trial was recorded and expressed as a percentage of total trials. Rats in both treatment groups selected the morphine-paired side more following conditioning than during pre-conditioning tests. Specifically, rats in the 5 mg/kg morphine group selected the morphine-paired arm 75% post-conditioning compared to 45.3% pre-conditioning and rats in the 20 mg/kg group selected the morphine-paired arm 74.4% post-conditioning as compared to 43.8% during pre-conditioning. In contrast, saline treated mice did not change the percentage of arm selection (45% vs. 43.8%) following the procedure. This early study is possibly the first to demonstrate withdrawal CPA since it could be assumed that rats not having received their daily morphine injection prior to the saline conditioning session were

undergoing spontaneous withdrawal. Thus, the selection of the morphine paired arm could also be interpreted as an avoidance of the withdrawal-paired arm. Beach also demonstrated in the same paper conditioned place preference to morphine in non-dependent rodents. Morphine (1 – 5 mg/kg i.p.) was conditioned daily for 12 days, reaching the high dose (5 mg/kg) on day five and being maintained at that dose for the remaining conditioning days. Rats exhibited a greater percentage of entries into the morphine-paired arm (70.8%) post-training than during pre-training (46%). These studies by Beach were the first to demonstrate the place conditioning effects of drugs. Subsequently Rossi and Reid (1976) modified the procedure to examine the amount of time spent in each context and this measure has been adopted in most place conditioning procedures.

The negative motivational properties of opioid antagonism were elucidated by Mucha et al. (1982). Rats conditioned with naloxone (0.1 – 45 mg/kg i.v.) exhibited a CPA behavior following 4 conditioning sessions for each treatment condition (saline vs. naloxone) and when morphine-dependent rats were used (75 mg pellets, 72 h prior to first conditioning session), much lower doses of naloxone (0.004 and 0.0025 mg/kg i.v.) produced CPA in these animals. This study demonstrated the differential sensitivity to naloxone in morphine-pelleted animals and therefore naloxone-precipitated morphine withdrawal CPA.

Hand et al. (1988) was the first to demonstrate central mediation of CPA of precipitated opioid withdrawal. Methylnaloxonium administered intracerebroventricularly (i.c.v.; 200-1000 ng) prior to placement of rats in compartments with distinct contextual cues produced a significant avoidance behavior to the withdrawal-paired compartment following conditioning in dependent, but not non-dependent, rats. Methylnaloxonium, which does not readily cross the

blood-brain barrier, did not produce CPA when administered s.c. in dependent rats at lower doses and only produced CPA at the highest dose (10 mg/kg), at which point some central blockade of mu opioid receptors may have occurred. Stinus et al. (1990), using a similar procedure, determined that the nucleus accumbens followed by the amygdala and periaqueductal grey were the most sensitive regions to methylnaloxonium, with intracerebral microinfusions of 250 ng (NAc) and 500 ng (amygdala, PAG) producing CPA behavior. Furthermore, the brain regions which were most sensitive, i.e. nucleus accumbens, amygdala, and PAG, support a role for multiple loci in the acquisition of withdrawal CPA. Later studies demonstrated that microinjections of methylnaloxonium into the NAc and amygdala elicited only a subset of somatic withdrawal signs at relatively high doses as compared to LC and PAG; e.g. (lowest significant dose; ED₅₀) NAc: mastication (500 ng; ED₅₀ = 5000 ng); Amygdala: mastication (31 ng; ED₅₀ = 112 ng), ptosis (62 ng; ED₅₀ = 94 ng), teeth chattering (250 ng; ED₅₀ = 1024 ng). These are in contrast to LC and PAG infusions where all withdrawal behaviors were observed. It is notable that methylnaloxonium (1000 ng) in the LC produced much greater jumping behavior in comparison to PAG where the effect was 10 fold lower, suggesting a greater role of LC in withdrawal jumping behavior (Maldonado *et al.*, 1992).

Indeed, higher doses of naloxone have consistently been shown to be required to precipitate quantifiable somatic withdrawal behaviors as compared with those necessary to produce CPA (Frenois *et al.*, 2002). In their study, Frenois et al. demonstrated a separation between naloxone dose and both the behaviors and brain regions activated in morphine-pelleted rats, thus showing a clear distinction between regions necessary for the expression of somatic and negative motivational aspects of withdrawal. The calculated ED₅₀ values of naloxone to elicit withdrawal weight loss (21.2 µg/kg), jumping (30 µg/kg), diarrhea (32.2 µg/kg), and salivation

(67.3 µg/kg) were higher than the ED₅₀ value to produce CPA (4.7 µg/kg). Furthermore, the specific brain regions (NAc and amygdala), which Stinus et al. had previously (1990) teased apart to mediate the negative motivational aspects of withdrawal behaviors, also showed greater sensitivity to naloxone induced increases in *cFos* expression than areas thought to mediate physical withdrawal aspects (e.g. locus coeruleus). While significant increases in *cFos* expression were observed at 7.5 µg/kg in the amygdala and 15 µg/kg in the nucleus accumbens, significant increases in *cFos* were only observed at doses of 30 µg/kg and higher.

Conditioned place avoidance as a model for the negative motivational aspects of opioid withdrawal has been well established and is currently used to assess the role of different neurotransmitter systems and brain regions during this process. Further discussion of opioid withdrawal CPA is presented in chapter 4, along with experiments examining the impact of inhibiting endocannabinoid catabolic enzymes in morphine withdrawal CPA.

3.1.6. Rationale and hypothesis

While inhibitors of endocannabinoid catabolism block somatic signs of morphine withdrawal in mice, it is possible that these effects are due to an overall suppression of behavior rather than a specific attenuation of withdrawal itself. Furthermore, if these drugs are in fact attenuating somatic withdrawal signs via a specific anti-withdrawal mechanism, there are still multiple components (e.g. somatic signs vs. motivational aspects) to opioid withdrawal in rodents which involve multiple brain regions (Frenois *et al.*, 2002; Stinus *et al.*, 1990) so reductions in somatic withdrawal signs do not necessarily indicate a reduction in motivational aspects. Therefore, in order to determine the extent to which inhibitors of endocannabinoid catabolism can reduce opioid withdrawal, other behavioral assays which reflect

motivational/affective states are needed. The EPM, light/dark box, and novelty-induced hypophagia tests have been used to evaluate the potential anxiolytic-like and anxiogenic-like aspects of various pharmacological manipulations and have therefore been suggested to be models of affective behaviors, though it is important to ensure that effects in these assays are not confounded by effects such as locomotor activity. These tests have been used to evaluate drug withdrawal and are investigated here as potential assays in which to evaluate morphine withdrawal. Paradoxical effects of morphine withdrawal in the EPM (Buckman *et al.*, 2009; Hodgson *et al.*, 2008; Hodgson *et al.*, 2009) wherein mice exhibit increases in open-arm time have been suggested to reflect shifts in defensive behavior towards escaping. Assessment of mouse behavior during morphine withdrawal in the light/dark box here resulted in a similar behavior (increases in time spent in the light side) and so a novel procedure was developed to test the hypothesis that mice are exhibiting escape behavior. We hypothesized that mice undergoing morphine withdrawal would exhibit 1) decreases in the amount of time in the light side of the light/dark box; 2) decreases in the latency to escape a novel environment; 3) increases in digging behavior as assessed in the marble burying task; 4) increases in latency to consume food in a novel environment in the novelty-induced hypophagia test; and 5) greater conditioned place avoidance following naloxone conditioning than non-dependent mice.

3.2. Methods

3.2.1. Subjects

Male ICR mice (Harlan, Indianapolis, IN) with a body mass of 27-32 g were used for all experiments. Mice were group-housed (4 per cage) or single-housed on a 12/12 light/dark cycle (lights on at 0600 h) and given food and water *ad libitum* except during the experimental

sessions for the light dark box, the escape box, marble burying, and the CPA assay. Mice had access to water and the palatable food during the novelty-induced hypophagia test. All efforts were made to minimize animal suffering and number of animals used. All animal protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 2011).

3.2.2. *Drugs*

Morphine pellets (75 mg), placebo pellets, and morphine sulfate were obtained from the National Institute on Drug Abuse (Bethesda, MD) and diazepam and naloxone hydrochloride were purchased from Sigma Aldrich (St. Louis, MO). Naloxone HCl was dissolved in physiological saline (0.9% NaCl) and drug concentrations were calculated using the weight of the salt as used previously (Ramesh et al., 2011). Morphine sulfate was dissolved in physiological saline (0.9% NaCl) and drug concentrations were calculated based upon the free base. Diazepam was dissolved in ethanol and Emulphor-620 (Rhone-Poulenc, Princeton, NJ) and then diluted with physiological saline to form a vehicle mixture of ethanol:Emulphor-620:saline in a ratio of 1:1:18. Diazepam was administered intraperitoneally while naloxone HCl and morphine were administered subcutaneously. All drugs were administered in a volume of 10 μ l per g body mass.

3.2.3. *Light/dark box*

The light dark box (Bourin *et al.*, 2003) consisted of two distinct compartments: a light side measuring 20.5 \times 40 cm and a dark side measuring 10 \times 40 cm and walls 34 cm high. The middle wall separating the two sides contained a 7 \times 7 cm opening in the middle through which

the mice could travel between sides. The dark side had a UV penetrable lid blocking out the visible light while the light side had a 60 watt bulb above it. Fire-iTM digital cameras (Unibrain, San Ramon, CA) recorded video and the videos were analyzed using the ANY-mazeTM video tracking software (Stoelting Co., Wood Dale, IL)

Mice were implanted with morphine (75 mg/kg) or placebo pellets. Forty-eight hours later, mice were injected s.c. with naloxone 10 min prior to start of the experiment at which point they were placed in the front right corner of the light side and time spent on both sides and general activity were recorded for 5 min.

3.2.4. Escape box

Three variations of the escape box procedure were employed. The first involved opening the lid of a mouse cage and positioning it so that there was a gap through which mice could climb through to escape. The second and third procedures used a modified mouse cage in which a small hole (5×5 cm) was cut out of one end of the lid to narrow the escape route and a small grid mesh ramp was fitted over the lip to facilitate escape climbing. The height of this mesh lip was lowered in the third procedure to further facilitate escaping and the scoring of escape behavior in the absence of jumping.

3.2.4.1. Escape box procedure 1

The test chamber consisted of a standard plastic mouse cage (28×18×11.5 cm; $l \times w \times h$) wiped down with Pinesol (Clorox Co., Oakland, CA) diluted with water (1:10) which served as a novel odor. The plastic lid covering the cage was left ajar at one end leaving a 2.5 cm opening through which mice could climb through to escape. Mice were treated s.c. with naloxone and then 10 min later placed directly in the center of the box and observed for 15 min. Latency for

the mice to climb up and onto the inverted lid was recorded. Mice that did not escape during the 15 min were considered to not escape and their time was recorded as 900 s.

3.2.4.2. Escape box procedure 2

The test chamber described above was used; however, two modifications were made. First, the chamber (see Figure 8 for picture) was covered with a plastic lid that contained a 5x5 cm hole centered on one end. Three sides of the exit hole were blocked off by a 5 cm high plastic lip that prevented mice from climbing onto the top of the cage. The mice were able to escape from the apparatus by climbing up a grid wire mesh (0.7×0.3 cm) that was located 11.5 cm from the bottom of the cage. The wire mesh formed to a 15.5×4.5 cm ramp (42° from side of cage) that led down to a clean, escape cage containing fresh bedding and no novel odor. The apparatus was placed in a sound-attenuating chamber illuminated by an array of white LEDs (75 lux) and containing a ventilation fan for air circulation and masking noise. Mice were treated s.c. with naloxone and then 10 min later placed directly in the center of the box. Fire-iTM digital cameras (Unibrain, San Ramon, CA) captured video and video tracking software (ANY-mazeTM, Stoelting, Kiel, WI) recorded the latency of the mice to exit out through the escape hole by detection of mice entering the zone adjacent to the cage. Mice that did not escape during the 15 min were considered to not escape and their time was recorded as 900 s.

3.2.4.3. Escape box procedure 3

The escape box in procedure 2 was modified so that the climbing mesh was extended lower into the cage (9.5 cm from the bottom) to facilitate escape behavior so that rodents could escape without jumping. Mice were treated s.c. with the test compound and then 10 min later placed directly in the center of the box. Fire-iTM digital cameras (Unibrain, San Ramon, CA)

captured video and video tracking software (ANY-mazeTM, Stoelting, Kiel, WI) recorded the latency of the mice to exit out through the escape hole by detection of mice entering the zone adjacent to the cage. Subsequently, videos were observed for jumping behavior and whether mice jumped or climbed up the wire mesh to escape the cage was recorded. Mice that exhibited jumping were excluded from a second analysis to determine if jumping behavior was necessary for reduced latencies.

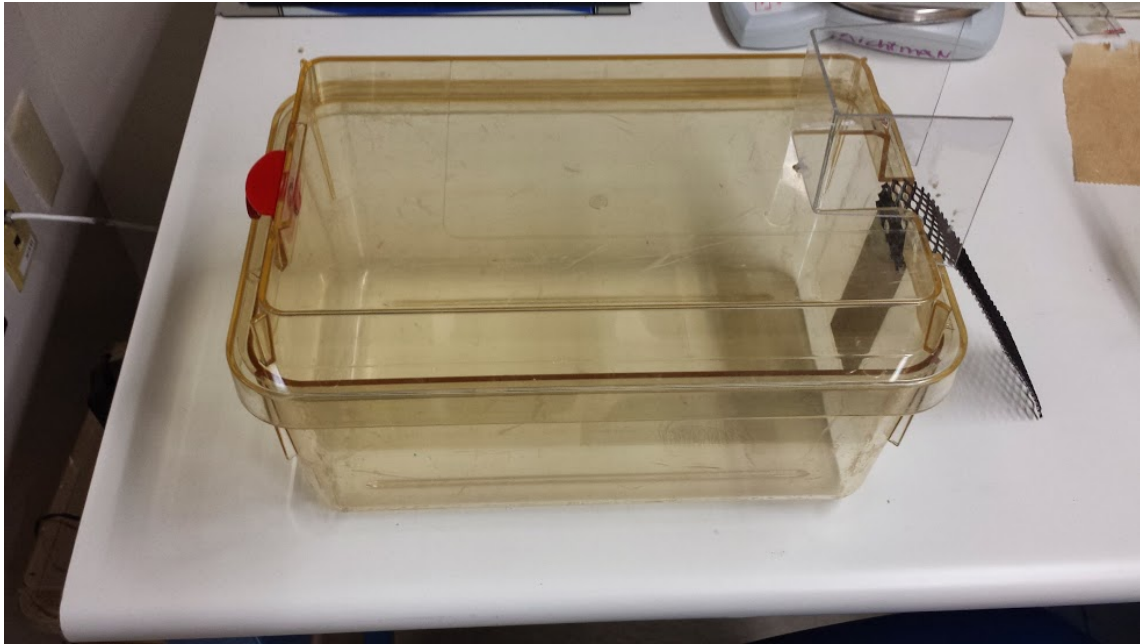


Figure 8. The escape box. The escape box is placed within a sound-attenuating cubicle that contains a fan for ventilation and masking noise. LED lights (75 lux) located on the side wall provide lighting. Mice are placed within the cage and the lid is closed and the door to the cubicle is shut. ANY-mazeTM software tracks movement of the mice and once a mouse climbs over the lip of the cage and the center of the animal has crossed the threshold between the edge of the cage and the ramp the latency to escape is recorded.

3.2.5. Marble Burying

Mice were acclimated to the test room for at least 1 h prior to start of the experiment. For spontaneous withdrawal experiments, mice were either 1) treated repeatedly with morphine (6.25 – 50 mg/kg b.i.d.) for 6 days and tested on day 7, 24 h after their last injection or 2) implanted with subcutaneous morphine (75 mg) or placebo pellets; 48 h later, baseline behaviors were measured for 20 min, then pellets were removed under light anesthesia (isoflurane), and mice were again tested at 4 and 8 h post-pellet removal. For naloxone-precipitated withdrawal, mice were injected with naloxone (0.03, 0.1 and 0.3 mg/kg s.c.) 10 min prior to the start of the experiment 48 h after pellet implantation. The procedure for assessing marble burying was adapted from a published protocol (Kinsey *et al.*, 2011; Thomas *et al.*, 2009). The test cage was a polycarbonate mouse cage (33×21×19 cm; $l \times w \times h$) filled with pine wood chip bedding (Harlan Sani-Chip, Indianapolis, IN) to a depth of 5 cm. The cage was first gently shaken to flatten the surface of the bedding and then 20 marbles were placed in a 4×5 grid pattern on top of the bedding. Individual mice were placed in each cage which was subsequently covered with a clear plexiglass lid containing holes for ventilation. The cage was placed in a sound-attenuating chamber illuminated by an array of white LEDs (75 lux) and containing a ventilation fan for air circulation and masking noise. The test lasted for either 20 min (spontaneous withdrawal) or 5 min (precipitated withdrawal), after which the cages were removed and mice were placed back into their home cages. The session length for precipitated withdrawal was shortened to 5 min due to control animals burying most of the marbles within the 20 min time limit. The number of marbles buried was determined by a blinded observer and a marble was counted as “buried” if half or more of the marble was covered by bedding.

3.2.6. Novelty-induced Hypophagia

The procedure was adapted from a previously established protocol (Gur *et al.*, 2007). Mice were single-housed for 1 week prior to the start of food acclimation. Mice were then given access to 5 peanut butter chips (partially defatted peanuts, sugar; partially hydrogenated vegetable oil (palm kernel and soybean oil); corn syrup solids, dextrose; reduced minerals whey (milk), salt; vanillin, artificial flavor; soy lecithin; Reeses®, The Hershey Company, Hershey, PA) for 15 min each day and the latency to consume the chips and the amount consumed were calculated until behaviors stabilized to less than 10% variability between days for three days and this training period occurred over the course of 12 days.

Mice were then implanted subcutaneously with either 75 mg morphine pellet or a placebo pellet. At 48 h and 72 h post-pellet implantation, mice were tested in either their home cage or a novel environment, the order of testing being counterbalanced within each treatment group. Mice received naloxone (0.056 mg/kg) or saline s.c. 10 min prior to the start of the experiment. Home cage testing occurred on a metal rack in the laboratory where mice had previously been exposed to the food for 12 days. Novel environment testing was carried out in a separate room within white cubicles containing a 60 watt overhead light and a ventilation fan for masking noise. Cages contained no bedding and were wiped down prior to each test with dilute Pinesol (1:10) which served as a novel odor. Mice were observed and the latency to approach and consume the food was recorded and the food was weighed before and after to determine the amount consumed.

3.2.7. Place conditioning

3.2.7.1. Apparatus

The place conditioning chambers (MED Associates, St. Albans, VT, USA) were comprised of three distinct compartments separated by manual sliding doors. A central grey compartment with a plastic floor separated a black compartment with a floor comprised metal bars (0.31 cm) placed in a parallel series and a white compartment with a metal grid mesh floor (0.625×0.625 cm). The dimensions (w×d×h) of each compartment were as follows: grey compartment (8.25×12.5×13 cm), black and white compartments (16.5×12.5×13cm).

3.2.7.2. Pre-conditioning and post-conditioning tests

Mice were group housed (4 per cage) with PVC pipe corner pieces placed in the cages for environmental enrichment for at least 3 days prior to the preconditioning test. Mice were handled for three days prior to the pre-conditioning test and administered daily saline (s.c.) injections during this period to acclimate them to the injection procedure.

For all place conditioning procedures, the pre-conditioning and post-conditioning tests were the same. The mice were placed gently into the central grey chamber with all the doors pre-opened and mice were allowed to freely ambulate between compartments for 20 min and time spent in each compartment was recorded by Med-PC (MED Associates, St. Albans, VT, USA). For the preconditioning test, mice that exhibited a strong unconditioned avoidance (25%) or preference (75%) for any compartment were excluded from the experiment. For post-conditioning tests, the amount of time spent on the treatment-paired side during the pre-conditioning test was subtracted from the amount of time on that same side during post-conditioning test (i.e. post-conditioning time – preconditioning time). From this

preference/avoidance scores were calculated so that statistically significant negative scores compared to control indicated avoidance whereas positive scores indicated preference.

3.2.7.3. Conditioned place preference

Following the pre-conditioning test, mice were counterbalanced to experimental groups and treatment was assigned to either the black or white side pseudo-randomly. The day following the pre-conditioning test, mice were injected with either morphine (10 mg/kg s.c.), cocaine (20 mg/kg i.p.) or saline immediately prior to being placed in the respective compartment. The conditioning session lasted 20 min and afterwards mice were returned to their home cage. The afternoon conditioning session occurred 4 h later and mice were treated with the opposite conditioning treatment and placed in the other side of the compartment for 20 min. One half the mice received their saline condition in the morning session and the other half received it in the afternoon session. The conditioning period lasted for three days resulting in a total of three saline conditioning sessions and three drug conditioning sessions (or 6 saline conditioning sessions for the saline control group). Following conditioning, mice were tested the next day in the post-conditioning test.

3.2.7.4. Conditioned place avoidance

Two procedures for CPA were used in these studies. The first procedure, adapted from Broseta et al. (2005) used repeated morphine injections for the induction of dependence and three naloxone pairings over a three day period for conditioning. The second procedure was developed so that morphine pellets could be used for the induction of dependence in order to provide a separation between the effects of naloxone in morphine dependent and non-dependent mice. In this procedure naloxone conditioning occurred during two sessions in a single day.

3.2.7.4.1. Conditioned place avoidance – Procedure 1

This procedure was adapted from Broseta et al. (2005), in which mice were treated twice daily s.c. (9AM and 7PM) with increasing doses of morphine: day 1 (12.5 mg/kg), day 2 (25 mg/kg) and day 3 (50 mg/kg) and maintained at this high dose for the duration of the experiment. The experiment began on day 4 with the pre-conditioning test. On day 4, and all subsequent days, mice were brought into the room and acclimated for at least 45 min prior to testing or conditioning. The dose of naloxone used for conditioning in this dependence procedure was 0.1 mg/kg s.c. in the first experiment and 1 mg/kg s.c. in the second experiment.

Mice were counterbalanced to treatment groups and the sides on which naloxone was paired were assigned pseudo-randomly within each group so that approximately half the mice received naloxone on the black side and half on the white side. After pre-conditioning, the conditioning phase began and lasted 6 days. On the first day, mice were conditioned to the saline-paired side 2 h after the morning injection of morphine. Mice were injected s.c. and immediately placed in the corresponding conditioning chamber for 20 min, after which mice were returned to their home cage and 24 h later mice were treated with naloxone and placed in the other compartment for 20 min. This was repeated for four more days, so that mice received a total of three naloxone conditioning sessions and three saline conditioning sessions (or six saline conditioning sessions for the saline control group). The day after the final conditioning day mice were assessed in the post-conditioning test. An initial experiment using this exact procedure occurred first to assess the dose-response relationship of naloxone (0.1, 0.3 and 1 mg/kg) to produce CPA on its own. In this experiment, mice did not receive repeated morphine or saline treatments prior to conditioning.

3.2.7.4.2. *Conditioned place avoidance – Procedure 2*

On day 1, and all subsequent days, mice were brought into the room and acclimated for at least 45 min. The mice were placed gently into the central grey chamber with all the doors pre-opened and mice were allowed to freely ambulate between compartments for 20 min. Med-PC software recorded the time spent in each compartment and the number of beam breaks to estimate locomotor activity. Mice were then returned to their home cage. On day 2 (saline conditioning; non-withdrawal day), mice were injected s.c. with saline and placed directly in the chamber designated as the saline side where they remained for 30 min. Mice were returned to their home cage and remained in the lab until the second conditioning session of the day which repeated the same procedure 4 h later. On day 3, mice were implanted with a 75 mg morphine pellet under anesthesia (see section 2.2.4. for pellet implantation surgery). At 48 h post-pellet implantation, mice were injected with either naloxone or saline (placebo+saline controls) and placed into the naloxone-designated compartment for 30 min. The naloxone conditioning procedure was then repeated 4 h later, i.e. 52 h post pellet implantation. On day 6, mice were tested for the expression of CPA.

In an initial experiment, mice were treated with saline instead of naloxone following pellet implantation to determine if the presence of the 75 mg morphine pellet could serve as an unconditioned stimulus to produce conditioned place preference. The results of this experiment were used to guide subsequent experiments as to whether the modified version of the CPA procedure was justified. The procedure was developed to have pellet implantation occur after the saline (non-withdrawal) conditioning out of concern for a potential confound in which it would be difficult to discern whether the mice exhibited a preference for the side in which mice were

exposed to morphine from the s.c. implanted pellets or exhibited an aversion to the side in which they received the naloxone injection.

3.2.8. *Somatic withdrawal signs*

A naloxone dose response experiment was conducted in mice 48 h after implantation of a 75 mg morphine pellet. Somatic withdrawal signs were scored, as previously described (Schlosburg *et al.*, 2009). In brief, mice were placed in white acrylic chambers (20x20 cm), with a clear acrylic front panel and a mirrored back panel for a 30 min acclimation period. The chambers were enclosed in sound-attenuating cabinets that contained an indirect filtered LED light source and fans for air circulation and white noise. The mice were briefly removed from the chambers for naloxone administration and immediately returned to the chambers for a 30 min observation period. Behavior was recorded using a series of Fire-iTM digital cameras (Unibrain, San Ramon, CA), and the videos were saved using the ANY-mazeTM video tracking software (Stoelting Co., Wood Dale, IL). Chambers were changed between tests and cleaned at the end of testing with an ammonia based cleaner and left to dry for two days, to allow for odors to dissipate. The recorded videos were randomized and scored by a trained observer, who was blinded with respect to treatment condition. The primary behavioral signs of interest were frequency of jumps and front paw tremors (including single and double paw flutters and twitches, which are not commonly displayed by naïve mice). The occurrence of diarrhea during the testing period was noted. All behaviors were recorded as new incidences when separated by at least 1 s or interrupted by any other normal behavior. In addition, mice were weighed before and immediately after the 30 min test session to assess body weight loss.

3.2.9. Statistical Analysis

All data are reported as mean \pm SEM. Data were analyzed using either Student's t-test, one-way between measures analysis of variance (ANOVA) for dose response studies and two-way ANOVA for all 2x2 designs. Dunnett's test was used to compare drug treatments with vehicle and Newman-Keuls post-hoc test was employed for comparisons between various treatments. For two-way ANOVAs, Bonferroni's post-hoc analysis was used. In addition, planned comparisons were used to compare differences in naloxone CPA between dependent and non-dependent mice. Differences were considered statistically significant at $p < 0.05$.

3.3. Results

3.3.1. Light/dark box

3.3.1.1. *Naloxone increases time spent in the light side of the light dark box in morphine-pelleted, but not placebo-pelleted, mice*

To determine if naloxone-precipitated morphine withdrawal would affect amount of time in either side of the light/dark box, we examined the dose-response relationship of naloxone in morphine-pelleted and placebo-pelleted mice. Naloxone produced significant increases in time spent in the light side [$F(4,35) = 13.6$; $p < 0.0001$; Figure 9A] while having no effect on distance traveled [$p = 0.84$; Figure 9B] or any effect on time spent in the light side [$p = 0.42$; Figure 10A] or distance traveled [$p = 0.77$; Figure 10B] in placebo-pelleted mice.

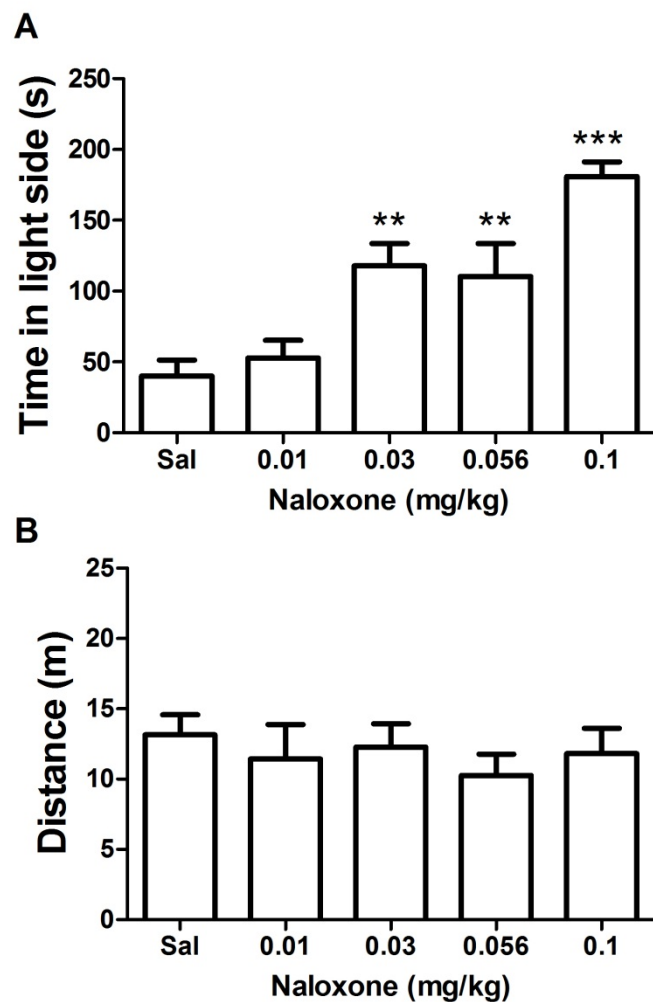


Figure 9. Naloxone-precipitated morphine withdrawal increased the amount of time spent in the light side of the light dark box, but did not affect locomotor activity. Naloxone (0.03, 0.056, or 0.1 mg/kg) administered s.c. in morphine-pelleted mice prior to the start of the session (A) increased the amount of time spent on the light side of the light dark box but (B) did not affect distance traveled. $n=8$ per group. Data are expressed as mean \pm SEM. ** $p<0.01$, *** $p<0.001$ compared to saline.

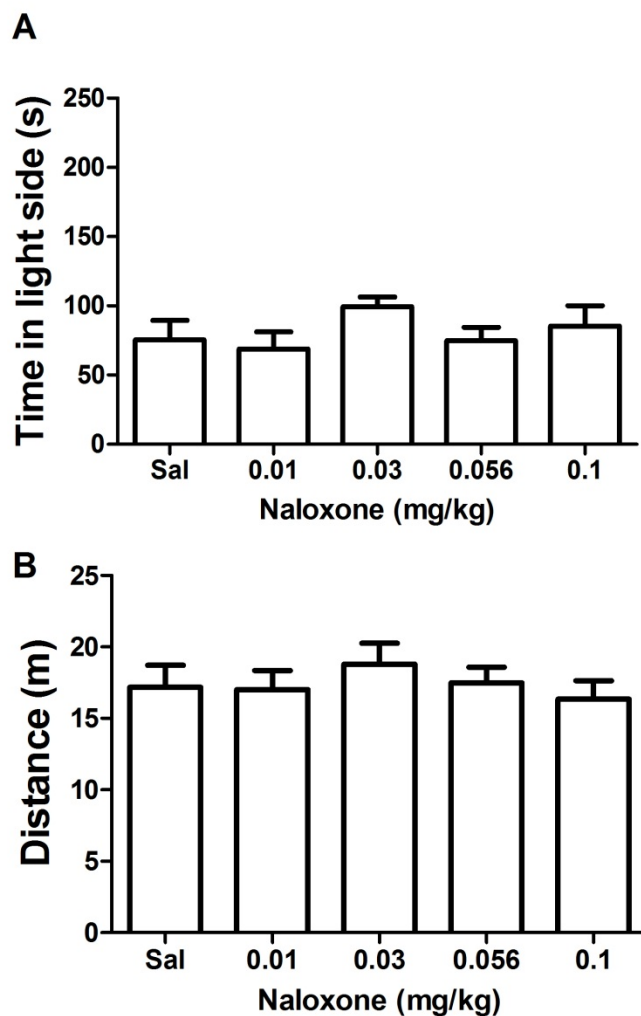


Figure 10. Naloxone did not affect the amount of time spent in the light side of the light dark box or locomotor activity in placebo-pelleted mice. Naloxone administered s.c. to placebo-pelleted mice did not (A) affect the amount of time spent on the light side of the light dark box or (B) affect distance traveled. $n=8$ per group. Data are expressed as mean \pm SEM.

3.3.2. *Escape paradigm*

3.3.2.1. *Naloxone reduces the latency to escape a novel environment in morphine-pelleted mice, but not in placebo-pelleted mice*

In order to ascertain if changes in preference for sides in the light dark box and increases in time spent in the open-arms of the elevated plus maze as reported in the literature were related to escape behavior, we assessed the latency of mice to climb out of an empty clean cage with the lid ajar 2.5 cm. In morphine-pelleted mice, but not placebo-pelleted mice, naloxone significantly reduced the latency to escape [$F(4,31) = 29.0$; $p < 0.0001$; Figure 11].

3.3.2.2. *Naloxone reduces the latency to escape a novel environment in morphine-pelleted, but not placebo-pelleted mice*

We designed a modified mouse cage in which a 4x4 cm hole was cut out from one end of the cage where a climbing mesh was located that led to an exit ramp. This design narrowed the opening so that random jumping was less likely to result in an escape and provided a means by which mice could climb up and out via the wire mesh ramp. In morphine-pelleted mice, naloxone significantly reduced the latency to escape [$F(4,35) = 3.7$; $p < 0.05$; Figure 12A] but did not affect escape latencies in placebo-pelleted mice [$p = 0.29$; Figure 12B].

3.3.2.3. *Naloxone reduces the latency to escape a novel environment in morphine-pelleted mice independent of jumping behavior*

Because the height of the climbing mesh in the previous experiment was sufficiently high enough to require that mice had to hop up to reach it, the escape cage was further modified by extending the climbing mesh lower into the cage to allow mice an option to climb out of the box. In morphine-pelleted mice, (A) naloxone (0.056 mg/kg) significantly reduced the latency to escape [$t = 3.1$; $p < 0.01$; Figure 13A]. Exclusion of mice ($n = 3$) that exhibited jumping behavior

still yielded a significant reduction in escape latency as compared with placebo-pelleted controls [$t=2.8$; $p<0.05$; Figure 13B].

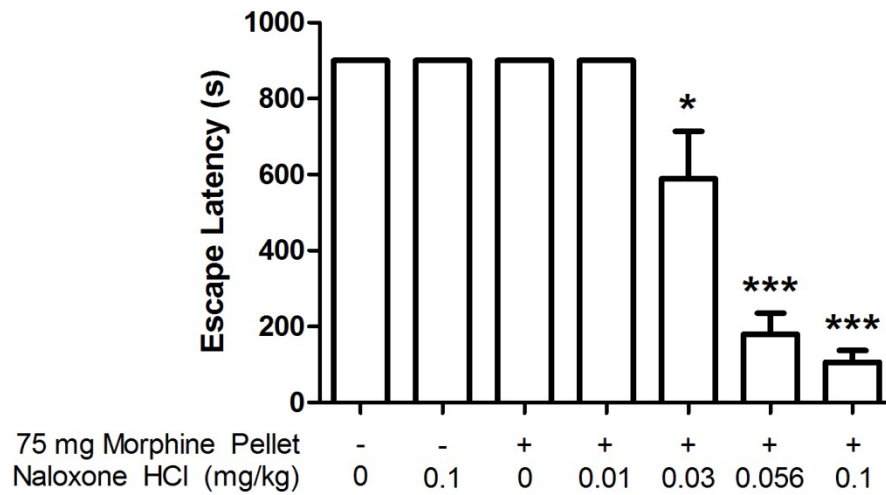


Figure 11. Naloxone challenge reduced escape latency in morphine-pelleted mice. Mice pelleted with morphine pellets, but not placebo pellets, exhibited escape behavior as indicated by a reduced latency to exit a novel cage upon treatment with naloxone (0.03 – 0.1 mg/kg). $n=6-8$ per group. Data are expressed as $\text{mean} \pm \text{SEM}$. $*p < 0.05$, compared to placebo+saline mice.

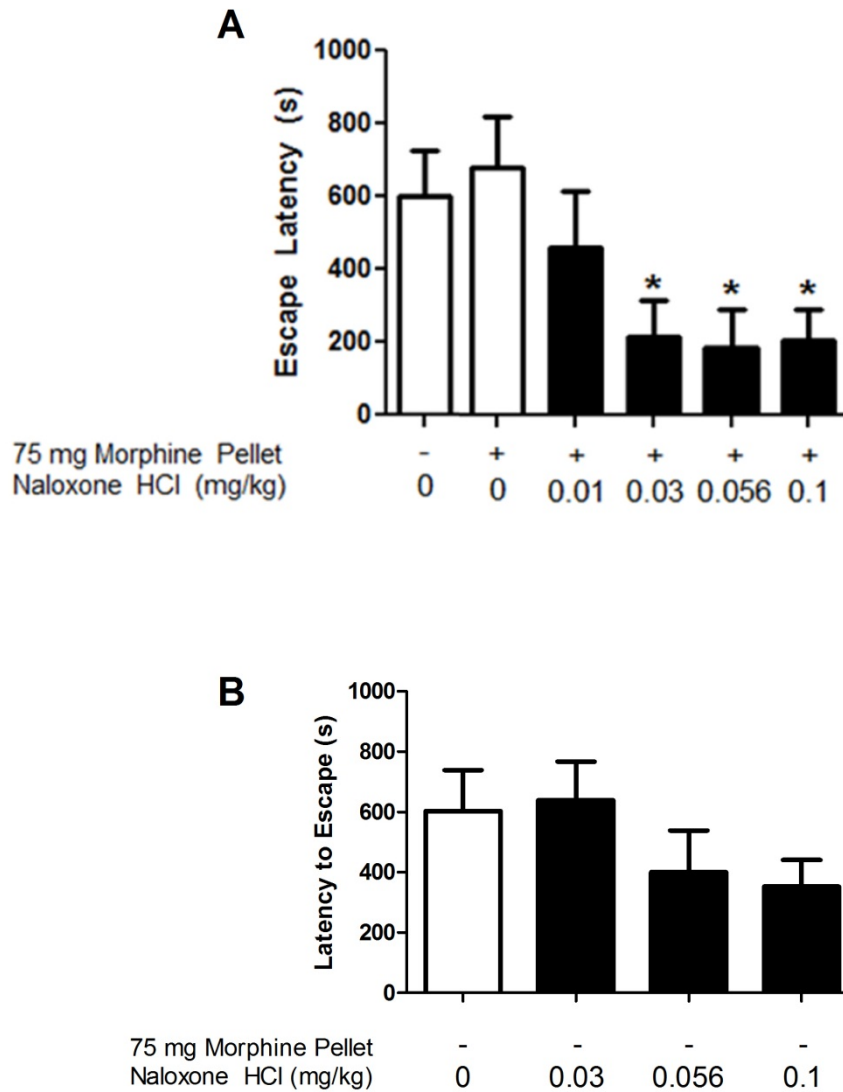


Figure 12. Naloxone-precipitated morphine withdrawal reduced escape latency. Naloxone reduced the latency to escape in (A) morphine-pelleted but not (B) placebo-pelleted mice. $n=7-9$ per group. Data are expressed as mean \pm SEM. * $p<0.05$, compared to placebo+saline mice.

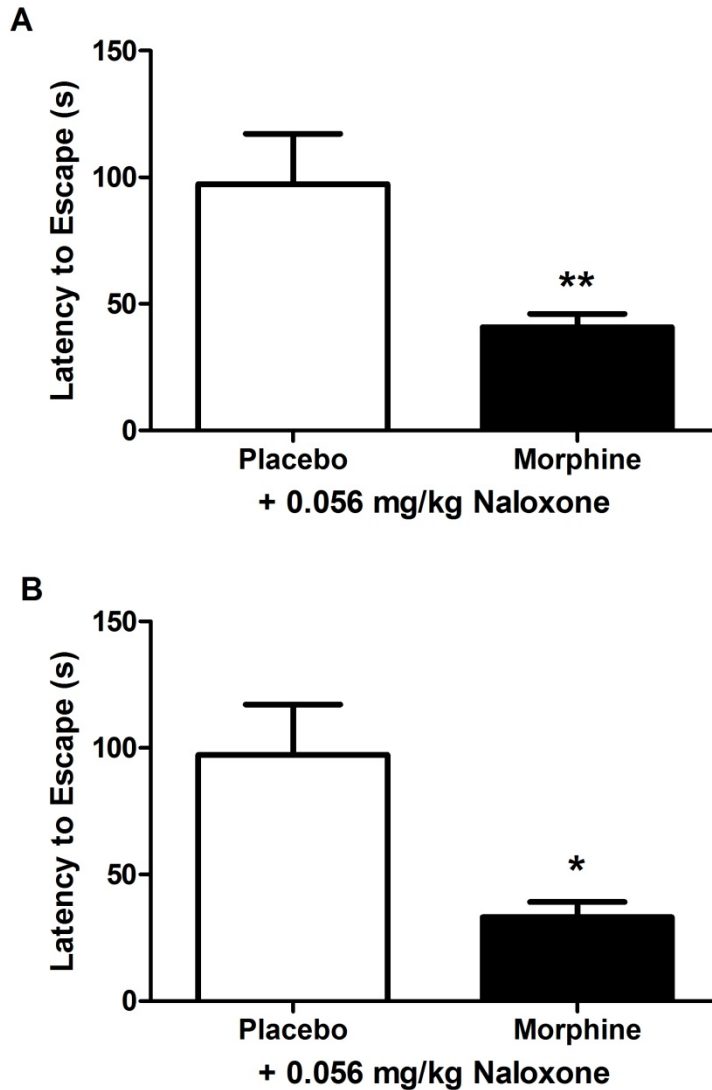


Figure 13. Mice undergoing precipitated-morphine withdrawal exhibit reduced latencies to escape independent of jumping behavior. Morphine-pelleted (75 mg) mice treated with naloxone (0.056 mg/kg s.c.) exhibited (A) decreased latencies to escape compared to placebo-pelleted mice. Exclusion of mice (3 mice) that exhibited jumping behavior did not affect (B) the reduced latency to escape in morphine-pelleted mice. $n=5-8$ per group. Data are expressed as mean \pm SEM. * $p<0.05$, ** $p<0.01$ compared to placebo-pelleted mice.

3.3.3. Marble Burying

3.3.3.1. Mice undergoing spontaneous morphine withdrawal did not exhibit changes in digging behavior as measured by number of marbles buried during 20 min test.

Mice treated repeatedly with morphine (6.25 – 50 mg/kg s.c. b.i.d.) for 6 days were tested 24 h after their last injection for the effects of spontaneous morphine withdrawal on digging behavior in the marble burying test. No significant differences were found for the number of marbles buried between morphine-treated and saline-treated mice [$p=0.09$; Figure 14].

3.3.3.2. Mice undergoing spontaneous withdrawal from morphine pellets do not exhibit changes in digging behavior as assessed by marble burying.

Mice implanted with morphine pellets exhibited less digging compared to placebo-pelleted control mice during baseline and 4 h following pellet removal [main effect of morphine treatment: $F(1,20) = 14.6$; $p<0.01$; Figure 15] but this effect was absent at 8 h following pellet removal. Mice buried fewer marbles after multiple exposures to the test [main effect of time: $F(2,20) = 6.5$; $p<0.01$; Figure 15] but no interaction effect was observed [$p=0.22$].

3.3.3.3. Mice undergoing precipitated withdrawal from morphine pellets do not exhibit changes in digging behavior as assessed by 5 min marble burying test.

In order to prevent ceiling effects with respect to control mice burying most of the marbles, the test was shortened to 5 min to allow for detection of increases and decreases in the number of marbles buried. Morphine-pelleted mice treated with naloxone (0.03 – 0.3 mg/kg s.c.) 72 h after pellet implantation did not exhibit differences in digging behavior as assessed in the marble burying test [Figure 16], nor did placebo-pelleted mice receiving naloxone (0.3 mg/kg s.c.).

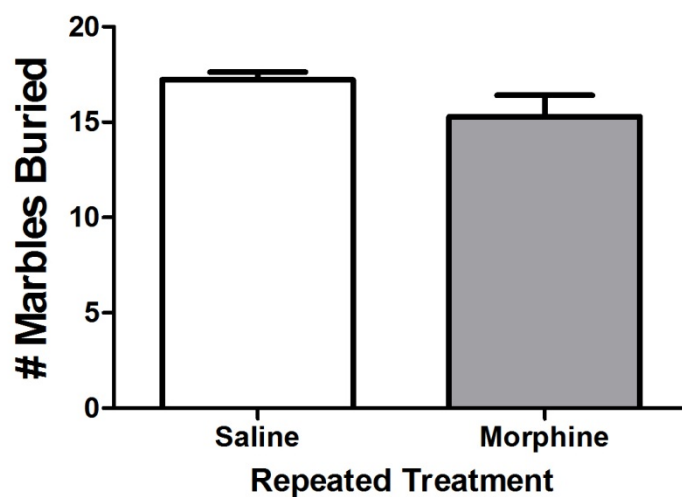


Figure 14. Mice undergoing spontaneous morphine withdrawal did not exhibit changes in digging behavior as measured by number of marbles buried during 20 min test. Following repeated twice-daily injections of morphine (6.25 – 50 mg/kg s.c.) or saline for 6 days, mice were tested 24 h after their last morphine or saline injection. The number of marbles buried did not differ between morphine-treated mice and saline-treated mice. $n=7-9$ mice per group. Data are expressed as mean \pm SEM.

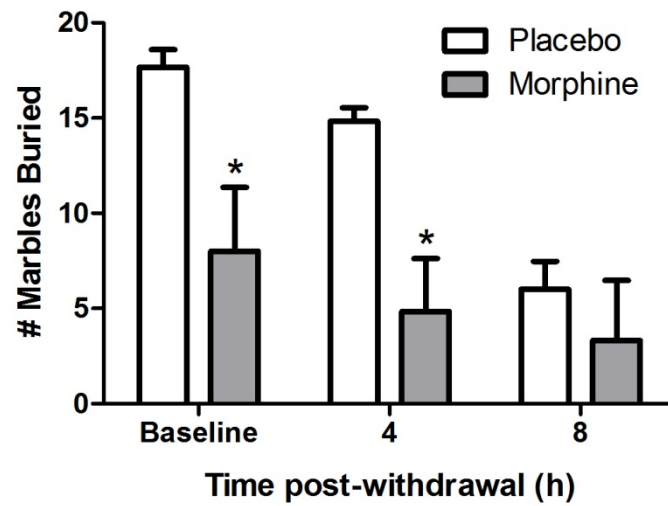


Figure 15. No effect of spontaneous withdrawal on number of marbles buried during 20 min tests. Mice pelleted for 72 h were tested 4 h and 8 h after pellets were removed. Morphine (75 mg) pelleted mice initially buried fewer marbles than placebo-pelleted mice but at 8 h there was no difference between these groups. $n=8$ per group. $*p<0.05$ compared to placebo. Data are expressed as $\text{mean} \pm \text{SEM}$.

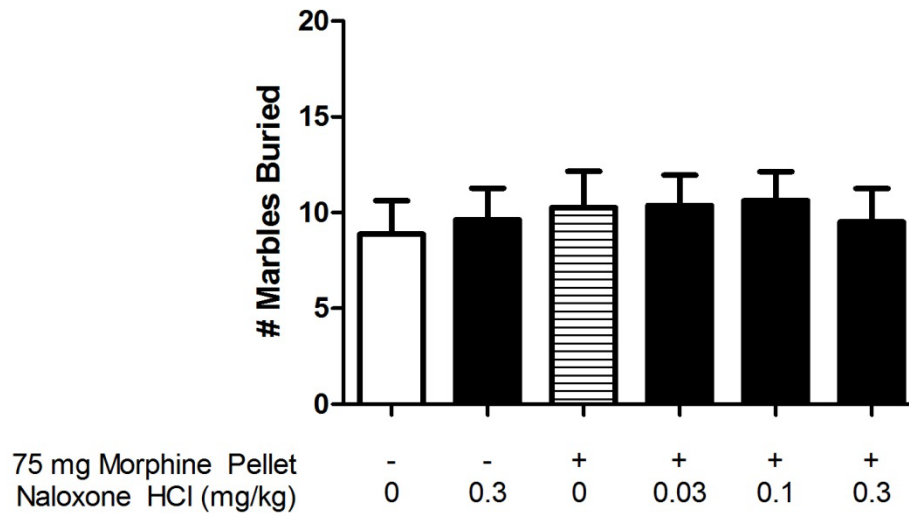


Figure 16. Naloxone does not affect number of marbles buried in placebo-pelleted or morphine-pelleted mice during a 5 min test. Mice implanted with morphine (75 mg) or placebo pellets for 72 h were challenged with naloxone (0.03 – 0.3 mg/kg) and placed in the marble burying apparatus for 5 min. No differences were detected for any treatment group. n=8 per group. Data are expressed as mean \pm SEM.

3.3.4. Novelty-induced hypophagia

3.3.4.1. Diazepam reduces the latency to consume food in a novel environment

Initially, mice were exposed for 12 days to the peanut butter chips in their home cages and latency to approach and consume and amount consumed were recorded [Figure 17]. To first assess a drug which has been shown to produce effects in the novelty-induced hypophagia test, diazepam (3 mg/kg) was tested as a positive control. Diazepam did not affect the latency to consume [Figure 18A] or the amount consumed [Figure 18B] during the home cage test; however, diazepam reduced the latency to consume food [$t=2.3$; $p<0.05$; Figure 18C] and increased the amount consumed [$t=3.7$; $p<0.01$; Figure 18D] in the novel cage test.

3.3.4.2. Naloxone does not affect latency or food consumption in placebo or morphine-pelleted mice in either home or novel cage

To determine if naloxone-precipitated morphine withdrawal would elicit increases in latency to consume food in a novel environment, morphine-pelleted and placebo-pelleted mice were challenged with saline or naloxone (0.056 mg/kg s.c). In morphine-pelleted mice, there was no effect between the familiar and novel test cage [$p=0.93$], naloxone treatment [$p=0.16$] or interaction [$p=0.32$] on latency to approach and consume food [Figure 19A]. Also, there were no significant effects of test cage [$p=0.27$], naloxone treatment [$p=0.08$] or interaction [$p=0.89$] on the amount consumed [Figure 19B]. In placebo-pelleted mice, there was an effect of cage [$F(1,20) = 19.3$; $p<0.001$], but no effect of naloxone treatment [$p=0.35$] or interaction [$p=0.24$] on latency to approach and consume [Figure 19C]. For amount of food consumed, placebo-pelleted mice consumed less food in the novel cage than the familiar cage [$F(1,20) = 52.7$; $p<0.0001$], but no effects of naloxone treatment [$p=0.61$] or interaction [$p=0.08$; Figure 19D] were detected.

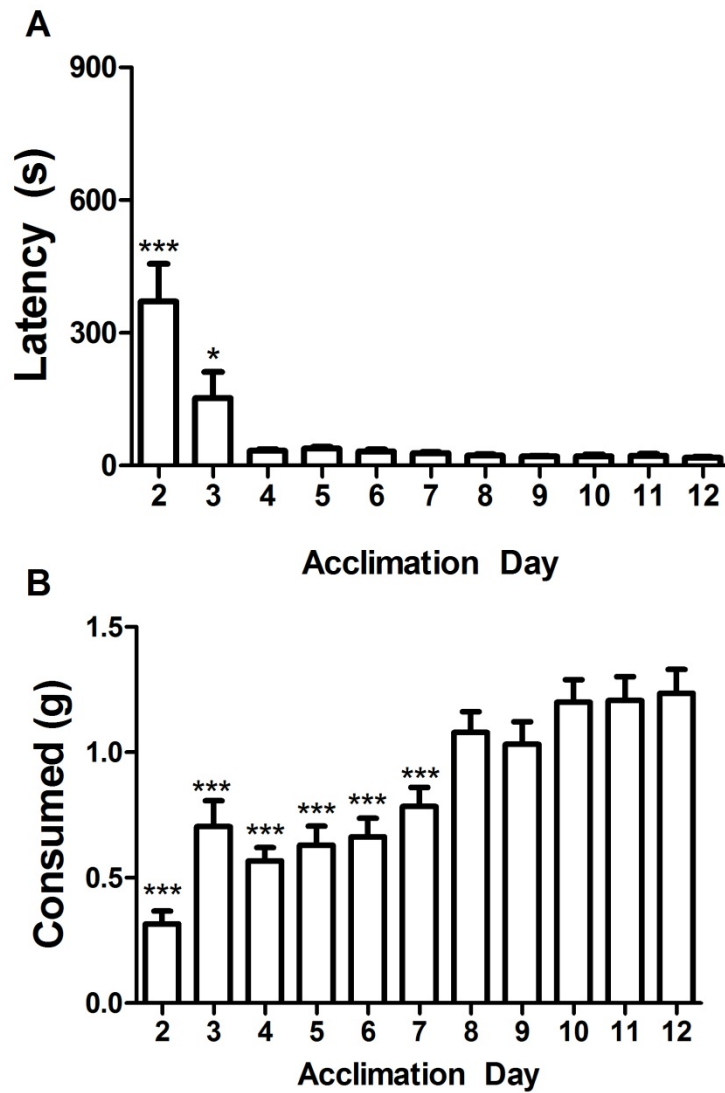


Figure 17. Baseline latencies and consumption data for mice exposed to peanut butter chips in their home cage. Mice given access to peanut butter chips in their home cage for 15 minutes exhibit rapid decreases in (A) latency to approach and consume food and (B) increases in amount of food consumed during acclimation period over 12 days. $n=24$ per group. Data are expressed as $\text{mean} \pm \text{SEM}$. *** $p < 0.001$, * $p < 0.05$ compared to day 12.

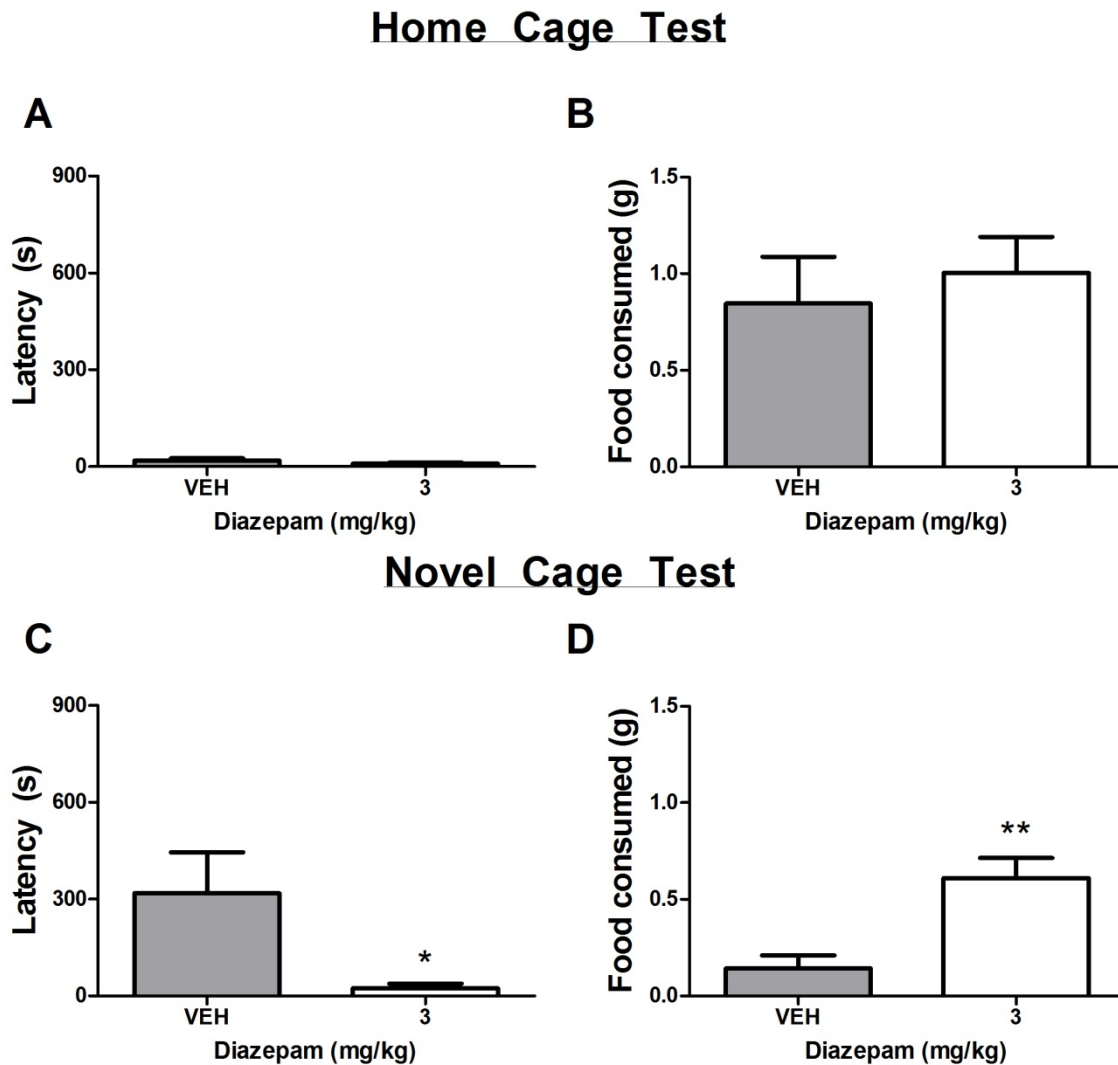


Figure 18. Effects of diazepam in the novelty-induced hypophagia test. Diazepam (3 mg/mg i.p.) did not affect (A) latency or (B) amount of food consumed in the home cage test, but reduced the (C) latency and increased the (D) amount of food consumed in the novel environment. n=6 per group. Data are expressed as mean \pm SEM, or as a percent of animals that exhibited the specified behavior. *p<0.05, **p<0.01 compared to VEH.

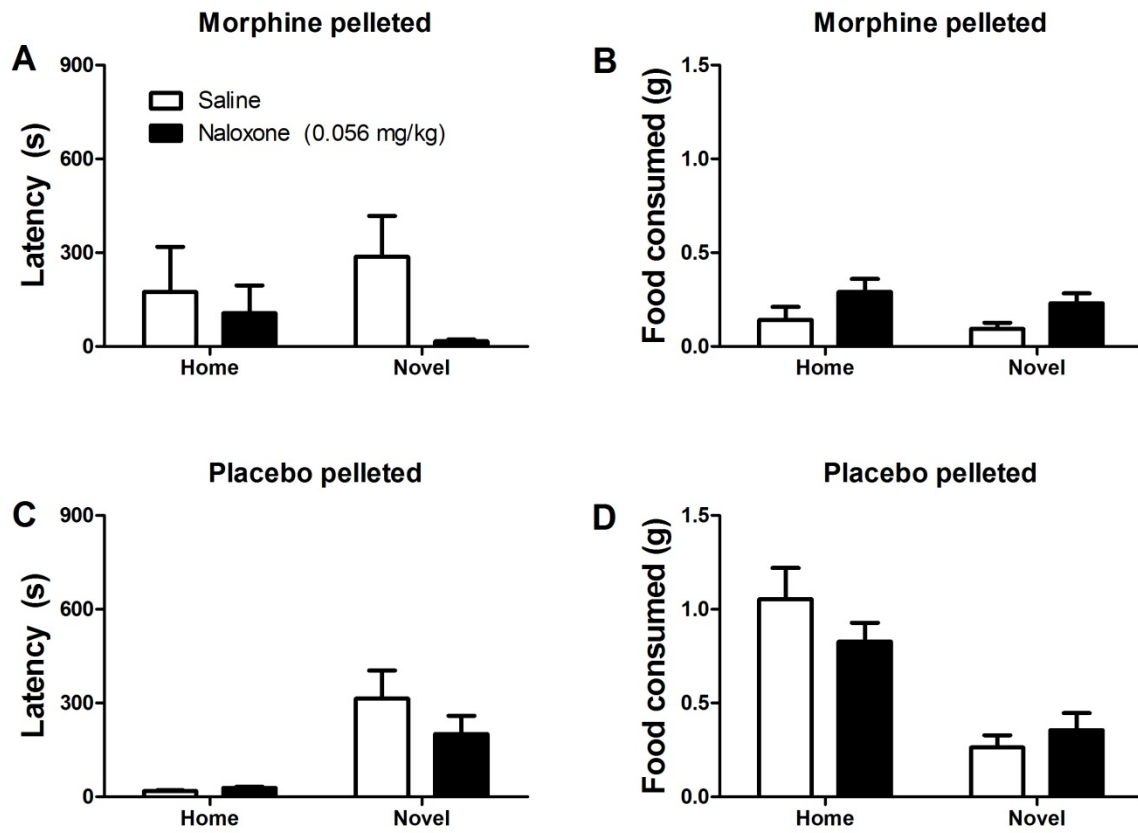


Figure 19. Effects of naloxone or saline in morphine-pelleted and placebo-pelleted mice in the novelty induced hypophagia test. There was no effect of naloxone or test cage in morphine-pelleted mice on (A) latency to approach and consume food or (B) amount of food consumed. In placebo-pelleted mice, there was an increase in (C) latency to approach and consume food in the novel environment and a (D) decrease in the amount of food consumed, but there was no effect of naloxone in any treatment. $n=6-11$ per group. Data are expressed as mean \pm SEM. * $p<0.01$ compared to saline.

3.3.5. Conditioned place avoidance – Procedure 1

These experiments sought to establish the place conditioning procedure by validating the apparatus as unbiased (i.e. mice do not have a preference for the black or white side) and replicating CPP effects of morphine and cocaine. Additionally, these experiments sought to replicate a published procedure for producing naloxone-precipitated morphine withdrawal CPA. We conducted a naloxone dose response curve experiment in non-dependent mice to determine its propensity to produce CPA on its own. We then selected the naloxone dose for the first CPA experiment based upon these findings. Upon lack of significant CPA following this dose in mice treated repeatedly with morphine, we tested a higher dose to see if it would produce greater CPA in morphine-treated than vehicle-treated mice.

3.3.5.1. Male ICR mice do not have initial preference to white or black side

To determine whether the place conditioning apparatus was biased for either side, time spent on each side was analyzed from baseline data. Mice showed no preference for the white side (446.6 ± 11.8 s) vs. black side (441.5 ± 11.7 s), but spent significantly less time in the central grey chamber (312.0 ± 11.4 s) [$F(2,189) = 42.9$; $p < 0.0001$; Figure 20].

3.3.5.2. Morphine and cocaine produce conditioned place preference

To ensure that place conditioning would be readily established, an initial study employed morphine and cocaine as positive controls in the place preference assay. Both morphine and cocaine produced a significant conditioned place preference [$F(2,23) = 4.4$; $p < 0.05$; Figure 21A] and there was no significant effect of conditioning treatment on locomotor activity during the expression test [$F(2,23) = 3.2$; $p = 0.06$; Figure 21B].

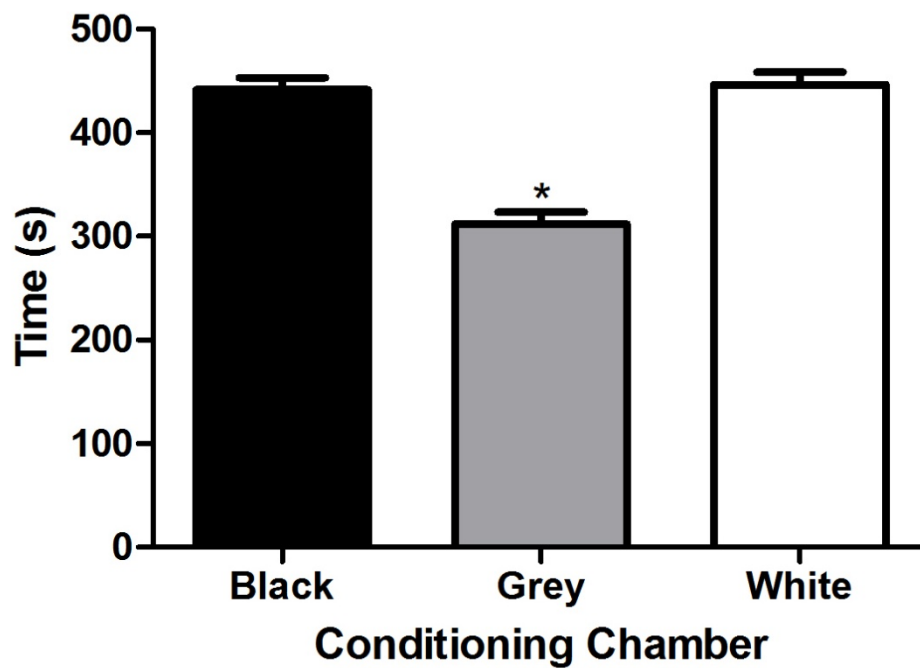


Figure 20. Male ICR mice do not exhibit a preference for the white versus black side during 20 min access but spend less time in the central grey chamber. * $p < 0.05$ compared to black side. $n = 64$ mice per group. Data are expressed as mean \pm SEM, or as a percent of animals that exhibited the specified behavior.

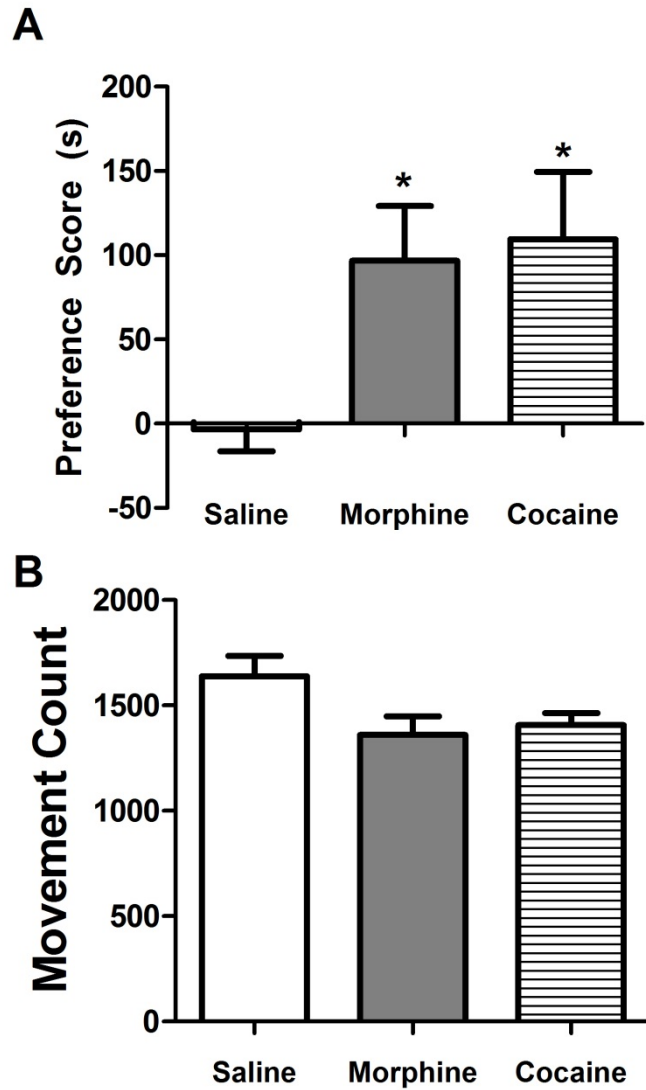


Figure 21. Conditioned place preference to morphine and cocaine. Pretreatment with morphine (10 mg/kg s.c.) or cocaine (20 mg/kg i.p.) produced (A) conditioned place preference in male ICR mice but (B) neither group of conditioned mice exhibited a significant change in locomotor activity during the expression test. $n=8-9$ mice per group. Data are expressed as mean \pm SEM. * $p<0.05$ compared to saline.

3.3.5.3. Naloxone produces conditioned place avoidance in naïve mice

To determine whether naloxone would produce CPA in non-dependent mice, a dose-response study for naloxone was conducted. Naloxone (0.3 and 1 mg/kg) produced significant CPA [$F(3,53) = 5.5$; $p < 0.01$; Figure 22A] but there was no effect on locomotor activity during the expression test [$p = 0.74$; Figure 22B].

3.3.5.4. Low dose Naloxone (0.1 mg/kg) does not produce conditioned place avoidance in mice given repeated s.c. injections of morphine or saline

Mice that received repeated administration of morphine (12.5 – 50 mg/kg s.c. b.i.d.) or saline and conditioned with naloxone (0.1 mg/kg) did not exhibit significant CPA [$p = 0.21$; Figure 23A] but there was a main effect of morphine treatment showing an increase in locomotor activity during the test session [$F(1,56) = 20.2$; $p < 0.0001$; Figure 23B].

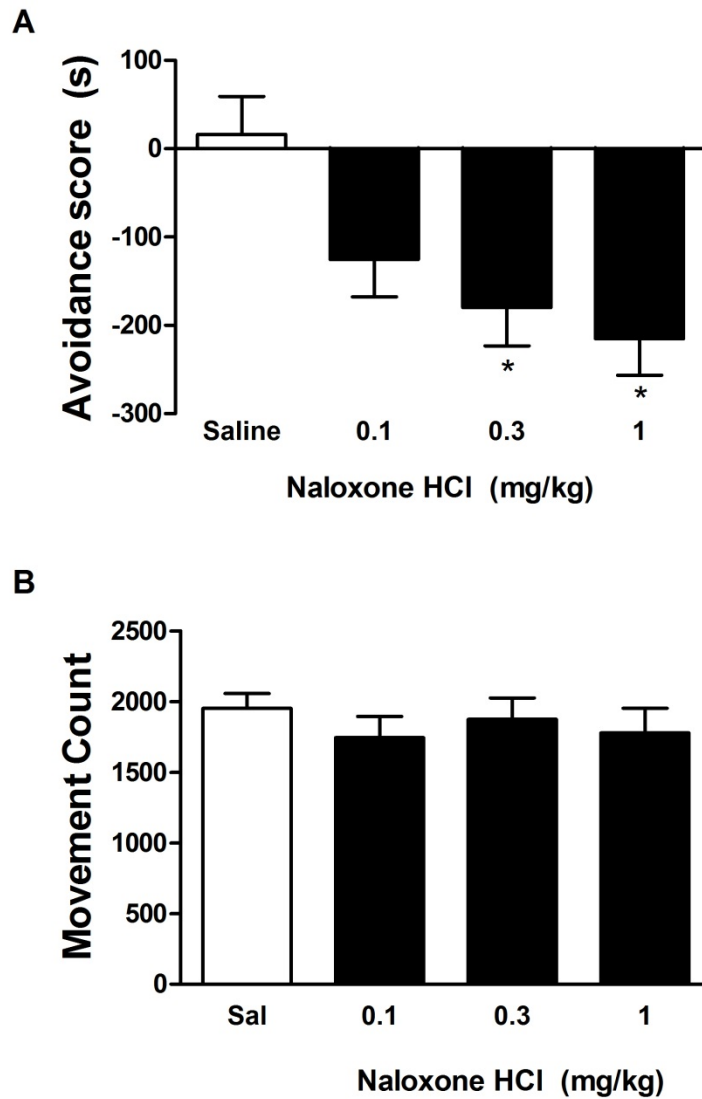


Figure 22. Repeated injections of naloxone produces conditioned place avoidance in mice. Naloxone (0.3 - 1 mg/kg s.c.) produces (A) CPA in mice after 3 conditioning sessions (20 min) as compared with saline but did not affect (B) locomotor activity during the expression test. n=13-15 per group. Data are expressed as mean \pm SEM. *p<0.05 compared to saline.

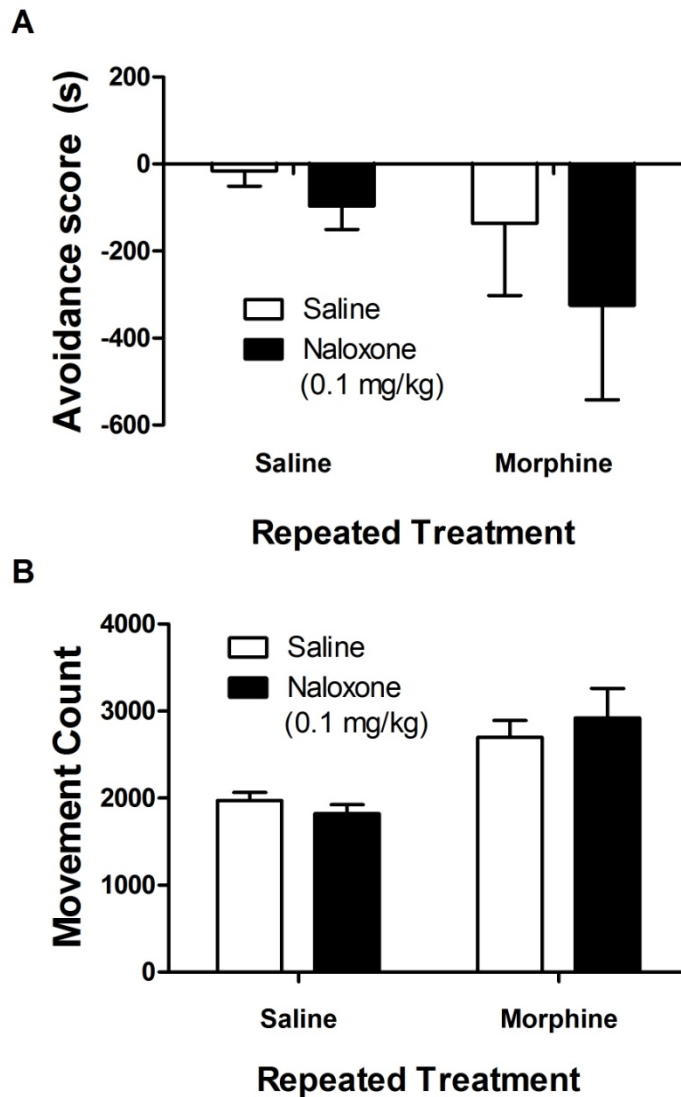


Figure 23. Low dose naloxone (0.1 mg/kg) does not produce significant conditioned place avoidance in mice treated repeatedly with vehicle or morphine. Mice treated repeatedly with morphine (12.5 – 50 mg/kg s.c. b.i.d.) or saline and conditioned with naloxone (0.1 mg/kg) did not exhibit (A) CPA behavior; however, mice receiving repeated morphine exhibited significant (B) increases in locomotor activity during the post-conditioning test. $n=8-9$ per group. Data are expressed as $\text{mean} \pm \text{SEM}$.

3.3.5.5. High dose naloxone (1 mg/kg) produces conditioned place avoidance in mice treated repeatedly with either escalating doses of morphine or saline

Both morphine-treated (12.5 – 50 mg/kg s.c. b.i.d.) and saline-treated mice conditioned with naloxone (1 mg/kg) exhibited significant CPA [$F(1,35) = 33.0$; $p < 0.0001$; Figure 24A], but there was no effect of morphine treatment [$p = 0.27$] nor was there an interaction effect [$p = 0.76$]. Therefore, no dissociation between naloxone's effects and precipitated withdrawal could be inferred. There was a main effect of morphine treatment [$F(1,33) = 69.0$; $p < 0.0001$; Figure 24B] on locomotor activity during the expression test but no effect of naloxone [$p = 0.52$] or interaction [$p = 0.66$].

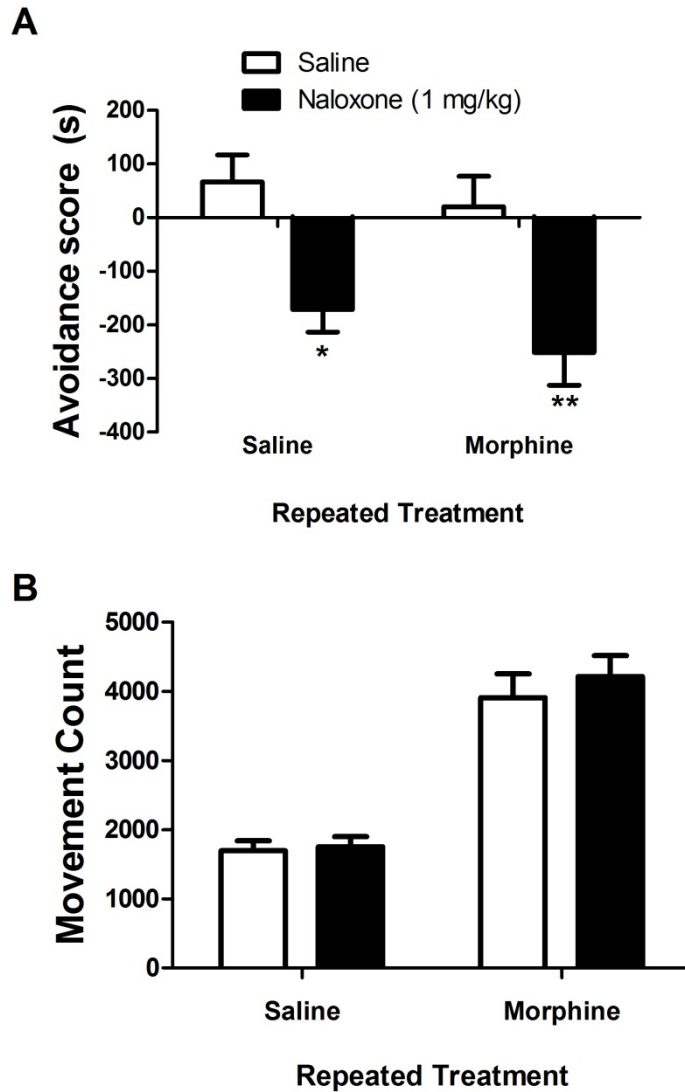


Figure 24. Naloxone (1 mg/kg s.c.) produced conditioned place avoidance in mice treated repeatedly with morphine and saline. Mice treated repeatedly with morphine (12.5 – 50 mg/kg s.c. b.i.d.) or saline and conditioned with naloxone (1 mg/kg) exhibited (A) CPA behavior. Mice receiving repeated morphine exhibited significant (B) increases in locomotor activity during the post-conditioning test. $n=8-11$ per group. * $p<0.05$, ** $p<0.01$ compared to saline. Data are expressed as $\text{mean} \pm \text{SEM}$.

3.3.6. Conditioned place avoidance – Procedure 2

In the previous CPA procedures, we used repeated injections of morphine (12.5 – 50 mg/kg s.c. b.i.d.) to induce dependence and conditioned mice over the course of 6 days during which they received one daily conditioning session, either naloxone or saline, for a total of three conditioning sessions each. The dose of naloxone (0.1 mg/kg) that did not produce CPA on its own was insufficient to produce CPA in morphine-treated mice. We tested a higher dose of naloxone (1 mg/kg) in order to precipitate a greater withdrawal response; however, the CPA produced by naloxone was not significantly different between mice treated repeatedly with morphine or saline. Thus, the dependence induction procedure used in these experiments was not sufficient to allow for naloxone to elicit CPA that was contingent upon morphine dependence. The following studies employed a morphine pellet in order to induce a greater degree of dependence in mice thus facilitating the separation of naloxone's effects and those of precipitated morphine withdrawal. In these studies, mice received two 30 min saline (non-withdrawal conditioning) sessions on the day prior to pellet implantation and then received two naloxone conditioning sessions 48 h and 52 h post pellet implantation.

3.3.6.1. 75 mg morphine pellets produce conditioned place preference

In an initial experiment, we tested if implantation of the morphine pellet would elicit a conditioned place preference. A finding in which morphine alone elicited a place preference would have important implications for the naloxone precipitated withdrawal place avoidance experiments. Specifically, morphine in the absence of naloxone might elicit a place preference, which would confound interpretation of the withdrawal experiments since it would be unclear whether mice would be expressing avoidance to the side paired with withdrawal or a preference to the side paired with morphine alone. Accordingly, morphine-pelleted mice were assessed in

the conditioned place procedure using a similar procedure employed for the withdrawal studies. Prior to pellet implantation, mice were conditioned on one side of the apparatus and then following pellet implantation, during what would be the withdrawal conditioning session, each mouse received a subcutaneous injection of saline. A t-test revealed a significant increase in time spent on the side paired with the morphine pellet [Figure 25A] suggesting that the continuous morphine exposure from the pellet produced CPP. There was no effect of morphine pellet on locomotor activity during the expression test [Figure 25B]. In order to remove the potential confound of morphine CPP, we implanted the morphine pellets after the saline conditioning (non-withdrawal) day.

3.3.6.2. Naloxone-precipitated morphine withdrawal produces conditioned place avoidance behavior at a dose that does not elicit this effect in non-dependent mice

To determine the doses of naloxone that produced CPA in dependent and non-dependent mice, we examined the dose-response relationship of naloxone (0.03, 0.056, and 0.1 mg/kg) in morphine- and placebo-pelleted mice. Naloxone (0.056 and 0.1 mg/kg) produced a significant CPA in morphine-pelleted mice [$F(3,74) = 4.3$; $p < 0.01$; Figure 26A]. In contrast, only naloxone (0.1 mg/kg) produced CPA in placebo-pelleted animals [$F(3,80) = 4.3$; $p < 0.01$; Figure 26B] and a planned comparison t-test revealed this to be a significantly lower than that observed in the morphine-pelleted animals [$t = 2.6$, $p < 0.05$]. Additionally, morphine-pelleted mice receiving naloxone exhibited reduced locomotor activity during the expression test [$F(3,74) = 7.2$; $p < 0.001$; Figure 26C] whereas placebo-pelleted mice receiving naloxone showed no change in activity [$p = 0.34$; Figure 26D]. Since naloxone (0.056 mg/kg) did not produce significant CPA in non-dependent mice, this dose was selected for all subsequent studies to ensure that the avoidance behavior observed was due to morphine withdrawal alone.

3.3.6.3. *Naloxone precipitates somatic withdrawal signs in mice*

In morphine-pelleted mice, naloxone elicited significant paw flutters [$F(5,26) = 12.0$; $p < 0.0001$; Figure 27A], head shakes [$F(5,26) = 12.9$; $p < 0.0001$; Figure 27B], jumps [$F(5,26) = 17.6$; $p < 0.0001$; Figure 27C] and diarrhea [$z = -3.4$; $p < 0.001$; Figure 27D]. Naloxone (0.3 and 1 mg/kg) produced significant increases in paw flutters, head shakes, and diarrhea whereas naloxone (0.1, 0.3, and 1 mg/kg) produced significant increases in jumping.

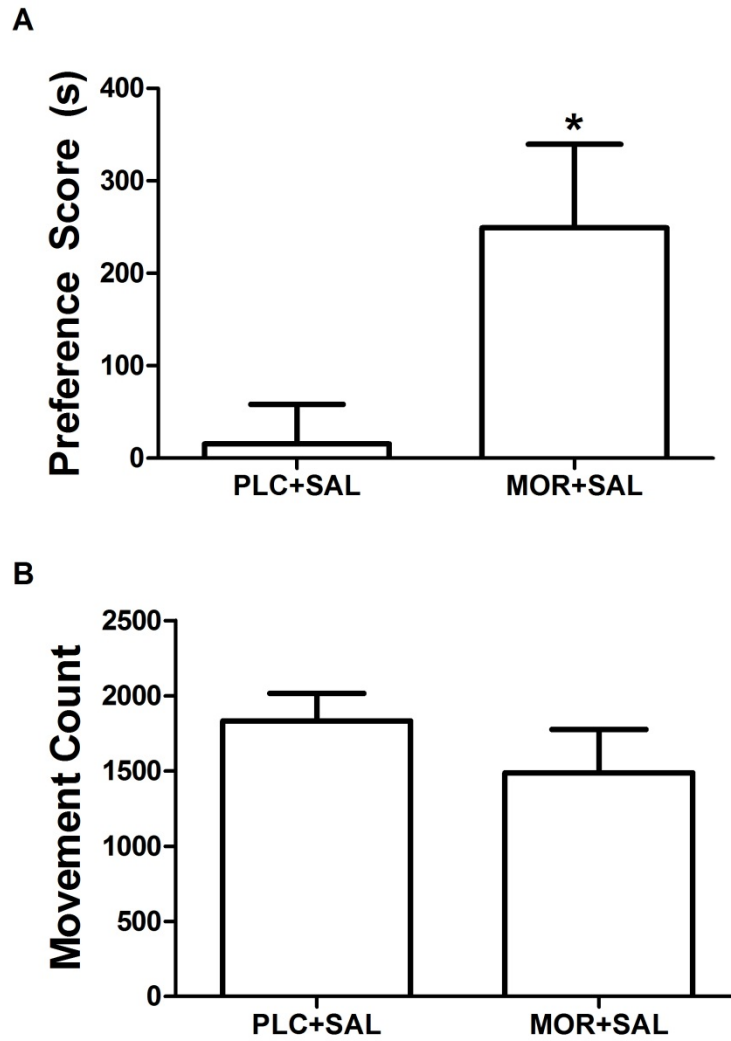


Figure 25. Conditioned place preference to 75 mg morphine pellet. Presence of a 75 mg morphine pellet produced (A) conditioned place preference in mice receiving saline but (B) no effect on locomotor activity during the expression test. $n=8-9$ mice per group. Data are expressed as $\text{mean} \pm \text{SEM}$. * $p < 0.05$

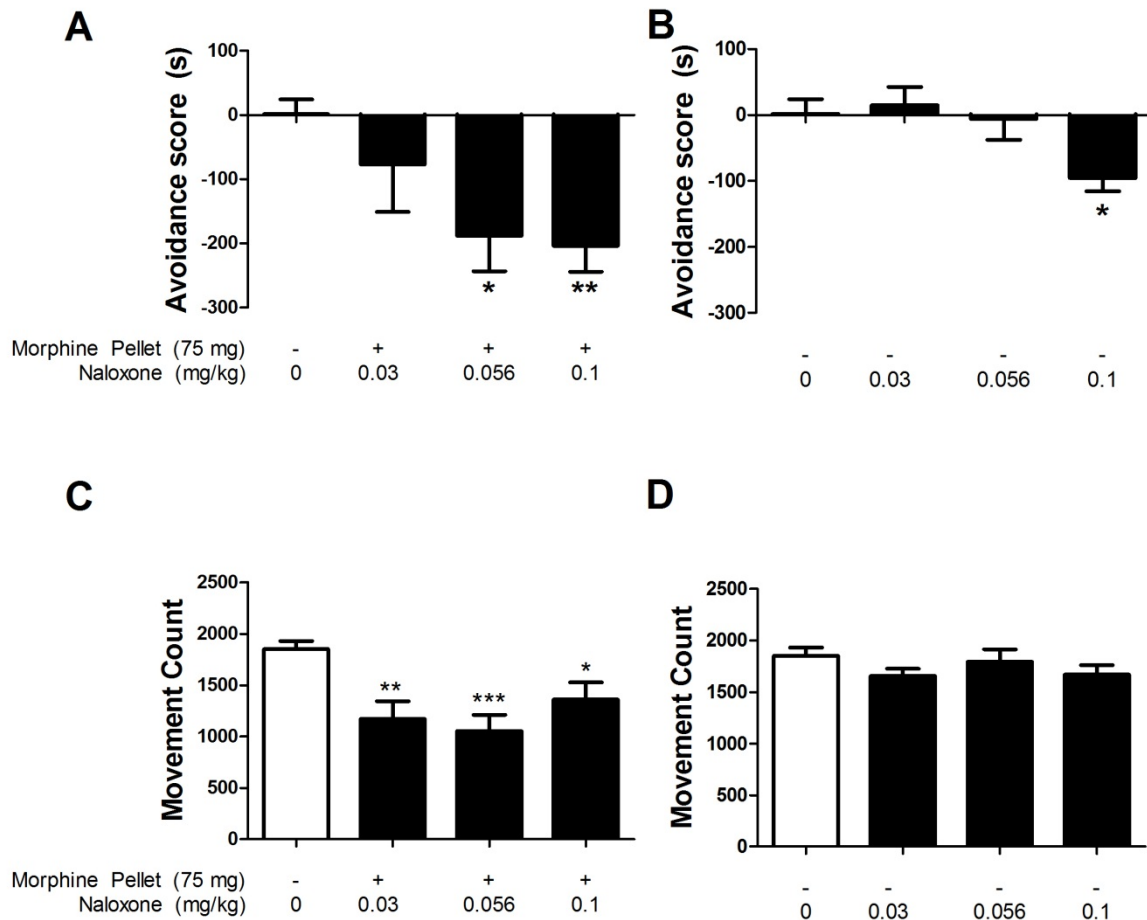


Figure 26. Naloxone-precipitated morphine withdrawal produced conditioned place avoidance behavior in mice. In (A) morphine-pelleted mice, naloxone (0.056 and 0.1 mg/kg) produced a significant CPA behavior whereas in (B) placebo-pelleted mice, only naloxone (0.1 mg/kg) produced CPA. Naloxone conditioning did not have a significant effect on locomotor activity during the expression test in (D) placebo-pelleted mice. $n=16-26$ mice per group. Data are expressed as mean \pm SEM, or as a percent of animals that exhibited the specified behavior. * $p<0.05$, ** $p<0.01$ compared to Placebo+SAL control.

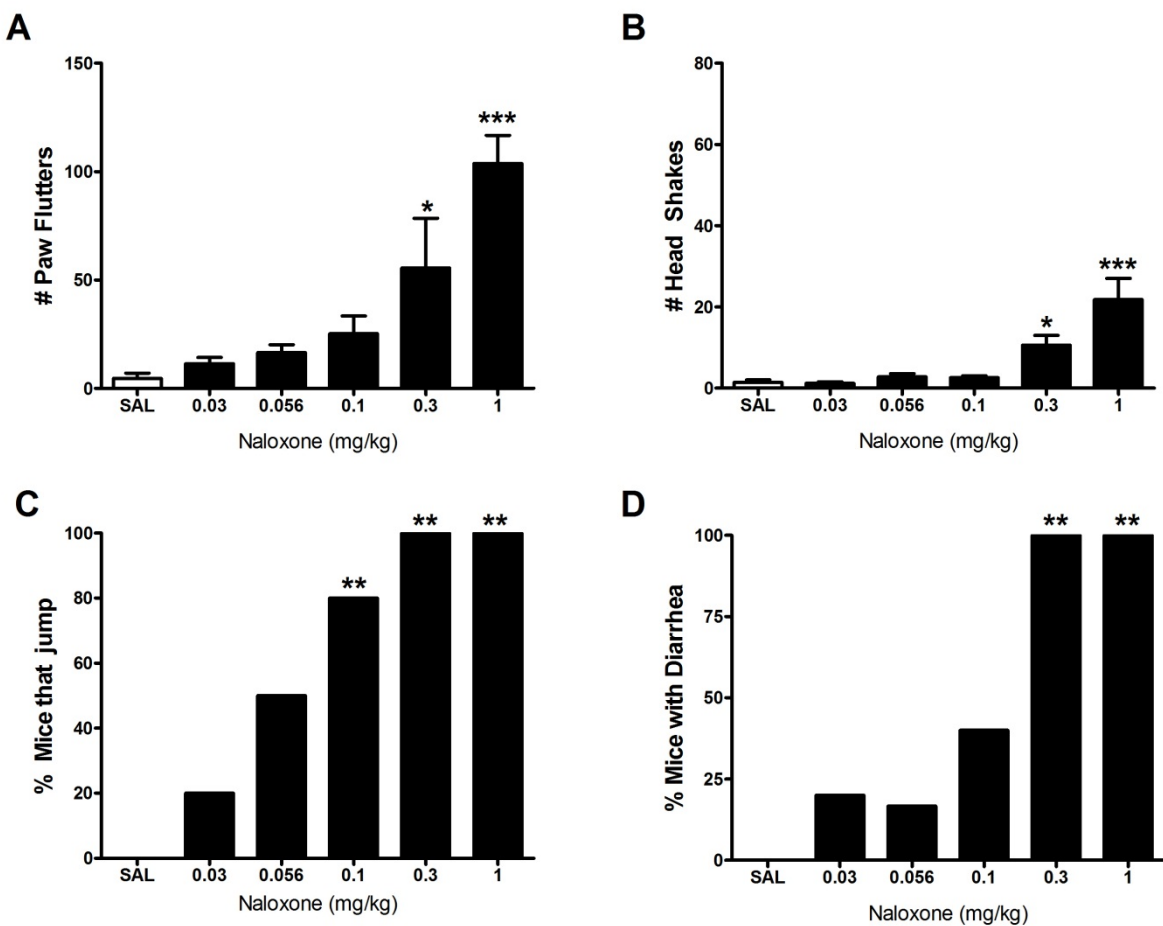


Figure 27. Naloxone elicits withdrawal behaviors in mice when administered s.c. 48 h following implantation of a 75 mg morphine pellet. Behaviors scored were (A) paw flutters, (B) head shakes, (C) presence of jumping behavior, and (D) presence of diarrhea. $n=5-6$ per group. Data are expressed as mean \pm SEM, or as a percent of animals that exhibited the specified behavior.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

3.4. Summary

These studies sought to evaluate the effects of morphine withdrawal in multiple established behavioral tests as well as a novel assay developed to examine escape behavior. In the light/dark box, naloxone significantly increased the amount of time spent in the light side in morphine-pelleted [Figure 9] but not placebo-pelleted mice [Figure 10], such that there was essentially no preference for one side or the other. The finding that naloxone affected only morphine-pelleted mice suggests that this behavior was the result of naloxone-precipitated morphine withdrawal rather than naloxone alone. To determine if increases in time spent in the light side might reflect an escape behavior, we tested the effects of morphine withdrawal in a novel procedure developed to model escape. In a preliminary study, naloxone significantly reduced the latency to escape a mouse cage with the lid ajar in morphine-pelleted, but not placebo-pelleted, mice [Figure 11]. Subsequently, the design of the mouse cage was modified so that the lid was closed and a 5x5 cm hole was cut out of one end of the lid with a wire mesh ramp leading out. Naloxone reduced the latency for the mice to escape in this procedure in morphine-pelleted mice but not placebo-pelleted mice [Figure 12]. To further determine if escape behavior was a consequence of jumping, the procedure was modified to facilitate escaping so that mice could easily reach the wire mesh. Morphine-pelleted mice treated with naloxone (0.056 mg/kg) exhibited significant reductions in escape latency and this effect was maintained when mice that exhibited jumping were excluded [Figure 13]. These data suggest that morphine withdrawal led to increases in escape behavior.

In marble burying, neither spontaneous morphine withdrawal [Figure 14 and 15] nor naloxone treatment in morphine-pelleted mice [Figure 16] affected digging behavior. In the novelty-induced hypophagia test, diazepam increased the amount of food consumed and

decreased the latency to consume in the novel environment, but not in the home cage, an effect that has been previously established thus serving as a positive control [Figure 18]. There was no effect of naloxone in morphine-pelleted or placebo-pelleted mice. However, implantation of 75 mg morphine pellets resulted in reductions in food consumption during the home cage test as compared with placebo-pelleted mice [Figure 19].

In place conditioning, mice exhibited no preference for the white vs. black side [Figure 20] demonstrating an unbiased apparatus. Morphine (10 mg/kg) and cocaine (20 mg/kg) served as positive controls to establish the general conditioning procedure and both produced CPP [Figure 21]. An established procedure in which daily conditioning sessions were carried out for six days was adapted for the initial experiments (Broseta *et al.*, 2005). First, naloxone was tested alone to determine doses which could produce CPA and naloxone (0.3 – 1 mg/kg) produced significant CPA [Figure 22]. Using the dependence procedure from a published CPA protocol (Broseta *et al.*, 2005), we tested the effects of naloxone (0.1 mg/kg) in saline and morphine treated mice, but found no acquisition of CPA [Figure 23]. The next experiment used naloxone (1 mg/kg), the dose that was used in the study this procedure was adapted from, but found that no distinction between CPA in morphine treated and saline treated mice could be made [Figure 24]. In order to induce a greater level of dependence to facilitate a separation between naloxone's effects and those of precipitated morphine withdrawal, we developed a new procedure in which mice were implanted subcutaneously with 75 mg morphine pellets. Due to concerns about the presence of morphine during the saline (non-withdrawal) conditioning trials, we tested whether the presence of 75 mg morphine pellets could serve as an unconditioned stimulus to produce CPP and found that this was indeed the case [Figure 25]. Thus, the design of the CPA experiment was modified such that morphine pellets were implanted after the saline

conditioning (non-withdrawal) sessions. Naloxone (0.03 – 0.1 mg/kg) conditioning trials were conducted 2 days after morphine pellet or placebo pellet implantation. Naloxone (0.056 mg/kg) produced significant CPA in morphine-pelleted mice, but did not elicit a preference in placebo-pelleted mice [Figure 26], suggesting that conditioning to naloxone-precipitated morphine withdrawal was producing an avoidance behavior in these mice. Finally, to determine the degree to which these doses of naloxone were precipitating somatic withdrawal signs, we assessed naloxone (0.03 – 1 mg/kg) during a 30 min session in which behaviors were recorded 48 h after pellet implantation, which corresponded to the time point of the first conditioning session in CPA. Naloxone (0.056 mg/kg) produced negligible increases in withdrawal signs [Figure 27]. Based on these studies we selected the CPA procedure to further evaluate the effects of endocannabinoid catabolic inhibitors on morphine withdrawal.

Chapter 4: Effects of endocannabinoid catabolic inhibitors on negative motivational aspects of morphine withdrawal

4.1. Introduction: Conditioned place avoidance to opioid withdrawal in rodents

In Chapter 2 it was demonstrated that dual inhibition of FAAH and MAGL reduces both spontaneous and naloxone-precipitated morphine withdrawal signs in mice. As well, the effects of cannabinoids including endocannabinoid catabolic inhibitors have been well established in the literature in regards to their effects on somatic signs of opioid withdrawal [see Table 1]. However, it remains to be established whether cannabinoids are also reducing the motivational aspects of opioid withdrawal. In Chapter 3, the effects of morphine withdrawal in a number of behavioral assays were reported and among them the CPA procedure produced a clear withdrawal-related effect that has also been well characterized in the literature. From these experiments the CPA procedure was selected to assess endocannabinoid catabolic inhibitors for their ability to reduce the negative motivational aspects of morphine withdrawal.

Naloxone-precipitated morphine withdrawal CPA is a highly sensitive measure of withdrawal with very low doses producing this withdrawal conditioned behavior as compared with those doses necessary to elicit physical withdrawal signs (Caillé *et al.*, 1999; Frenois *et al.*, 2002). Early studies in mice (Mucha *et al.*, 1987) and rats (Hand *et al.*, 1988; Mucha *et al.*, 1982) first demonstrated the phenomena that naloxone produced enhanced CPA in morphine exposed rodents, suggesting a withdrawal component to this effect. Subsequently the brain regions important for morphine withdrawal CPA were elucidated by administering microinjections of the opioid antagonist methylnaloxonium through stereotaxically implanted guide cannulae aimed at

the nucleus accumbens and amygdala in rats treated repeatedly with morphine (Stinus *et al.*, 1990).

While both humans and rodents exhibit overt symptoms following opioid withdrawal, e.g. diarrhea, it is more difficult to ascertain aspects of opioid withdrawal that relate to the aversive qualities. As discussed in Chapter 1, humans undergoing opioid withdrawal experience both physical and psychological effects that accompany withdrawal. For example, people have been reported to experience dysphoria, anhedonia, nausea, anxiety, and panic attacks during withdrawal from an opioid (Farrell, 1994; Gossop, 1988; Jasinski, 1981; Wesson *et al.*, 2003). In rodents, the aversive effects of opioid withdrawal can be inferred through behavioral assays which employ the withdrawal state as an unconditioned stimulus and pair it with a neutral context (the conditioned stimulus) in a Pavlovian paradigm called conditioned place avoidance or conditioned place aversion (CPA) (Tzschentke, 2007). Opioid withdrawal serving as an unconditioned stimulus in this procedure has successfully been modeled in mice (Broseta *et al.*, 2005; Maldonado *et al.*, 2003; Olson *et al.*, 2006; Sato *et al.*, 2005; Shoblock *et al.*, 2005) and rats (Gracy *et al.*, 2001; Hand *et al.*, 1988; Parker *et al.*, 1992; Schnur *et al.*, 1992; Stinus *et al.*, 2000; Stinus *et al.*, 1990; Watanabe *et al.*, 2003).

Comparison of the ability of naloxone to produce CPA and somatic withdrawal signs by Schulteis *et al.* (1994) first demonstrated that naloxone was more potent at producing withdrawal effects in behaviors including disruption of operant responding for food, elevating ICSS thresholds and producing CPA at doses from 0.004 – 0.01 mg/kg. In contrast 0.01 mg/kg naloxone, which produced withdrawal-related effects in all measures, did not significantly increase somatic withdrawal behaviors.

Since distinct brain regions mediate both the negative motivational and somatic aspects of withdrawal, our previous work demonstrating that inhibition of endocannabinoid catabolism reduces somatic signs of morphine withdrawal does not indicate that the aversive aspects of withdrawal are also being reduced. Thus, we investigated if endocannabinoid catabolic inhibitors would reduce the negative motivational aspects of opioid withdrawal as assessed in the CPA paradigm.

4.1.1. Rationale and Hypothesis

The CPA procedure is a well established model of aversive effects of opioid withdrawal (Broseta *et al.*, 2005; Frenois *et al.*, 2002; Maldonado *et al.*, 2004; Sato *et al.*, 2005; Stinus *et al.*, 2000; Stinus *et al.*, 1990; Watanabe *et al.*, 2002) and has been shown to be sensitive to pharmacological treatments (e.g. clonidine) (Kosten, 1994; Schulteis *et al.*, 1998a) which are known to be effective at reducing opioid withdrawal in humans (Gold *et al.*, 1978; Gossop, 1988). Thus, we selected this procedure to determine if inhibition of endocannabinoid catabolism would be effective at reducing the negative motivational aspects of opioid withdrawal in mice.

We hypothesized that inhibition of endocannabinoid catabolism would reduce the acquisition of CPA to naloxone-precipitated morphine withdrawal.

4.2. Methods

4.2.1. Subjects

Male ICR mice (Harlan, Indianapolis, IN) with a body mass of 27-32 g were used for all experiments. Mice were group-housed (four per cage) on a 12/12 light/dark cycle (lights on at 0600 h) and given food and water *ad libitum*. All efforts were made to minimize animal suffering and number of animals used. All animal protocols were approved by the Virginia

Commonwealth University Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 2011).

4.2.2. *Drugs*

Morphine pellets (75 mg), placebo pellets, morphine sulfate, delta-9-tetrahydrocannabinol (THC) and clonidine HCl were obtained from the National Institute on Drug Abuse (Bethesda, MD) and naloxone hydrochloride was purchased from Sigma Aldrich (St. Louis, MO). JZL184, PF-3845, and SA-57 were provided by the Cravatt laboratory (Scripps, La Jolla, CA). Naloxone HCl and clonidine were dissolved in physiological saline (0.9% NaCl) and drug concentrations were calculated using the weight of the salt as used previously (Ramesh *et al.*, 2011). Morphine sulfate was dissolved in physiological saline (0.9% NaCl) and drug concentrations were calculated based upon the free base. JZL184, PF-3845, SA-57 and THC were dissolved in ethanol and Emulphor-620 (Rhone-Poulenc, Princeton, NJ) and then diluted with physiological saline to form a vehicle mixture of ethanol:Emulphor-620:saline in a ratio of 1:1:18. JZL184, PF-3845, and SA-57 were administered intraperitoneally, THC, clonidine, and naloxone HCl was administered subcutaneously, and all drugs were administered in a volume of 10µl per g body mass.

4.2.3. *Conditioned place avoidance*

The place conditioning chambers (MED Associates, St. Albans, VT, USA) were comprised of three distinct compartments separated by manual sliding doors. A central grey compartment with a plastic floor separated a black compartment with a floor comprised metal bars (0.31 cm) placed in a parallel series and a white compartment with a metal grid mesh floor

(0.625×0.625 cm). The dimensions ($w \times l \times h$) of each compartment were as follows: grey compartment (8.25×12.5×13 cm), black and white compartments (16.5×12.5×13cm).

The conditioning procedure was an unbiased (counterbalanced) design, i.e. mice were paired with naloxone on either the black or white side. Mice were handled for 3 days prior to start of the experiment to acclimate them to the experimenter. On day 1, and all subsequent days, mice were brought into the room and acclimated for at least 45 min. The mice were placed gently into the central grey chamber with all the doors pre-opened and mice were allowed to freely ambulate between compartments for 20 min. Med-PC software recorded the time spent in each compartment and the number of beam breaks to estimate locomotor activity. Mice were then returned to their home cage. On day 2 (saline conditioning; non-withdrawal day), mice were injected s.c. with saline and placed directly in the chamber designated as the saline side where they remained for 30 min. Mice were returned to their home cage and remained in the lab until the second conditioning session of the day which repeats the same procedure 4 h later. On day 3, mice were implanted with a 75 mg morphine pellet under anesthesia (see section 2.2.5. for pellet implantation surgery). On day 5, mice were pretreated either 2 h prior to the first conditioning session (JZL184, PF-3845, SA-57) or 30 min prior to both conditioning sessions (THC, clonidine, morphine). At 48 h post-pellet implantation, mice were injected with either naloxone HCl or saline (placebo+saline controls) and placed into the naloxone-designated compartment for 30 min. The naloxone conditioning procedure was then repeated 4 h later, i.e. 52 h post pellet implantation. On day 6, mice were tested for the expression of CPA during a 20 min test.

Avoidance scores were calculated by subtracting the amount of time spent on the naloxone paired side during the pre-conditioning test from the time spent on the post-conditioning test. Thus, a negative score indicated that the animal spent less time on that side

following the conditioning procedure and this value reflected a change from the animal's initial preference for that side. Positive scores are considered to reflect CPP and are used to infer the rewarding effects of drugs while negative scores are considered to reflect an aversive state.

4.2.4. Statistical Analysis

All data are reported as mean \pm SEM. Data were analyzed using either Student's t-test or one-way between measures analysis of variance. Dunnett's test was used to compare drug treatments with vehicle and Newman-Keuls post-hoc test was employed for comparisons between various treatments. The z-test of two proportions was used to analyze jumping data. Differences were considered statistically significant at $p < 0.05$.

4.3. Results

4.3.1. Morphine reduces both withdrawal jumping and acquisition of naloxone-precipitated morphine withdrawal conditioned place avoidance

As a positive control, morphine (30 mg/kg) was administered s.c. 30 min prior to each naloxone conditioning session. Morphine pretreatment significantly attenuated naloxone-precipitated morphine withdrawal CPA [$F(2,23)=9.6$; $p < 0.001$; Figure 28A] and withdrawal jumping [$p < 0.01$; Figure 28B]. There was an effect of conditioning treatment on locomotor activity during the expression test, with mice that were pretreated with saline, but not morphine, exhibiting reduced movement counts with respect to placebo mice [$F(2,23) = 4.2$; $p < 0.05$; Figure 28C].

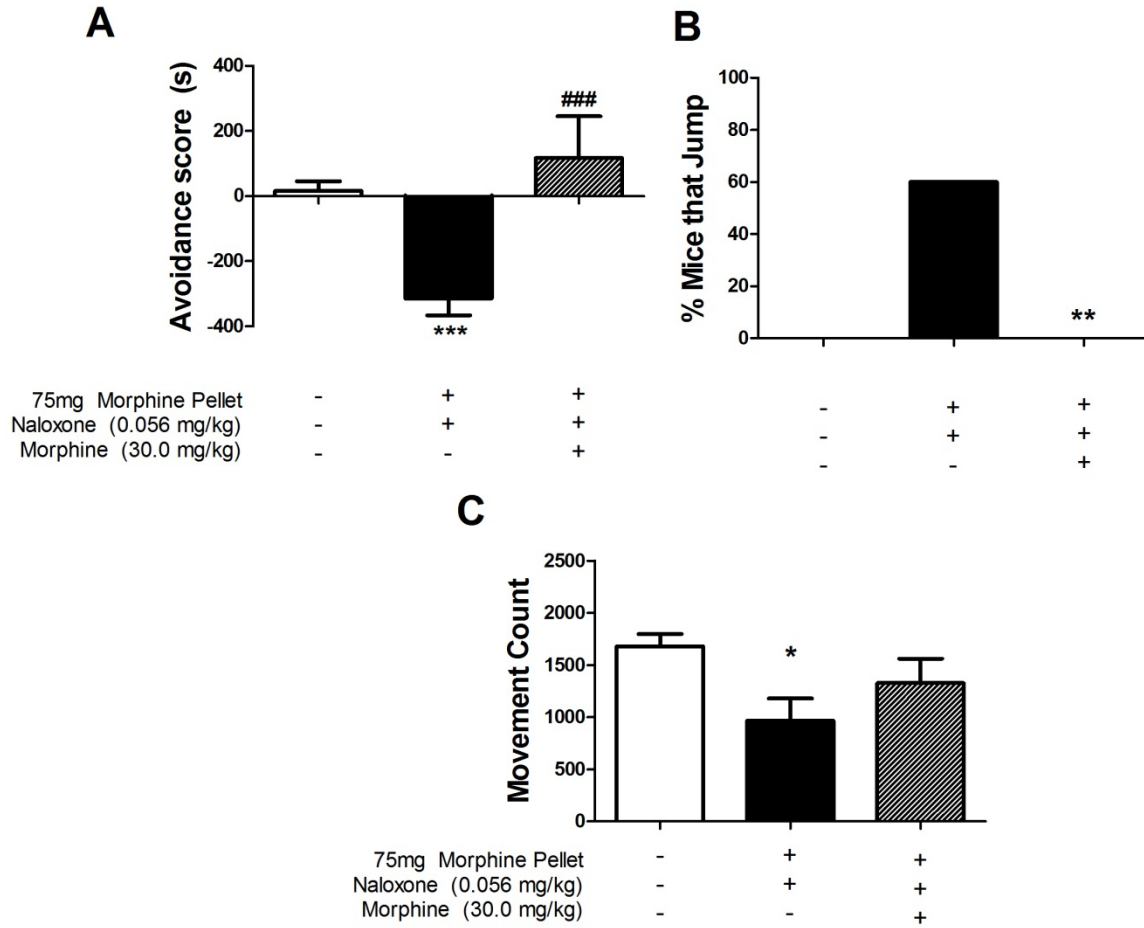


Figure 28. Morphine pretreatment blocked both naloxone-precipitated morphine withdrawal CPA and jumping behavior. Morphine (30 mg/kg) administered 30 min prior to naloxone conditioning blocked (A) withdrawal CPA and (B) withdrawal jumping. $n=6-10$ per group. Data are expressed as mean \pm SEM, or as a percent of animals that exhibited the specified behavior. ** $p<0.01$, *** $p<0.001$ compared to Placebo+SAL control for (A,C) and compared to MOR+NLX for (B), ### $p<0.001$ compared with the MOR+NLX group.

4.3.2. Clonidine reduces acquisition of conditioned place avoidance to naloxone-precipitated morphine withdrawal but not withdrawal jumping

We tested whether clonidine, the α_2 adrenergic agonist would reduce the acquisition of naloxone-precipitated morphine withdrawal induced CPA. Clonidine (0.1 and 0.3 mg/kg) pretreatment significantly reduced CPA [$F(4,69) = 4.5$; $p < 0.001$; Figure 29A], but did not significantly affect withdrawal jumping [Figure 29B]. Clonidine (0.1 and 0.3 mg/kg) attenuated the hypomotility during the expression test [$F(4,49) = 4.8$; $p < 0.01$; Figure 29C].

4.3.3. JZL184 does not produce conditioned place preference or avoidance

To determine if inhibition of MAGL and the resulting elevation of 2-AG might produce either CPP or CPA on its own, naïve mice were tested with JZL184 (4 or 40 mg/kg) or vehicle. JZL184 was administered i.p. 2 h prior to the conditioning session. Following conditioning, mice did not display a significant preference or avoidance to the JZL184-paired side [Figure 30A]. Additionally, conditioning with JZL184 did not affect locomotor activity during the expression test [Figure 30B].

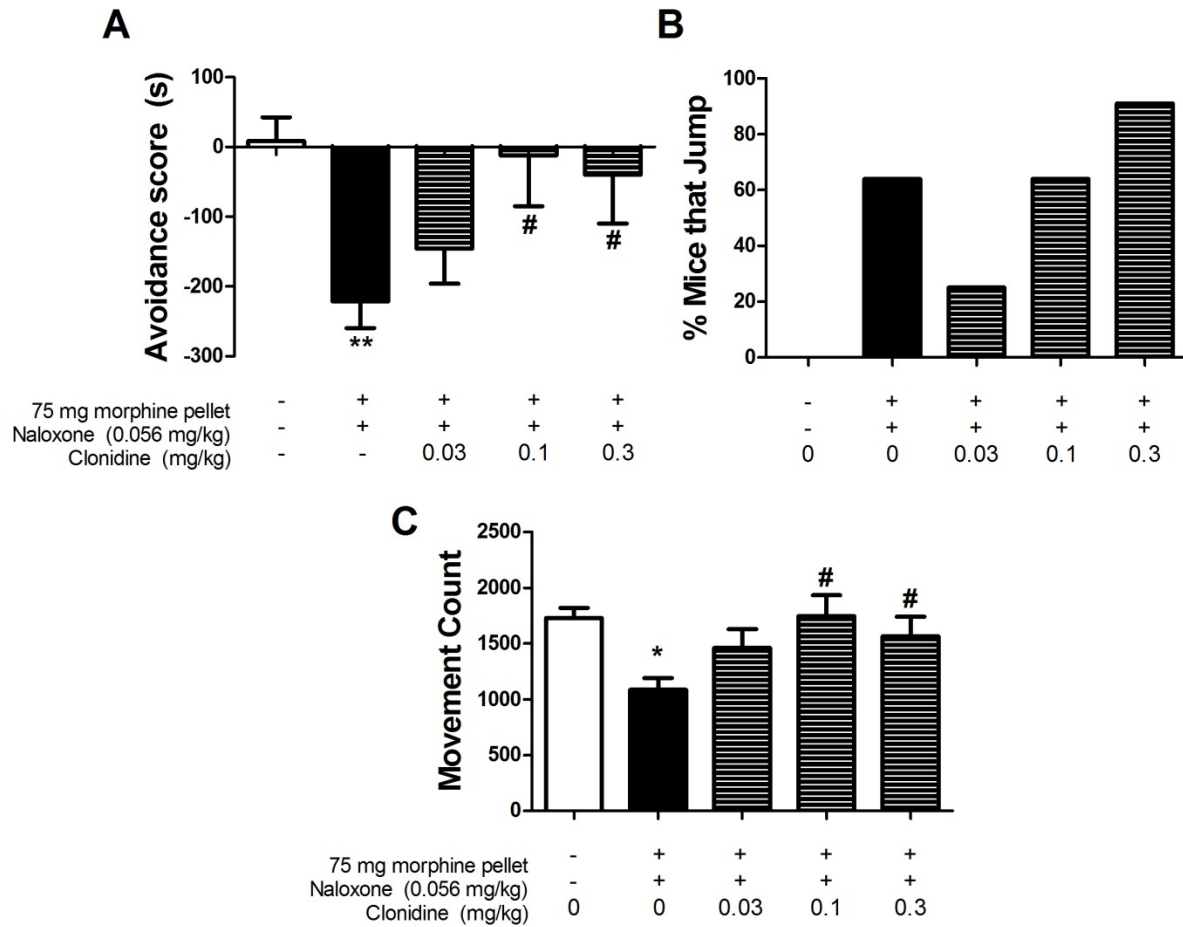


Figure 29. The alpha2 adrenergic agonist clonidine reduced naloxone-precipitated morphine withdrawal CPA, but not withdrawal jumping. Clonidine (0.1 and 0.3 mg/kg) administered s.c. 30 min prior to the first conditioning session reduced (A) acquisition of naloxone-precipitated morphine withdrawal CPA but did not affect (B) the percentage of mice that exhibited jumping behavior. Clonidine (0.1 mg/kg) administered prior to conditioning also significantly reduced (C) hypomotility during the expression test. n=11-20 per group. Data are expressed as mean±SEM, or as a percent of animals that exhibited the specified behavior. * p<0.05, ** p<0.01, compared to Placebo+SAL control for (A,C), # p<0.05, compared with the MOR+NLX group.

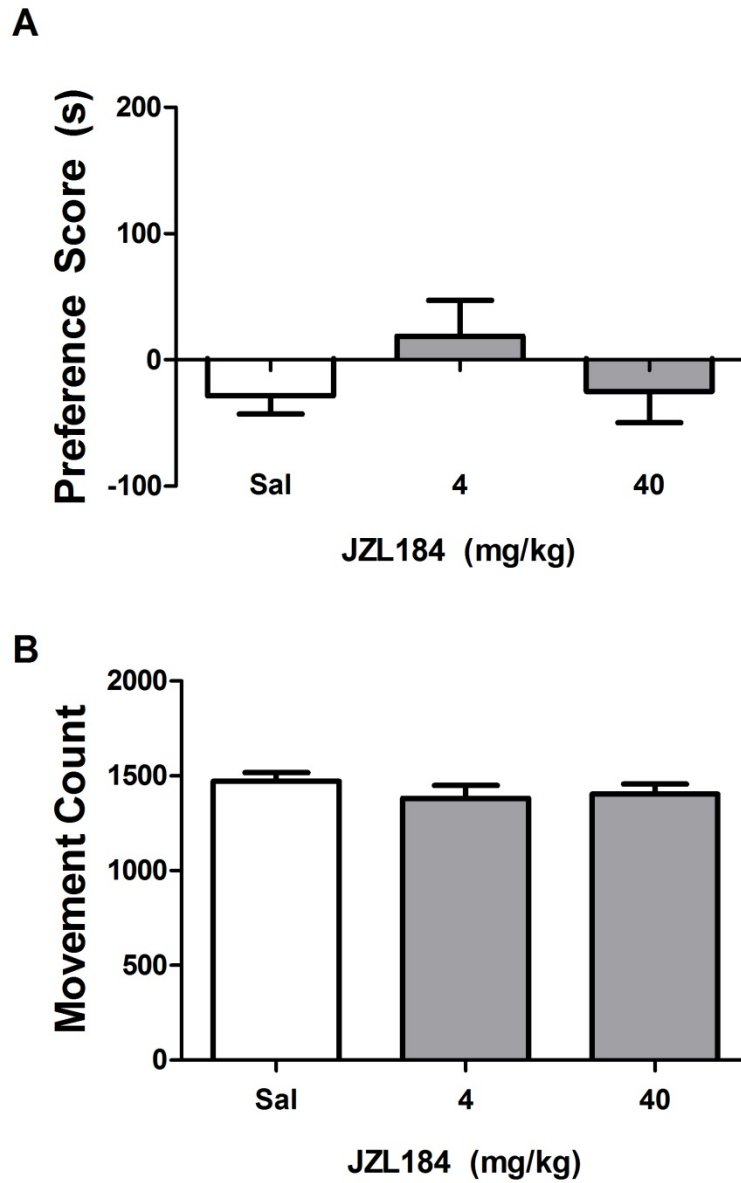


Figure 30. JZL184 did not produce conditioned place preference or avoidance in mice. JZL184 (4 or 40 mg/kg i.p.) was administered 2 h prior to the first conditioning session. Upon testing for expression, mice exhibited no (A) preference or avoidance behavior or (B) differences in locomotor activity. $n=7-8$ per group. Data are expressed as mean \pm SEM.

4.3.4. JZL184 reduces withdrawal jumping but does not affect acquisition of conditioned place avoidance to morphine withdrawal

In order to determine if inhibition of MAGL would affect the negative motivational consequences of morphine withdrawal, JZL184 was tested in the CPA procedure. JZL184 (4 or 40 mg/kg) was administered i.p. 2 h prior to the first conditioning session. Since JZL184 maintains elevated 2-AG for up to 8 h (Long *et al.*, 2009a), only a single injection of the compound was administered because the second conditioning session occurred 6 h post JZL184 pretreatment which is within this time frame. Naloxone-precipitated withdrawal produced a CPA response [$F(3,49) = 7.4$; $p < 0.001$; Figure 31A] which was not affected by JZL184. In contrast to JZL184's lack of effects on acquisition of CPA, the high dose of 40 mg/kg significantly reduced the number of mice that exhibited jumping behavior during the conditioning sessions [Figure 31B]. Mice that received morphine pellets all had equivalent reductions in locomotor activity during the expression test [$F(3,50) = 6.6$; $p < 0.001$; Figure 31C].

4.3.5. PF-3845 does not affect withdrawal jumping nor does it affect acquisition of conditioned place avoidance to naloxone-precipitated morphine withdrawal

To test whether inhibition of FAAH would affect the negative motivational consequences of morphine withdrawal, PF-3845 was tested in the CPA procedure. PF-3845 (1, 3 or 10 mg/kg) was administered i.p. 2 h prior to the first conditioning session. Since PF-3845 maintains elevated AEA for up to 8 h (Ahn *et al.*, 2009), a single injection of the compound was necessary since the second conditioning session occurred 6 h after PF-3845 pretreatment. Naloxone-precipitated withdrawal produced a CPA response [$F(4,50) = 7.7$; $p < 0.0001$; Figure 32A] which was not affected by PF-3845. Furthermore, PF-3845 (10 mg/kg) did not significantly affect the number of mice that exhibited jumping behavior during the conditioning sessions [$p = 0.27$;

Figure 32B]. All mice receiving morphine pellets exhibited reduced locomotor activity during the expression test [$F(4,50) = 9.2$; $p < 0.0001$; Figure 32C].

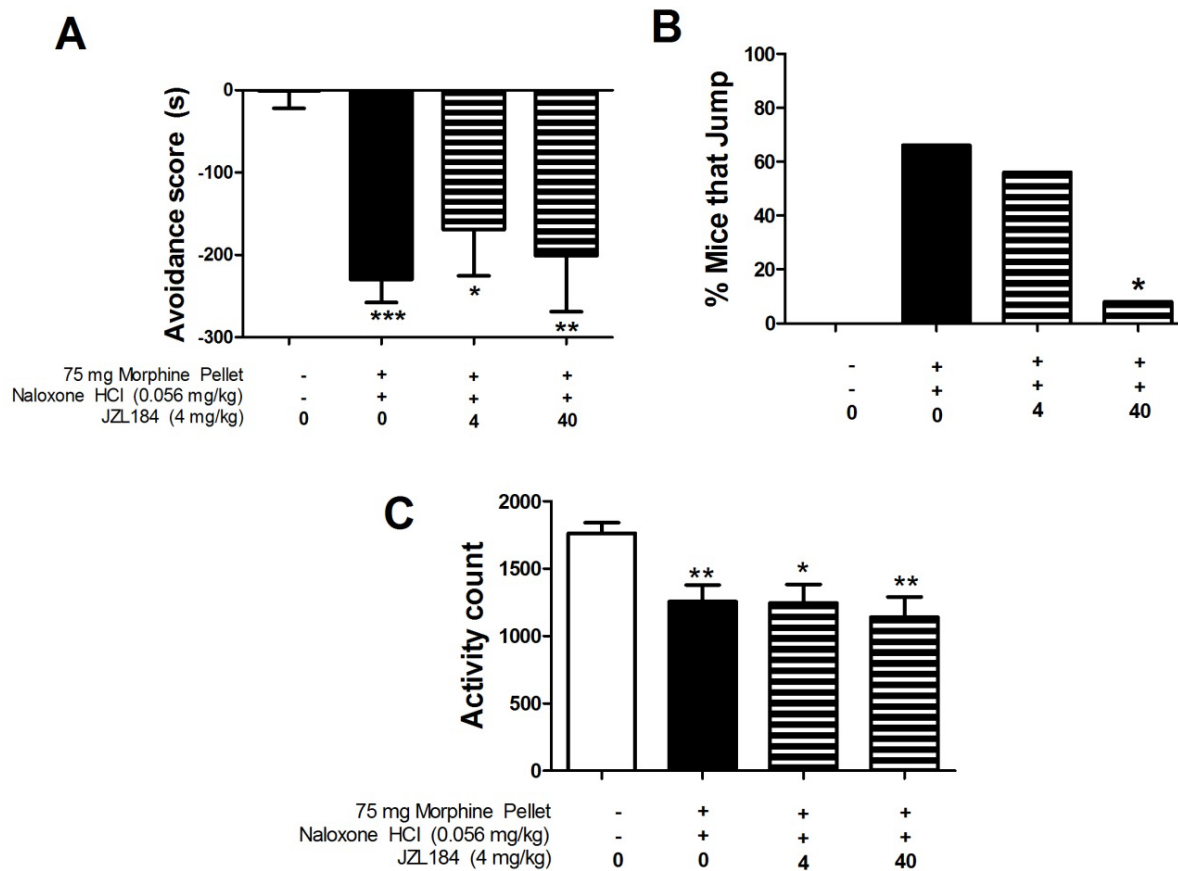


Figure 31. JZL184 reduced naloxone-precipitated morphine withdrawal jumping but not acquisition of CPA. JZL184 did not affect (A) acquisition of CPA to naloxone-precipitated morphine withdrawal but reduced (B) percent of mice that exhibited jumping behavior. There was no effect of JZL184 on (C) locomotor activity during the expression test. $n=9-17$ per group. Data are expressed as mean \pm SEM, or as a percent of animals that exhibited the specified behavior. * $p<0.05$, ** $p<0.01$ compared to Placebo+SAL control for (A,C) and compared to MOR+NLX for (B).

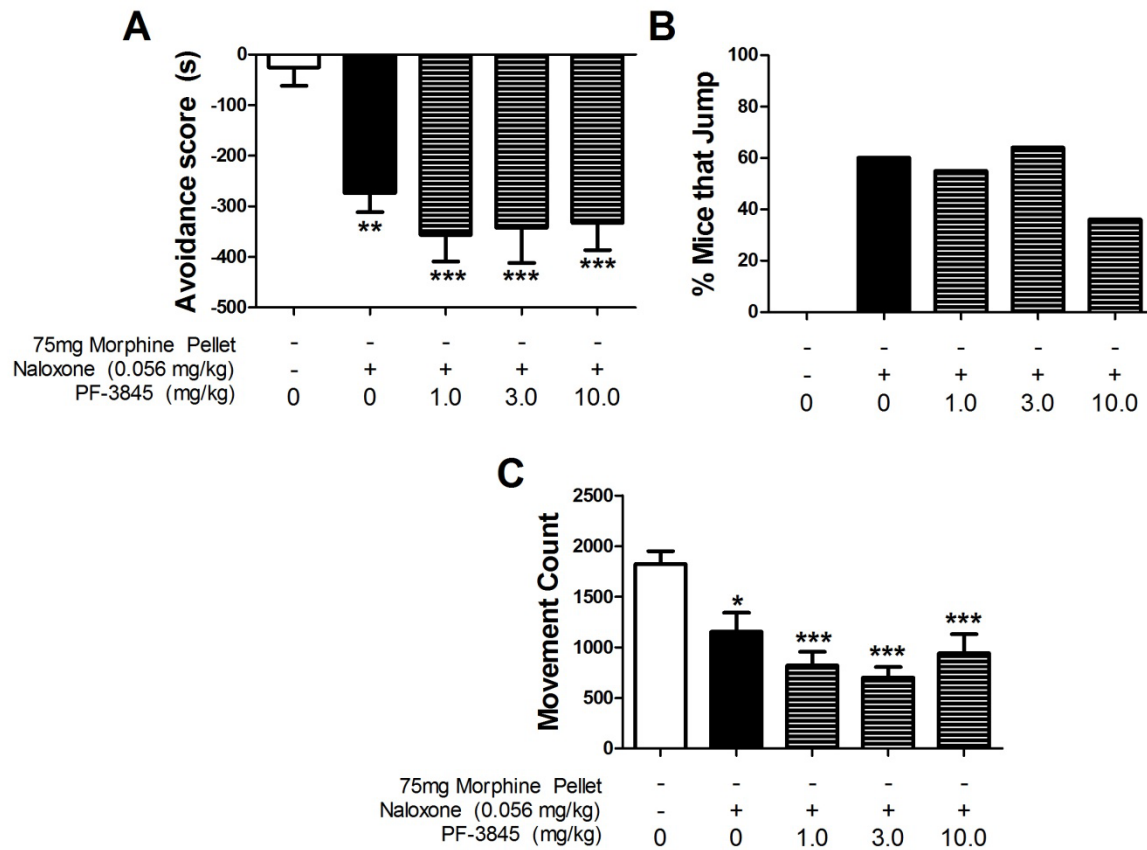


Figure 32. PF-3845 did not affect jumping nor CPA associated with naloxone-precipitated morphine withdrawal. PF-3845 (1, 3, or 10 mg/kg) did not affect (A) acquisition of naloxone-precipitated morphine withdrawal CPA and did not significantly reduce (B) the number of mice exhibiting jumping behavior. Morphine-pelleted mice all exhibited (C) reduced locomotor activity during expression test. PF-3845 treatment did not result in significant effects on locomotor activity on test day. $n=10-12$ per group. Data are expressed as mean \pm SEM, or as a percent of animals that exhibited the specified behavior. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to Placebo+SAL control.

4.3.6. SA-57 reduces withdrawal jumping but does not affect acquisition of naloxone-precipitated morphine withdrawal conditioned place avoidance

Next we tested whether dual inhibition of FAAH/MAGL would block the negative motivational aspects of morphine withdrawal in the CPA paradigm. Naloxone-precipitated morphine withdrawal produced a robust CPA response [$F(4,53)=8.0$, $p<0.0001$; Figure 33A], but this was not affected [$p=0.58$] by pretreatment with SA-57 (1.25, 5, or 12.5 mg/kg). In contrast, SA-57 produced a significant reduction in number of mice exhibiting withdrawal jumping behavior [Figure 33B]. Mice that received morphine pellets exhibited reduced locomotor activity during the expression test, which was not affected by previous SA-57 treatment [$F(4,48) = 7.2$; $p<0.0001$; Figure 33C].

4.3.7. THC reduces withdrawal jumping but not acquisition of conditioned place avoidance to naloxone-precipitated morphine withdrawal

To test if a direct agonist of cannabinoid receptors would reduce acquisition of naloxone-precipitated morphine withdrawal CPA we tested the prototypical phytocannabinoid THC which has previously been shown to reduce behavioral signs of morphine withdrawal in rats and mice. Naloxone produced a significant CPA behavior [$F(4,23)=8.8$; $p<0.001$; Figure 34A], but THC (1, 3 and 10 mg/kg) did not affect acquisition of CPA. In contrast, THC (3 and 10 mg/kg) significantly reduced naloxone-precipitated morphine withdrawal jumping behavior in mice [Figure 34B]. There was no significant effect of THC on locomotor activity [$p=0.06$; Figure 34C].

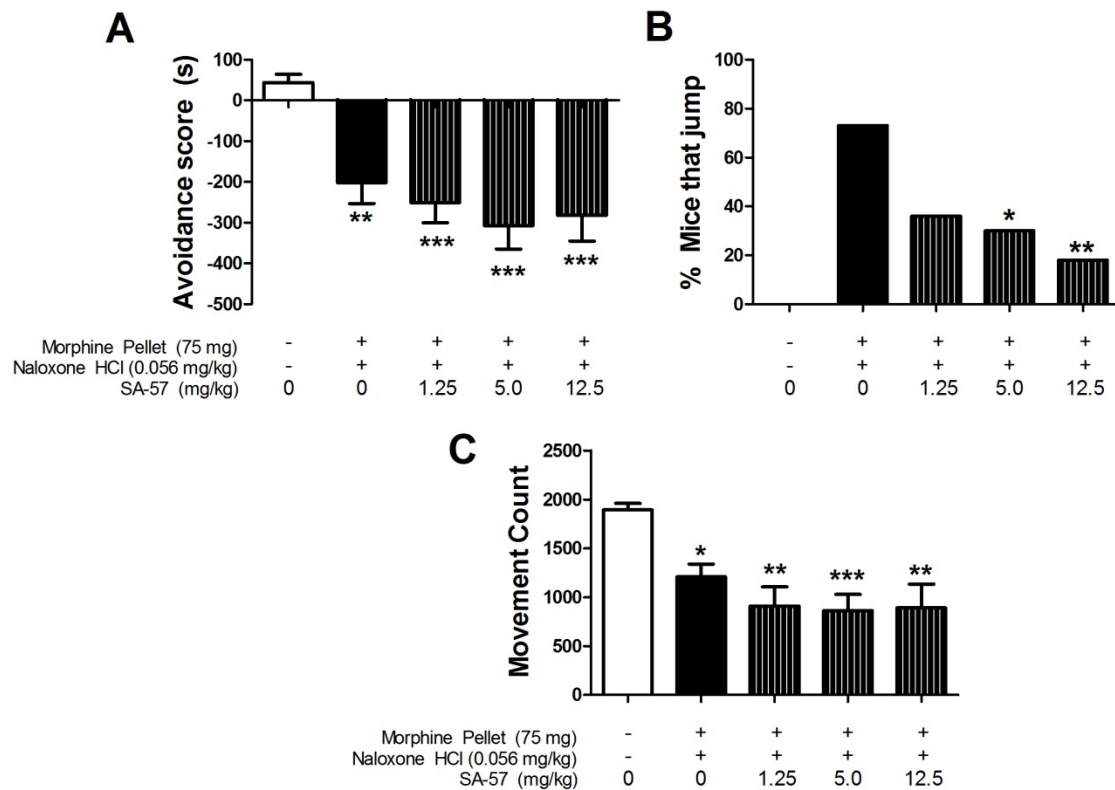


Figure 33. The mixed FAAH/MAGL inhibitor reduced naloxone-precipitated morphine withdrawal related jumping, but not CPA. SA-57 (1.25, 5.0, and 12.5 mg/kg) administered i.p, 2 h prior to the first conditioning session did not affect (A) acquisition of naloxone-precipitated morphine withdrawal CPA but reduced (B) the percentage of mice that exhibited jumping behavior. SA-57 (1.25 and 12.5) also significantly reduced overall activity during the expression test. n=9-12 per group. Data are expressed as mean±SEM, or as a percent of animals that exhibited the specified behavior. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to Placebo+SAL control for (A,C) and compared to MOR+NLX for (B).

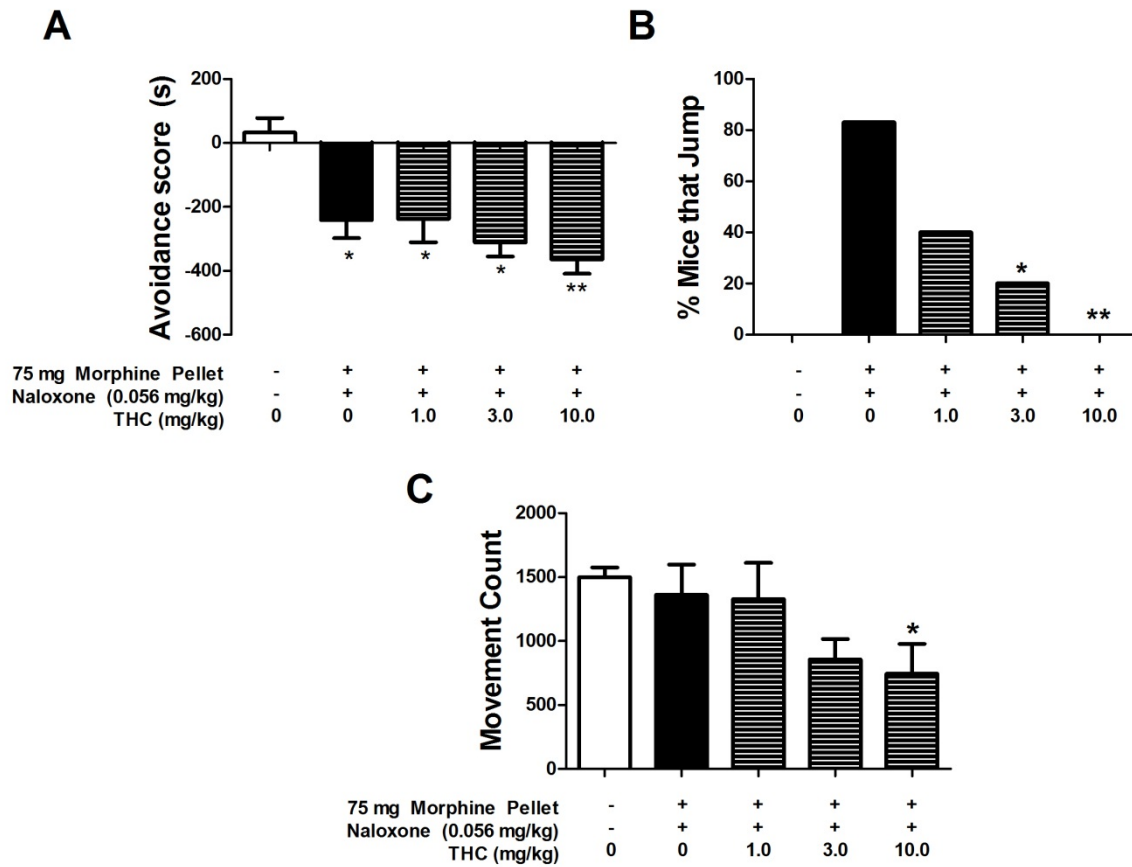


Figure 34. THC reduced jumping, but not CPA, associated with naloxone-precipitated morphine withdrawal. THC (1, 3, or 10 mg/kg) did not affect (A) acquisition of naloxone-precipitated morphine withdrawal CPA but significantly reduced (B) the percentage of mice exhibiting jumping behavior. Mice pretreated with THC (10 mg/kg) exhibited (C) reduced locomotor activity during the expression test. $n=5-6$ per group. Data are expressed as mean \pm SEM, or as a percent of animals that exhibited the specified behavior. * $p<0.05$, ** $p<0.01$, compared to Placebo+SAL control for (A,C) and compared to MOR+NLX for (B).

4.4. Summary

These experiments tested the hypothesis that inhibition of endocannabinoid catabolism would reduce the negative motivational signs of morphine withdrawal as assessed in the CPA paradigm. Morphine (30 mg/kg) significantly blocked the acquisition of withdrawal CPA and significantly reduced the percentage of mice that jumped [Figure 28], serving as the first positive control. We also found that the alpha-2 adrenergic agonist clonidine (0.1 and 0.3 mg/kg) reduced acquisition of morphine withdrawal CPA and this served as our non-opioid positive control [Figure 29]. The MAGL inhibitor JZL184 which elevates the endocannabinoid 2-AG did not affect CPA but did significantly reduce the percentage of mice that jumped [Figure 31] and had no effects on place conditioning by itself [Figure 30]. Additionally, the FAAH inhibitor PF-3845 which elevates the endocannabinoid AEA did not affect CPA or the percentage of mice that exhibited jumping behavior [Figure 32]. We also tested the dual FAAH/MAGL inhibitor SA-57 which more potently inhibits FAAH than MAGL but found that even the highest dose tested (12.5 mg/kg), which maximally elevates 2-AG and AEA, did not affect CPA; however, SA-57 reduced the percentage of mice that jumped [Figure 33]. We also tested whether THC, the cannabinoid partial agonist, would block the acquisition of morphine withdrawal CPA. We found that while THC did not affect CPA it reduced the percentage of mice that jumped [Figure 34].

Chapter 5: Discussion

It is well established that drugs acting upon the endocannabinoid system are effective at reducing somatic withdrawal signs in rodents (Bhargava, 1976; Hine *et al.*, 1975; Ramesh *et al.*, 2011; Vela *et al.*, 1995; Yamaguchi *et al.*, 2001) and recent data suggest that cannabis use during methadone maintenance treatment is associated with lower withdrawal severity (Scavone *et al.*, 2013b). However, direct acting agonists, e.g. THC, have been reported to elicit dysphoria and anxiety in humans (Martin-Santos *et al.*, 2012) and also elicit behavioral/physiological effects in mice including hypomotility, catalepsy and hypothermia (Varvel *et al.*, 2005). Inhibition of FAAH and MAGL reduces somatic signs of opioid withdrawal in mice, but only full inhibition of MAGL produces maximally efficacious reductions in all withdrawal measures (Ramesh *et al.*, 2011). Additionally, the high dose of the MAGL inhibitor JZL184 that produces these pronounced effects also elicits a subset of cannabimimetic effects (hypomotility, hypothermia) (Long *et al.*, 2009a) and upon repeated administration leads to dependence liability and functional CB₁ receptor tolerance (Schlosburg *et al.*, 2010b). Since full FAAH inhibition is without cannabimimetic effects (hypomotility, catalepsy, hypothermia) and does not lead to functional tolerance or cannabinoid dependence (Falenski *et al.*, 2010; Long *et al.*, 2009c; Schlosburg *et al.*, 2010b), we sought to determine if full FAAH inhibition combined with partial MAGL inhibition would reduce morphine withdrawal signs without eliciting cannabimimetic effects.

While inhibition of endocannabinoid catabolism reduces the somatic signs of opioid withdrawal, it is not established whether these effects extend to negative motivational aspects of withdrawal. As previously discussed, the motivational and somatic aspects of withdrawal are dissociable by brain region and naloxone dose (Frenois *et al.*, 2002; Hand *et al.*, 1988; Stinus *et*

al., 1990) suggesting that distinct regions mediate the expression of somatic withdrawal signs and aversive aspects of withdrawal. While endocannabinoid catabolic inhibitors may reduce somatic signs of morphine withdrawal, it is not known whether they will also reduce the aversive aspects of withdrawal. In order to determine the extent to which inhibition of endocannabinoid catabolism reduces opioid withdrawal, other behavioral assays are necessary that reflect these states. In Chapter 3, we examined the effects of opioid withdrawal in several behavioral assays that have been suggested to reflect affective/aversive states, including the light/dark box, a novel escape procedure, the marble burying test, the novelty-induced hypophagia test and the conditioned place avoidance (CPA) procedure. The morphine withdrawal CPA assay resulted in a reproducible and robust conditioned measure of withdrawal-related effects and this behavioral procedure was selected to evaluate the endocannabinoid catabolic inhibitors.

Thus, these studies tested the hypothesis that inhibition of FAAH and/or MAGL would attenuate somatic signs as well as the development of CPA to morphine withdrawal in mice.

5.1. Dual inhibition of endocannabinoid catabolism attenuates signs of opioid withdrawal without producing cannabimimetic effects

We demonstrated that partially blocking MAGL in combination with full FAAH blockade produced an enhanced reduction in a subset (jumps and diarrhea) of somatic opioid withdrawal signs than either of these inhibitors elicited alone. Specifically, combined administration of low dose of the MAGL inhibitor JZL184 (i.e., 4 mg/kg) and high dose of the FAAH inhibitor PF-3845 (i.e., 10 mg/kg) reduced precipitated [Figure 2] and spontaneous [Figure 3] somatic morphine withdrawal signs and these effects were CB₁ mediated [Figure 4]. Additionally, the dual FAAH/MAGL inhibitor SA-57 [Figure 5] significantly reduced paw flutters, head shakes, jumps, diarrhea, and weight loss in morphine-pelleted mice undergoing

spontaneous withdrawal. Importantly, these studies have shown that dual inhibition of FAAH/MAGL at doses of SA-57 that only partially (3-5 fold) elevated 2-AG [Figure 6] did not produce detectable changes in measures of cannabimimetic activity [Figure 7].

High dose JZL184 (40 mg/kg) significantly blocked measured somatic opioid withdrawal signs, while high dose PF-3845 (10 mg/kg) reduced only a subset of these effects (Ramesh *et al.*, 2011). Problematically this high dose of JZL184 produces a subset of cannabimimetic effects (hypomotility, hypothermia) (Long *et al.*, 2009a). Although FAAH inhibitors do not elicit observable cannabimimetic side-effects, PF-3845 was less efficacious than JZL184 in reducing spontaneous and naloxone-precipitated opioid withdrawal signs (Ramesh *et al.*, 2011). Taken together, these results suggest that FAAH and MAGL are both potential targets for treating opioid dependence. However, selective inhibitors of these enzymes are associated with respective challenges related to efficacy and potential side effects. In order to circumvent these limitations, we investigated whether a combination of partial MAGL inhibition and complete FAAH inhibition would achieve enhanced efficacy in attenuating opioid withdrawal signs, without cannabimimetic side effects associated with simultaneously and completely blocking both endocannabinoid catabolic enzymes (Long *et al.*, 2009c). The reduction in somatic morphine withdrawal signs by this combination coupled with the lack of cannabimimetic effects are important findings supporting further development of dual FAAH/MAGL inhibitors. Notably, the dual inhibitor SA-57, which much more potently inhibits FAAH ($IC_{50} < 10$ nM) than MAGL ($IC_{50} = 410$ nM) (Niphakis *et al.*, 2012), displayed differential potency in elevating brain AEA ($ED_{50} = 0.4$ mg/kg) and 2-AG ($ED_{50} = 5.0$ mg/kg) levels [Figure 6] and dose-dependently prevented spontaneous withdrawal in morphine-pelleted mice [Figure 5].

Importantly, SA-57 did not produce cannabimimetic effects at doses that reduced opioid withdrawal signs [Figure 7]. Specifically, SA-57 (1.25 – 5 mg/kg) did not produce hypomotility, catalepsy, antinociception, or hypothermia during an 8 h period which corresponds to the period of time spontaneous opioid withdrawal signs were scored. The relative increase in brain AEA and 2-AG levels at 2.5 and 5 mg/kg SA-57 were similar to those obtained by combined administration of high PF-3845 (10 mg/kg) and low JZL184 (4 mg/kg) (Ramesh *et al.*, 2013). SA-57 at 2.5 and 5 mg/kg elevated 2-AG levels approximately 3- and 7-fold respectively whereas the combination of PF-3845 and JZL184 elevated 2-AG levels approximately 4-fold while both treatments fully elevated AEA. Additionally, the combination of high PF-3845 and low JZL184 did not produce cannabimimetic effects in the tetrad (Ramesh *et al.*, 2013). The lack of effects of combined JZL184 and PF-3845 or SA-57 (1.25 – 5 mg/kg) in the cannabinoid tetrad are in agreement with other published findings. First, complete FAAH blockade does not produce THC-like effects in drug discrimination and does not elicit hypothermia, catalepsy, or hypomotility (Ahn *et al.*, 2008; Long *et al.*, 2009c). Additionally, low dose JZL184 (4 mg/kg) produces minimal THC-like effects, while full blockade of MAGL elicits hypomotility and full inhibition of both FAAH and MAGL elicits robust cannabimimetic effects (hypomotility, catalepsy, hypothermia, and antinociception) (Long *et al.*, 2009c).

Although FAAH and MAGL are well established to be the primary hydrolytic enzymes of AEA (Cravatt *et al.*, 2001b) and 2-AG (Dinh *et al.*, 2002) respectively, non-endocannabinoid substrates of these enzymes and their receptor targets are also affected by inhibitors of these enzymes. In particular, MAGL plays a major role in the biosynthesis of free arachidonic acid in brain from its precursor, 2-AG (Nomura *et al.*, 2011). Also, FAAH metabolizes other bioactive fatty acid amides, including *N*-palmitoyl ethanolamine, *N*-oleoyl ethanolamine, and oleamide

(Cravatt *et al.*, 2001a; Cravatt *et al.*, 1996), as well as *N*-acyl taurines (Leung *et al.*, 2006), which activate various TRP channels and PPAR α receptors (Jhaveri *et al.*, 2008; Marzo *et al.*, 2010; Smart *et al.*, 2000). Thus, combined blockade of FAAH and MAGL produces many neurochemical alterations that could affect morphine withdrawal responses. Nonetheless, the observation that the CB₁ antagonist rimonabant completely prevents all reductions of somatic withdrawal signs produced by combined administration of JZL184 and PF-3845 in morphine-pelleted mice indicates that endocannabinoid activation of CB₁ receptors plays a necessary role in these actions. Similarly, rimonabant, but not the CB₂ receptor antagonist SR144528, prevented the protective effects of JZL184 on naloxone-precipitated withdrawal signs in morphine-pelleted mice (Ramesh *et al.*, 2011).

Although compelling evidence supports the idea that AEA and 2-AG play pivotal roles in mediating the reduction in somatic withdrawal signs elicited by dual inhibition of their primary catabolic enzymes, the manner by which these two endocannabinoids interact remains to be established. One explanation is that dual enzyme inhibition produces enhanced reductions in somatic withdrawal signs (jumps and diarrhea) by increasing total endocannabinoid brain levels to stimulate CB₁ receptors. While the observation that 2-AG levels in brain are at least 200-fold higher than AEA brain levels (Ahn *et al.*, 2009; Long *et al.*, 2009a) could argue against this idea of mass action, the difference in relative levels of AEA and 2-AG in interstitial space is more narrow. Long *et al.* reported baseline NAc interstitial levels of AEA and 2-AG in C57BL/6 mice as 0.5 ± 0.1 nM and 4.6 ± 0.7 nM respectively (Long *et al.*, 2009a). Thus, additive effects of elevated 2-AG and AEA might account for the enhanced effects on somatic withdrawal signs. Alternatively, it is plausible that simultaneous inhibition of MAGL and AEA may produce augmented effects because distinct CB₁ receptor-mediated circuits are activated by the respective

endocannabinoids. Consistent with this idea is that FAAH is predominantly expressed on post-synaptic terminals (Gulyas *et al.*, 2004) and MAGL is expressed on presynaptic terminals (Dinh *et al.*, 2002). Considering the localization of CB₁ receptors in LC both presynaptically on glutamatergic afferents and post-synaptically with mu opioid receptors (Scavone *et al.*, 2010), it is possible elevating either 2-AG or AEA by inhibition of their catabolic enzymes may preferentially activate these pools of CB₁ receptors. Furthermore, while phasic endocannabinoid signaling is mediated by 2-AG, tonic endocannabinoid signaling involves both 2-AG (Hashimoto *et al.*, 2007) and AEA (Kim *et al.*, 2010). Also, as discussed above, other consequences of enzyme inhibition may further contribute to the reduction in somatic withdrawal signs (e.g., decreased brain levels of arachidonic acid or increased brain levels of non-cannabinoid fatty acid amides). Indeed, phospholipase A2, cyclooxygenase-1, cyclooxygenase-2, and 5-lipoxygenase inhibitors were shown to decrease naloxone-precipitated opioid withdrawal induced contractions of guinea-pig isolated ileum, suggesting arachidonic acid and its metabolites (e.g. prostaglandins and leukotrienes) are involved in the expression of opioid withdrawal (Capasso *et al.*, 1997).

While these results highlight the potential of the endocannabinoid catabolic enzyme inhibitors to treat opioid withdrawal, it will be important to assess this strategy on other components of opioid addiction, including self-administration of opioids, extinction, and reinstatement. The CB₁ receptor antagonist rimonabant attenuates heroin self-administration (Navarro *et al.*, 2001) and reinstatement of heroin self-administration in rats (Fattore *et al.*, 2005), while CB₁ receptor agonists such as CP55,940 and WIN55,212-2 restore heroin self-administration in rats (Fattore *et al.*, 2003). Thus, it will be important to determine the degree to which elevating endocannabinoids may affect resumption of opioid self-administration.

Furthermore, although somatic withdrawal signs represent an important component of opioid withdrawal, it remains to be established whether manipulations of the endocannabinoid system also attenuate the negative motivational aspects of opioid withdrawal (Frenois *et al.*, 2002). Chapter 4 followed this line of investigation by assaying for behaviors indicative of negative motivational aspects of opioid withdrawal using the CPA paradigm.

5.2. Morphine withdrawal produces conditioned place avoidance but paradoxical effects in the light/dark box

Inhibitors of endocannabinoid catabolism reduce somatic signs of morphine withdrawal in mice suggesting that these drugs may be effective at reducing opioid withdrawal in clinical setting. It is not known whether endocannabinoid catabolic inhibitors reduce other aspects of opioid withdrawal such as those related to negative motivational or aversive aspects. Additionally, it might be argued that reductions in somatic withdrawal signs may be subject to non-specific behaviorally disruptive effects of drugs and not fully reflect whether treatment is reducing opioid withdrawal. Thus, it is important that the extent to which endocannabinoid catabolic inhibitors reduce other aspects of opioid withdrawal be established. These experiments investigated the effects of morphine withdrawal in five distinct *in vivo* assays in order to develop behavioral procedures sensitive to opioid withdrawal that may be used to infer the internal emotional state of the animal. Specifically, we examined the effects of morphine withdrawal in the marble burying test, the novelty induced hypophagia test, the light/dark box, a novel test for escape behavior, and CPA.

5.2.1. Light/dark box

We investigated the effects of naloxone-precipitated morphine withdrawal on mouse behavior in the light/dark box. Naloxone significantly increased the amount of time spent in the light side in morphine-pelleted [Figure 9] but not placebo-pelleted [Figure 10] mice. Furthermore, morphine-pelleted mice that received the highest dose of naloxone (0.1 mg/kg) spent more time in the light side than placebo-pelleted mice receiving the same naloxone dose. However, it must be noted that these mice did not exhibit a preference for one side or the other as approximately half the time was spent in the light side and half in the dark. These data are in contrast to the effects of morphine withdrawal observed in rats in the light/dark box where reductions in time spent in the light side were observed (Castilho *et al.*, 2008). Rats treated twice daily with ramping doses of morphine (10 – 50 mg/kg) for 10 days were challenged with naloxone (0.1 mg/kg) and tested in the light/dark box for 10 min. Rats undergoing precipitated-morphine withdrawal exhibited a significant decrease in the time spent on the light side (~150 s) compared to saline control animals (~250 s) as well as decreased transitions from the dark to light side (~6 transitions vs. 10 transitions). Furthermore, similar effects were observed in the EPM where naloxone-precipitated withdrawal decreased the amount of time spent in the open-arms (~12%) as compared to control rats (~26%).

While there are minimal published data regarding the effects of opioid withdrawal in the light/dark box, several studies have examined the effects of opioid withdrawal in the EPM, though the majority of these studies have been conducted in rats. Similar to the effects of morphine withdrawal in the light/dark box reported by Castilho where decreases in the amount of time spent in the light side were observed, decreases in the time spent in the open-arms of the EPM in rats have also been reported (Bhattacharya *et al.*, 1995; Castilho *et al.*, 2008; Schulteis *et*

al., 1998b; Zhang *et al.*, 2008). In contrast, similar to the effects we observed in the light/dark box, mice have been reported to exhibit increases in the amount of time spent in the open-arms following morphine withdrawal (Buckman *et al.*, 2009; Hodgson *et al.*, 2008; Hodgson *et al.*, 2009). Increased time in the open-arms is typically observed following acute treatment with anxiety-reducing compounds such as benzodiazepines and is therefore interpreted by investigators as an anxiolytic-like effect. Observations of rats undergoing morphine withdrawal in the EPM exhibiting decreases in the amount of time in the open-arms suggests an anxiogenic-like effect which is in accord with the reported increased anxiety in humans as well as the elevations in stress hormones that accompany withdrawal in both rats (Budziszewska *et al.*, 1996; Gonzalez *et al.*, 1994) and humans (Gil-Ad *et al.*, 1985). Mice also exhibit increases in plasma corticosterone following morphine withdrawal (Hodgson *et al.*, 2008; Rabbani *et al.*, 2009) which makes the increases in time spent in the open-arms of the EPM and the light side of the light/dark box surprising. Since mice treated with anxiolytic compounds typically exhibit increases in the time spent in the open-arms (Lister, 1987) of the EPM or light side of the light/dark box (Crawley *et al.*, 1979), the observation that morphine withdrawal increases time in the open-arms of the EPM or light side of the light/dark box in mice is paradoxical. Since morphine withdrawal appears to produce species-dependent effects in both the EPM and light/dark box, these data raise questions regarding the interpretation of behavior in these paradigms.

Hodgson *et al.* (2008) were the first to report that naloxone-precipitated morphine withdrawal increased time spent in the open-arms of the EPM in mice and suggested that these increases in time spent in the open-arms might reflect shifts in defensive strategies due to an increased motivation to escape. This would cause a decrease in the inhibitory avoidance

exhibited by rodents toward the open-arms of the EPM and thus increased time spent in this area of the apparatus. The suggestion that mice may be exhibiting increases in escape behavior led to the development of the escape assay in Chapter 3 in order to test the hypothesis that morphine withdrawal increases escape behavior in mice.

5.2.2. *Escape box*

The escape box was designed to examine mouse behavior during morphine withdrawal in an environment in which mice can escape. In an initial experiment, a clear plastic mouse cage was set on top the lab bench where the experimenter could observe the animal's behavior. The cage lid was left ajar in order to allow mice to exit the cage at one end. Morphine-pelleted and placebo-pelleted mice were then challenged with naloxone to precipitate withdrawal and the latency for them to exit the cage was recorded. Control animals did not escape during the 15 min test session, however morphine-pelleted mice receiving naloxone (0.03 – 0.1 mg/kg) did escape [Figure 11]. However, the height of the edge of the cage required that mice jump up to climb out and because mice undergoing precipitated morphine withdrawal exhibit jumping behavior in the absence of an opening through which they can escape, it is possible that escaping was simply a consequence of increased jumping. To facilitate escape behavior in mice and narrow the opening to prevent random escape via non-directed jumping, a second apparatus was designed that contained a 5x5 cm opening at one end with a wire mesh ramp recessed over the lip of the cage so that mice could climb out much easier. This design resulted in control mice exhibiting escape behavior as well suggesting that the resulting jumping behavior of morphine withdrawal was not necessary for mice to escape. Additionally, naloxone-precipitated morphine withdrawal further lowered this latency [Figure 12] suggesting that morphine withdrawal was facilitating escape in

this procedure. In an effort to optimize the likelihood that the escape behavior was not simply a consequence of withdrawal-induced jumping, the procedure was slightly modified so that the wire mesh extended further (2 cm) into the cage so that mice could easily climb out and further reduce the chances that jumping behavior was contributing to this effect. Mice were closely observed during the procedure and scored as to whether any jumping behavior was exhibited. Naloxone (0.056 mg/kg) significantly reduced the escape latency in morphine-pelleted mice as compared to placebo-pelleted controls and when mice that exhibited any jumping during the session were excluded (n=3), the effect remained statistically significant [Figure 13]. This last experiment suggested that mice undergoing morphine withdrawal would indeed escape with a lower latency than control mice and that this behavior was not dependent upon the presence of jumping behavior. These experiments taken together suggest that morphine withdrawal decreases the latency of mice to escape from a novel cage independently of effects on jumping behavior.

Previous morphine withdrawal studies have characterized jumping behavior in rodents as an “escape behavior” (Frederickson *et al.*, 1976; Noda *et al.*, 2004; Stinus *et al.*, 1990). The results of the present study support this interpretation by demonstrating that mice during morphine withdrawal are exhibiting a goal-directed behavior (i.e. they are actively escaping rather than simply emitting a jumping behavior). While it seems likely that the increased jumping behavior during opioid withdrawal is directly related to the escape behavior as we have defined it in our study, the observation that naloxone-precipitated morphine withdrawal results in mice exiting a novel environment more rapidly than control mice lends support to the notion that increases in time spent in the open-arms of the EPM and light side of the light/dark box reflect an escape response rather than an anxiolytic effect. Further characterization of this experimental procedure is warranted including assessment of known anxiolytic (e.g. benzodiazepines) and

anxiogenic compounds (e.g. kappa agonists, GABA antagonists), manipulations of the environment (stressors) and testing in home cage vs. novel cage to determine their effects on escape behavior.

5.2.3. *Marble Burying*

It has previously been shown that morphine withdrawal increased the number of marbles buried by male albino Swiss mice (Umathe *et al.*, 2012). The present studies sought to determine if morphine withdrawal would produce similar increases in digging behavior as measured by marble burying in male ICR mice. Spontaneous morphine withdrawal following six days of morphine treatment (6.25 – 50 mg/kg s.c., b.i.d.) did not produce significant changes in the number of marbles buried during a 20 min test [Figure 14]. Following that study, a second experiment was conducted in which mice were implanted with subcutaneous morphine (75 mg) pellets for a greater induction of dependence. During the baseline test (72 h post pellet implantation), morphine-pelleted mice buried fewer marbles than placebo-pelleted mice did, suggesting the presence of morphine was reducing digging behavior in these mice [Figure 15]. Subsequently, pellets were removed and mice were retested at 4 h and 8 h after pellet removal. The difference between number of marbles buried between morphine treated and placebo treated mice persisted at 4 h post pellet removal, but by 8 h placebo-pelleted mice were burying fewer marbles and this difference between groups was no longer present [Figure 15]. The effects of morphine on marble burying represented a challenge to the interpretation of these results since there were baseline differences between morphine-pelleted and placebo-pelleted mice. It is possible that mice habituated to the marble burying apparatus and thus were less active and buried fewer marbles, yet mice that were morphine-pelleted did not exhibit any significant shifts

in digging behavior as measured by number of marbles buried following pellet removal. Increases in the number of marbles buried might have been apparent at the later time point had the animals not acclimated to the test cages, but the relative high number of marbles buried by the placebo animals likely would have precluded detection of shifts in this direction. Thus, the next experiment was modified to reduce the number of marbles buried by control animals by shortening the test session length. In this experiment, we assessed a wide range of doses of naloxone in order to test whether it produced a dose-related effect on marble burying in morphine-pelleted mice. Mice were implanted with morphine or placebo pellets and tested 72 h later following treatment with either saline or naloxone (0.03 – 0.3 mg/kg, s.c.). Placebo-pelleted mice buried fewer marbles during the 5 min test than in previous experiments using the 20 min session length, providing a window for detection of either increases or decreases in digging behavior. However, naloxone did not significantly affect the number of marbles buried at any dose tested and both morphine and placebo-pelleted mice buried a similar number of marbles [Figure 16].

To date, only one study has reported the effects of opioid withdrawal on marble burying in mice. Umathe et al. (2012) reported that in male Swiss mice (24-30 g) repeatedly treated with morphine (5 – 20 mg/kg, s.c., b.i.d.) for 10 days exhibited significant increases in the number of marbles buried at 8 h, 24 h, and 48 h following their last morphine injection. We examined marble burying at 8 h post-pellet removal but did not observe this increase in marble burying but this could be due to the dependence induction procedure. It is notable, however, that control mice in the Umathe study buried very few marbles (~10 marbles out of 20) during the 30 min test session as compared with the ICR mice in the present study during the 20 min test. In order to facilitate a detection of increases in marble burying, the test session was shortened to 5 min and

the precipitated withdrawal model was employed to induce withdrawal to varying degrees, however this did not yield significant effects of morphine withdrawal. Thus, the marble burying assay under the conditions we tested was not sensitive to alterations in digging behavior by morphine withdrawal. Future studies could further modify the marble burying procedure to reduce the number of marbles buried. Acclimation is one possible means to reduce the number of marbles buried, however when tested at 8 h post pellet removal, morphine withdrawn mice did not exhibit even a slight trend for an increase in this behavior although control animals exhibited a reduction in the number of marbles buried as compared to baseline. Further shortening the time of the procedure may reduce the number of marbles buried but it is unlikely large changes in digging behavior could be observed in such a short time period. The difference in numbers of marbles buried between these studies and those published by Umathe et al. is the primary concern regarding translation of morphine withdrawal effects since the differences in baseline behavior are so different that it may reflect a strain dependent effect. Other groups using the Swiss mouse and the same 30 min marble burying procedure (non-dependence procedures) have also reported that control mice bury approximately 10 marbles (Li *et al.*, 2006; Uday *et al.*, 2007). In contrast, some studies using ICR mice have reported more marble burying under control conditions. Ichimaru et al., using male ICR mice in a 30 min procedure with a total of 25 marbles, reported that the median number of marbles buried under control conditioned was 22 (Ichimaru *et al.*, 1995). Another study reported that baseline marble burying was approximately 70% buried (out of 25 marbles) during a 30 min test, equaling approximately 18 marbles (Young *et al.*, 2006). In another study using 20 marbles, control ICR mice buried approximately 15 marbles after 20 min (Sugimoto *et al.*, 2007). Though some these studies used longer session times, it is possible that strain differences on baseline digging behavior may account for lack of effects of morphine

withdrawal in these studies, since the experimental design (aside from test length), size of the mice, and time of day were similar between our studies and those of Umathe et al. A previous study examined multiple inbred strains of mice and found that marble burying did indeed vary across strains but did not correlate with other behavioral measures typically interpreted as anxiety-like. Mice of the 129S6 strain buried the fewest marbles (~6) whereas CBA mice buried the most (~15-16) during a 30 min period in which 20 marbles were present (Thomas *et al.*, 2009). This study suggested that strain does indeed play a role in digging behavior and also demonstrated that digging was not affected by the presence of marbles as compared to control tests where marbles were absent, thus supporting the argument that the marble burying assay is a measure of digging behavior rather than marble burying.

Another difference which may account for differences in marbles buried is the scoring criteria. We used a procedure that has previously been published from our lab (Kinsey *et al.*, 2011) which was adapted from an established procedure (Thomas *et al.*, 2009). In this procedure, marbles are scored as buried if >50% of the marble is covered by bedding whereas other studies have typically used the criteria of 2/3 buried. Thus, differences in scoring of the number of marbles buried might reflect the higher scores in our studies since the threshold for being “buried” is lower. Though this difference exists it is such a small difference that it seems unlikely to account for lack of effects observed following morphine withdrawal but it cannot be completely discounted.

Digging behavior aside from defensive burying has been observed in rats during withdrawal. Rubino implanted morphine (75 mg) pellets daily into rats for 5 days then administered naloxone (10 mg/kg i.p.) and observed increases in digging behavior in morphine-pelleted but not placebo-pelleted rats (2000) and Rasmussen et al. reported increases in digging

during a naltrexone-precipitated (10 mg/kg s.c.) morphine (2x75 mg pellets) withdrawal during a 60 min observation period in which withdrawal behaviors were scored while rats were observed in clear plastic cages lined with wood shavings (2005). It is notable that in both of these studies there was also a significant increase in jumping behavior in the withdrawal group which suggests that increases in one behavior did not limit the emergence of other behaviors through response competition. In summary, the lack of effects of morphine withdrawal on marble burying in our assay may be due to mouse strain or the dependence procedure and because there was no effect of withdrawal we chose not to pursue this behavioral procedure.

5.2.4. Novelty-induced hypophagia

The novelty-induced hypophagia protocol was adapted from an established procedure which is sensitive to nicotine withdrawal (Gur *et al.*, 2007). Initially, mice were acclimated to the novel food in their home cages over 12 days [Figure 17] and an initial control experiment tested the benzodiazepine diazepam. Typically, mice will show an increased latency to consume this food in a novel environment, but benzodiazepines and other drugs that produce anxiolytic-like effects decrease latency to consume the food and increase the amount of food consumed in a novel environment [Figure 18]. This established the procedure and demonstrated that diazepam produced effects that have been previously reported (Merali *et al.*, 2003). We next assessed the effects of naloxone in morphine-pelleted and placebo-pelleted mice in this procedure. When tested in the home cage, morphine-pelleted mice exhibited a decrease in the amount of food consumption as compared to placebo-pelleted mice [Figure 19] but there was not an effect of naloxone on the amount of food consumed and or the latency to consume food. The suppressive effects of morphine on latency and food consumption predominated in this experiment and made

conclusions regarding withdrawal effects difficult. Future studies might use a longer dependence period following pellet implantation to allow for the anorectic effects of morphine to undergo tolerance or use a repeated dosing procedure to avoid the inhibitory effects of morphine on food consumption. Thus the suppressive effects of morphine on food consumption occluded any possible effects of naloxone-precipitated withdrawal and further studies would have to assess the effects of withdrawal in rodents in which morphine does not suppress feeding during control tests. The duration of time between pelleting and testing could be lengthened so that the animals are tested once the food consumption of the morphine treated group matches that of the placebo-pelleted group. In summary, naloxone-precipitated morphine withdrawal did not produce effects that could be interpreted as withdrawal related in this procedure.

5.2.5. *Conditioned place avoidance*

The place conditioning paradigm employed in our studies used an unbiased apparatus. This was first established following examination of baseline preferences which demonstrated that mice did not prefer the white or the black side [Figure 20]. An initial experiment replicating CPP to morphine and cocaine served to establish the basic conditioning procedure. Both morphine (10 mg/kg) and cocaine (20 mg/kg) produced a significant CPP in ICR mice demonstrating that the basic methodology (i.e. handling, conditioning apparatus) was sufficient to produce an established CPP effect [Figure 21]. The next set of experiments sought to replicate an established procedure (Broseta *et al.*, 2005) for producing CPA to morphine withdrawal. First, a dose-response experiment for naloxone was conducted in order to determine the potential for naloxone to produce CPA on its own which is an effect that has previously been reported (Mucha *et al.*, 1987). Naloxone (0.3 and 1 mg/kg) produced a significant CPA effect in ICR mice

[Figure 22]. Thus, for the first experiment, naloxone (0.1 mg/kg) was selected as the dose to precipitate morphine withdrawal. Mice were treated repeatedly with morphine (12.5 – 50 mg/kg s.c. b.i.d.) and conditioned with naloxone (0.1 mg/kg) on one side and saline on the other side. Naloxone did not yield a significant CPA effect in either morphine or saline treated mice [Figure 23]. A follow up experiment was conducted in which the naloxone dose was increased in order to produce a greater degree of morphine withdrawal and thus a more robust unconditioned stimulus. Naloxone (1 mg/kg) produced a significant CPA in both saline treated and morphine treated mice but no significant difference in the degree of CPA was observed between these two groups [Figure 24]. Thus, the conditions used in this experiment did not lead to precipitated morphine withdrawal effects. This was problematic in terms of being able to assess the effects of endocannabinoid catabolic inhibitors on acquisition of naloxone-precipitated morphine withdrawal CPA. The naloxone dose in these studies appeared less important in terms of acquisition of naloxone-precipitated morphine withdrawal CPA than the dependence induction procedure. Accordingly, the next set of experiments sought to determine if acquisition of naloxone-precipitated morphine withdrawal CPA would occur in animals implanted with subcutaneous morphine pellets. The use of morphine pellets was selected because it would produce a greater degree of dependence in these animals and because it was also the method to produce dependence in our previous studies (Ramesh *et al.*, 2011). While other studies have implanted morphine pellets prior to conditioning (Mucha *et al.*, 1982; Schulteis *et al.*, 1994; Watanabe *et al.*, 2002) we were concerned that the constant presence of the morphine pellet presented a potential pitfall in that morphine could potentially be serving as an additional unconditioned stimulus in these rodents. Specifically, if morphine pellets were implanted prior to saline (i.e. non-withdrawal) conditioning sessions, the rewarding effects could possibly be paired

with the non-withdrawal side. This would confound the interpretation of our data as the mice could either be exhibiting CPP to morphine or CPA to morphine withdrawal. Indeed, morphine pellets alone produced CPP when present during conditioning on only one side of the apparatus [Figure 25]. It has been shown that treatment with the conditioning compound prior to the expression test can facilitate either CPP (in the case of morphine) or CPA (in the case of naltrexone) (Bespalov *et al.*, 1999). It is possible that if morphine pellets were removed prior to the expression test that mice may not have exhibited CPP, however we did not test this condition. Nonetheless, the observation that morphine pellets could produce CPP suggested that presence of the morphine pellet during saline (i.e. non-withdrawal) conditioning could confound our interpretations since increases in time spent in the saline (i.e. morphine-pelleted) side compared to decreases in the naloxone (i.e. withdrawal) side could be attributed to either avoidance to withdrawal or preference to morphine. Thus, we concluded that morphine pellets should not be present during the “non-withdrawal” conditioning session and performed pellet implantation the day following the saline/“non-withdrawal” conditioning sessions.

We next demonstrated that naloxone produced CPA in morphine-pelleted mice at doses that did not produce CPA in placebo-pelleted mice [0.056 mg/kg naloxone; Figure 26]. Furthermore, planned comparison Student’s t-test revealed that the CPA response of morphine-pelleted mice following naloxone (0.1 mg/kg) conditioning was greater than in placebo-pelleted mice, suggesting withdrawal contributed to the behavior rather than being solely a naloxone effect. Differences in locomotor activity were observed where morphine-pelleted mice, but not placebo-pelleted mice, exhibited reduced locomotor counts during the expression test [Figure 26C and 26D]. Additionally, CPA occurred in morphine-pelleted mice at doses that produced minimal somatic signs of opioid withdrawal [Figure 27]. The effects of naloxone by itself in

placebo-pelleted mice were consistent with a previous report in which naloxone (0.1-10 mg/kg) produced CPA in naïve CD-1 mice to a similar degree. The effects of naloxone on CPA in naïve animals is mediated by mu opioid receptors as mu opioid receptor knockout mice do not exhibit CPA following naloxone (10 mg/kg s.c.) as compared with wild-type controls (Skoubis *et al.*, 2001). In morphine (2 x 75 mg) pelleted rats, naloxone (1 mg/kg) produced a roughly 2-fold increase in the CPA effect as compared to placebo-pelleted rats (Mucha *et al.*, 1987). This is similar to the CPA observed in our study where morphine-pelleted mice exhibited an avoidance score approximately twice that of placebo-pelleted mice following naloxone (0.1 mg/kg) [Figure 26].

The doses of naloxone that produced CPA were considerably lower than those necessary to produce somatic withdrawal signs in mice. Indeed, naloxone (0.056 mg/kg) did not produce statistically significant increases in withdrawal behaviors as compared with control animals [Figure 27]. While 50% of the mice exhibited jumping behavior following naloxone (0.056 mg/kg), the average number of jumps was very low (3 ± 1.4 , mean \pm SEM) which was not statistically significant from controls. In fact, naloxone (0.3 mg/kg) was the lowest dose that significantly increased number of jumps.

These data taken together suggest that naloxone is more potent at producing CPA than it is at eliciting somatic withdrawal signs. The naloxone-precipitated morphine withdrawal CPA procedure produced a clear withdrawal effect which has been well established in the literature and used to evaluate the negative motivational consequences of opioid withdrawal (Broseta *et al.*, 2005; Frenois *et al.*, 2002; Maldonado *et al.*, 2004; Sato *et al.*, 2005; Schulteis *et al.*, 1998a; Stinus *et al.*, 2000; Stinus *et al.*, 1990; Watanabe *et al.*, 2002). Thus, we chose this procedure to further evaluate the endocannabinoid catabolic inhibitors on opioid withdrawal.

5.3. *Inhibition of endocannabinoid catabolism reduces jumping, but not conditioned place avoidance of morphine withdrawal*

These experiments tested the hypothesis that inhibition of endocannabinoid catabolism would reduce the negative motivational aspects of morphine withdrawal as assessed in the CPA paradigm. Morphine and clonidine were tested as positive controls to determine if the CPA assay was sensitive to drug treatments that are effective in clinical settings. Thus, morphine pretreatment might represent what could be considered a replacement therapy used in the clinic, which involves treatment with a direct agonist such as methadone or buprenorphine (Lobmaier *et al.*, 2010). We hypothesized that morphine sulfate would block both acquisition of CPA and somatic withdrawal signs as assessed by recording the percentage of mice that exhibited spontaneous jumping behavior, a typical behavioral sign that accompanies opioid withdrawal. This behavioral observation during conditioning was used as a secondary measure of drug effects for all experiments to ensure that previously efficacious doses of the endocannabinoid catabolic inhibitors were effective at reducing somatic aspects which could be used to rule out experimental problems as a cause for lack of effect on CPA.

As expected, morphine (30 mg/kg) blocked naloxone-precipitated withdrawal jumping [Figure 28]. Previously, morphine (25.6 mg/kg) was shown to inhibit withdrawal jumping approximately 80% following a very large dose of naloxone (25 mg/kg i.p.) in mice treated repeatedly with morphine (Iorio *et al.*, 1975) and a morphine dose of 40 mg/kg shifted the naloxone ED₅₀ value for precipitating withdrawal jumping from 0.1 mg/kg to 0.5 mg/kg (Suzuki *et al.*, 1989). Our morphine dose was much higher relative to our naloxone dose compared to the Iorio study in which the morphine and naloxone doses were similar. We selected a high morphine dose to ensure that we blocked both the withdrawal jumping and CPA since there is no

data on morphine doses necessary to block naloxone-precipitated morphine withdrawal CPA. Importantly, morphine prevented acquisition of naloxone-precipitated withdrawal CPA. The effect of morphine on withdrawal CPA demonstrates that the CPA paradigm is at the very least capable of detecting reductions in withdrawal responses as would be accomplished using a model of substitution therapy.

We also tested if clonidine was effective at reducing CPA in our procedure, as it is used clinically for the treatment of opioid withdrawal (Gold *et al.*, 1978; Gossop, 1988) and has been shown to reduce CPA to morphine withdrawal in rats (Kosten, 1994; Schulteis *et al.*, 1998a). Clonidine (0.025-0.4 mg/kg) when administered immediately prior to conditioning, was previously shown to significantly reduce CPA to morphine withdrawal (Schulteis *et al.*, 1998a) so we tested a similar range of doses. We found that clonidine (0.1 and 0.3 mg/kg) significantly reduced the acquisition of CPA to morphine withdrawal [Figure 29]. Previously, clonidine (0.05 mg/kg i.p.) has been shown to produce CPA in mice but not at lower doses (Zarrindast *et al.*, 2002) thus it is unlikely that clonidine masked naloxone-precipitated morphine withdrawal CPA by producing CPP on its own. Surprisingly, none of these doses (0.03-0.3) affected morphine withdrawal jumping. In the present study the jumping was scored as a nominal measure rather than counting the total number of jumps, so it is likely that our measure lacked the ability to detect changes in the number of jumps. Clonidine may reduce the number of jumps, but not necessarily its occurrence. Thus, this measure mainly served as a confirmation if drug treatments had a detectable effect on somatic withdrawal signs. Indeed, clonidine (0.01-0.16 mg/kg) has been shown at similar doses to reduce morphine withdrawal-induced body shakes, jumping, and paw tremors (Dehpour *et al.*, 2001; Fielding *et al.*, 1978; Valeri *et al.*, 1989). Interestingly, others have reported that while clonidine reduces the height of naloxone-

precipitated withdrawal jumps, it does not affect the number of jumps (Alguacil *et al.*, 1989). Thus, it is plausible that clonidine may reduce other aspects related to naloxone-precipitated jumping behavior that were not assessed in the current study. Importantly, clonidine significantly reduced withdrawal CPA and to our knowledge this is the first time clonidine has been shown to reduce CPA to morphine withdrawal in mice.

JZL184 (40 mg/kg) did not affect acquisition of naloxone-precipitated morphine withdrawal CPA [Figure 31]. While it reduced the percentage of mice that jumped, this lack of effect on CPA gave an early indication that elevation of endocannabinoids may lack efficacy in ameliorating the negative motivational aspects of withdrawal. Additionally, inhibition of FAAH with PF-3845 did not affect withdrawal jumping or CPA [Figure 32] and PF-3845 has been shown to be less effective than JZL184 at reducing withdrawal jumping (Ramesh *et al.*, 2011). As previously mentioned in regards to the lack of effects of clonidine on jumping, it may be that potential effects of PF-3845 are not detectable when jumps are scored nominally.

We next assessed the dual FAAH/MAGL inhibitor SA-57 on naloxone-precipitated morphine withdrawal CPA. SA-57 (5 and 12.5 mg/kg) reduced the percentage of mice that jumped during conditioning but did not affect withdrawal CPA [Figure 33]. These data suggest that maximal elevation of AEA and 2-AG, which occurs at the 12.5 mg/kg dose [Figure 6], is not sufficient to block the acquisition of CPA to morphine withdrawal.

Finally, we tested direct agonism of the CB₁ receptor with the phytocannabinoid THC, which is well-established to attenuate opioid withdrawal signs (Bhargava, 1976; Hine *et al.*, 1975; Ramesh *et al.*, 2011). THC reduced jumping associated with naloxone-precipitated morphine withdrawal but did not affect CPA. In this experiment, morphine-pelleted mice receiving vehicle pretreatment and the low dose of THC (1 mg/kg) did not exhibit reduced locomotor activity

unlike other experiments. The placebo-pelleted mice exhibited locomotor counts around 1500 which is slightly less than in previous experiments. Low doses of THC have been shown to produce CPP (Braida *et al.*, 2004; Ji *et al.*, 2006) while higher doses (e.g. ≥ 5 mg/kg) have been shown to produce CPA (Braida *et al.*, 2004; Cheer *et al.*, 2000; Hutcheson *et al.*, 1998; Zimmer *et al.*, 2001). Braida *et al.* showed in rats that THC administered i.p. 10 min prior to conditioning produces CPP at 0.075 – 0.75 mg/kg, no effect at 1 or 3 mg/kg but produces CPA at 6 mg/kg (2004). In mice, Zimmer *et al.* (2001) reported that THC (5 mg/kg) produced CPA and Valjent *et al.* reported that the same dose of THC produced CPA in mice as well (2000). Similarly, THC (5 mg/kg) was reported to produce CPA in mice while a lower dose of 1 mg/kg produced CPP (Soria *et al.*, 2004). Previously our lab published that THC (1 – 3 mg/kg) attenuated paw flutters and weight loss following naloxone-precipitated morphine withdrawal and near full blockade was observed at 10 mg/kg (Ramesh *et al.*, 2011). While the range of doses we tested against withdrawal CPA is the same that significantly attenuated somatic withdrawal signs, they do fall within the range of doses that produce CPA. Thus, it is possible that any effect of THC on reducing the CPA of morphine withdrawal may have been masked by this aversive effect of THC alone. However, 40 mg/kg JZL184 which was shown to be nearly as efficacious as THC (10 mg/kg) at reducing somatic withdrawal signs via CB₁ mechanism (Ramesh *et al.*, 2011) did not affect morphine withdrawal CPA and alone did not elicit CPP or CPA [Figure 30].

We proposed that endocannabinoid catabolic inhibitors would reduce naloxone-precipitated morphine withdrawal CPA based on interactions between the opioid and endocannabinoid systems in the nucleus accumbens and amygdala, regions important for opioid withdrawal CPA. Specifically, Zarrindast *et al.* reported that injection of naloxone into the central nucleus of the amygdala reduced the anxiolytic effects of the CB₁ agonist

arachidonylpropylamide (Zarrindast *et al.*, 2008) and FAAH has been localized with CB₁ receptors in the amygdala (Egertova *et al.*, 2003). This is an important region for opioid withdrawal CPA as injection of mu opioid antagonists into the amygdala have been shown to produce CPA and mild somatic withdrawal signs in morphine-dependent rodents (Maldonado *et al.*, 1992; Stinus *et al.*, 1990). It's also been shown that CB₁ and mu opioid receptors are colocalized in LC neurons (Scavone *et al.*, 2010), which would be consistent with the effects of cannabinoids on somatic signs of opioid withdrawal, however colocalization of CB₁ receptors with mu opioid receptors in amygdala is not as well established. There is some evidence suggesting the interactions of cannabinoids and opioids in the central nucleus of amygdala. Rezayof *et al.* (2011) demonstrated that injection of arachidonylcyclopropylamide, a synthetic CB₁ agonist, into the central nucleus of the amygdala enhanced morphine CPP and injection of the CB₁ antagonist AM251 into the central nucleus of the amygdala produced CPA and blocked morphine CPP, suggesting CB₁ receptors in the amygdala are important for morphine reward.

While the research presented in Chapter 4 is the first known investigation of cannabinoid effects on naloxone-precipitated morphine withdrawal CPA, other work has studied the interaction of opioid and endocannabinoid systems in CPP. Specifically, the full CB₁ agonist CP55,940 (0.02 mg/kg) produced CPP which was blocked by pretreatment with naloxone (Braida *et al.*, 2001). THC produced CPP at doses of 0.075 – 0.75 mg/kg which was fully blocked by naloxone (2 mg/kg i.p.) at a dose that did not produce CPA on its own (Braida *et al.*, 2004). Furthermore, the CB₁ receptor antagonist rimonabant (0.1 mg/kg) blocked CPP of morphine (4 mg/kg) while having no effect on its own (Chaperon *et al.*, 1998) and cannabinoid mediation of opioid CPP has reported elsewhere (Mas-Nieto *et al.*, 2001; Singh *et al.*, 2004).

Although cannabinoids and opioids appear to interact in reward processes as measured by CPP, we observed no effect of endocannabinoid catabolic inhibition on acquisition of naloxone-precipitated morphine withdrawal CPA. This suggests different mechanisms may contribute to reward related aspects of acute opioid exposure and negative motivational aspects of opioid withdrawal. The opponent process theory of addiction suggests two processes: the positive hedonic response and the affective/hedonic withdrawal response to drugs of abuse (Koob *et al.*, 2008b; Solomon *et al.*, 1974). Initially, the hedonic response (in this case the CPP to opioids) produces a rewarding state which in humans drives initial drug taking and undergoes tolerance such that a fixed amount of drug becomes less rewarding over time. The other response, the affective/hedonic withdrawal response, which occurs later and becomes more intense following continued exposure to drug, becomes the main driving force of drug taking later on (Koob *et al.*, 2008b; Solomon *et al.*, 1974). These two opponent processes are thought to be mediated by separate systems (Koob *et al.*, 1988) and while the endocannabinoid system appears to be involved in the reward aspects of opioids (Caille *et al.*, 2003; Chaperon *et al.*, 1998; Mas-Nieto *et al.*, 2001; Navarro *et al.*, 2001; Singh *et al.*, 2004) our data suggest it is not involved in the negative motivational response of opioid withdrawal as measured by CPA. This could be due to distinct mediators of these two aspects of the opponent processes. While the rewarding aspects of morphine are mediated by mu opioid receptors in the nucleus accumbens and VTA (Le Merrer *et al.*, 2009), there is a postulated “anti-reward” system which compensates for over activation of the reward system. The affective withdrawal response is suggested to involve this “anti-reward system” (Koob *et al.*, 2005) and has been suggested to include corticotropin-releasing factor (CRF), norepinephrine and dynorphin systems (Koob *et al.*, 2008a). Indeed, increased dynorphin has been observed in the amygdala of rats that underwent morphine withdrawal (Rattan *et al.*,

1992) and kappa agonism is known to be aversive to humans (Pfeiffer *et al.*, 1986) and rodents (Mucha *et al.*, 1985). Furthermore, CRF mRNA levels are increased during morphine withdrawal in the central nucleus of the amygdala of rats (Maj *et al.*, 2003) and CRF is known to be involved in fear and anxiety (Takahashi, 2001). Consistent with this idea, CRF1 knockout mice display a reduced CPA to opioid withdrawal and the increased dynorphin expression in the nucleus accumbens shell region following opioid withdrawal is reduced to control levels in CRF1 knockout mice (Contarino *et al.*, 2005). Furthermore, CRF1 antagonism has been shown to block acquisition of morphine withdrawal CPA in rats (Stinus *et al.*, 2004) and these effects appear mediated in the central nucleus of the amygdala (Heinrichs *et al.*, 1995).

Interestingly, CRF1 antagonism has also been shown to block the anxiogenic effects of CB₁ agonism in rats in the defensive withdrawal test (De Fonseca *et al.*, 1996). Additionally, AEA and THC have been shown to increase adrenocorticotrophic hormone (ACTH) and corticosterone (Weidenfeld *et al.*, 1994). Thus, it might be that while cannabinoids are reducing the somatic signs of opioid withdrawal they are producing similar effects to those of withdrawal in regions that mediate the negative motivational aspects through modulation of the HPA axis. Additionally, the negative motivational effects of THC are absent in dynorphin deficient mice (Zimmer *et al.*, 2001), suggesting that the aversive effects of cannabinoids are kappa mediated. Considering the data which suggest dynorphin involvement in the aversive aspects of opioid withdrawal, this commonality between cannabinoid agonism and opioid withdrawal presents a problem for use of cannabinoids for the treatment of opioid dependence.

In conclusion, cannabinoid receptor agonism either directly with THC or indirectly via inhibition of endocannabinoid catabolism does not appear effective at reducing the negative motivational consequences of opioid withdrawal in mice using the CPA paradigm. This assay

represents one among many in which opioid withdrawal produces effects which may reflect an aversive state. For example, opioid withdrawal elevates reward-thresholds in intracranial self-stimulation which may indicate an anhedonic-like effect (Altarifi *et al.*, 2011b; Kenny *et al.*, 2006; Liu *et al.*, 2004; Schaefer *et al.*, 1986; Schaefer *et al.*, 1983). Furthermore, opioid withdrawal produces anxiogenic-like effects in rats in the elevated plus maze (Schulteis *et al.*, 1998b; Zhang *et al.*, 2008). Future studies should examine the effects of cannabinoids on these behaviors related to opioid withdrawal as CPA represents only one measure of negative motivational aspects and endocannabinoid catabolic inhibitors may exhibit efficacy in these other measures. However, despite the reductions in somatic withdrawal signs by cannabinoids, the lack of effects of cannabinoids in morphine withdrawal CPA suggests that these may have limited efficacy in reducing affective withdrawal symptoms in humans. The lack of cannabinoid effects on opioid withdrawal CPA could be due to the effects of CB₁ agonism on CRF and dynorphin that are similar to those accompanying opioid withdrawal and/or insufficient CB₁ co-localization with mu opioid receptors in particular regions that mediate the negative motivational withdrawal response.

5.4. Possible mechanism for reductions in somatic withdrawal signs

We have shown that dual inhibition of the endocannabinoid catabolic enzymes FAAH and MAGL is effective at reducing the somatic aspects opioid withdrawal via CB₁ mechanism of action. Since CB₁ and mu opioid receptors are both G-protein coupled receptors that couple with G_{i/o} proteins, they share common downstream signaling events including mutual inhibition of adenylyl cyclase (Childers *et al.*, 1992) via activation of G_{i/o} proteins (Robledo *et al.*, 2008). Opioid withdrawal involves superactivation of adenylyl cyclase which results in cAMP

overshoot (Avidor-Reiss *et al.*, 1995) in regions (i.e. LC and PAG) known to mediate somatic signs of opioid withdrawal (Guitart *et al.*, 1992; Nestler *et al.*, 1988) and these changes are associated with the expression of physical signs of opioid withdrawal (Shaw-Lutchman *et al.*, 2002). This upregulation of the cAMP pathway leads to the hyper-excitability of LC neurons during withdrawal (Lane-Ladd *et al.*, 1997; Widnell *et al.*, 1994). This resulting hyperexcitability leads to increased noradrenergic outflow which largely contributes to the physical withdrawal signs (Crawley *et al.*, 1979; Van Bockstaele *et al.*, 2008). The ability of CB₁ receptors through activation of G_{i/o} proteins to inhibit adenylyl cyclase (Matsuda *et al.*, 1990; Vogel *et al.*, 1993) provides a means by which superactivation of adenylyl cyclase could be throttled, and the colocalization of CB₁ with mu opioid receptors in LC (Scavone *et al.*, 2010) and PAG (Wilson-Poe *et al.*, 2012) neurons demonstrates that CB₁ receptors are localized to important regions for the mediation of these withdrawal signs. Thus, inhibition of adenylyl cyclase resulting in a reduction in the cAMP overshoot that accompanies opioid withdrawal seems a likely mechanism through which cannabinoids attenuate the physical signs of opioid withdrawal. In addition, CB₁ receptors are located presynaptically on glutamatergic afferents to LC neurons and thus could inhibit glutamate release onto noradrenergic neurons, thereby inhibiting the release of norepinephrine (Scavone *et al.*, 2010).

5.5. Overall conclusions

These studies suggest that endocannabinoid catabolic inhibitors reduce the somatic signs, but not a negative motivational aspect of opioid withdrawal in the CPA paradigm. While the alpha-2 adrenergic agonists clonidine and fexofenadine are currently in use as non-opioid treatments for withdrawal, they have side effects such as hypotension, sedation and cognitive

impairment and in some patients are not fully effective at lessening the withdrawal symptoms (Gerra *et al.*, 2001; Gossop, 1988; Lobmaier *et al.*, 2010). Better treatment options are needed to assist patients undergoing detoxification from opioids and to improve patient retention in these programs. The efficacy of the endocannabinoid catabolic inhibitors at reducing somatic signs of opioid withdrawal in mice suggests they may be effective at reducing physical aspects of opioid withdrawal in humans (e.g. diarrhea). However, the lack of effects of endocannabinoid catabolic inhibitors on the acquisition of CPA suggests that their efficacy may not extend to affective aspects of opioid withdrawal, though these results are from a single experimental assay (CPA) and tested in a single mouse strain. Regardless, the observations that endocannabinoid catabolic enzyme inhibitors reduce somatic withdrawal signs argue for further investigation of these drugs. The ability of endocannabinoids to attenuate the expression of diarrhea also suggests that these effects are not simply non-specific reductions in behavior and a previous study demonstrating that endocannabinoid catabolic inhibitors reduce naloxone-precipitated increases in contractions of morphine treated ilea supports this (Ramesh *et al.*, 2011). Future studies should examine the ability of these compounds to reduce other behaviors that may reflect affective components of opioid withdrawal and also examine them in other models of drug abuse such as reinstatement of opioid self-administration in dependent rodents. Additionally, determination of the downstream signaling events which may mediate their effects will provide further evidence for interactions between endocannabinoid and opioid systems during opioid withdrawal. Conditional knockout of CB₁ receptors on mu opioid receptor containing neurons would provide a means for determining the mechanism of cannabinoid action on reducing somatic withdrawal signs. In conclusion, inhibition of endocannabinoid catabolism is effective at attenuating somatic opioid

withdrawal signs and further studies on their effects on other aspects of opioid withdrawal are warranted.

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Ph.D., Pharmacology and Toxicology

Dissertation: Differential effects of endocannabinoid catabolic inhibitors on opioid withdrawal in mice

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Thesis: Anandamide produces discriminative stimulus effects in FAAH KO mice

Advisor: Dr. Jenny Wiley

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Bibliography

Papers

Gamage, T.F., Damaj, M.I., and Lichtman, A.H. (in prep) Inhibition of endocannabinoid catabolism reduces somatic, but not negative motivational, signs of withdrawal

Gamage, T.F., Sticht, M.A., Crowley, J.P., and Lichtman, A.H. (in prep) Assessment of escape behavior during naloxone-precipitated morphine withdrawal in mice

Gamage, T.F., Ignatowska-Jankowska, B., Poklis, J., Thakur, G., Tichkule, R., Ross, R.A., Pertwee, R.G., and Lichtman, A.H. (accepted) In vivo pharmacological evaluation of the CB1 receptor allosteric modulator, Org-27569, submitted September 2013 to *Behavioural Pharmacology*

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Abstracts

- Gamage, T.F. and Lichtman, A.H. Inhibition of endocannabinoid metabolism reduces somatic signs, but not conditioned place avoidance, to morphine withdrawal. *To be presented at The College on Problems of Drug Dependence, 2013*
- Gamage, T.F., Ramesh, D., Abdullah, R., Niphakis, M.J., Cravatt, B., and Lichtman, A.H. Endocannabinoid metabolic inhibitors for the treatment of opioid withdrawal. *Presented at Carolina Cannabinoid Collaborative 2012*
- Gamage, T.F., Ramesh, D., Abdullah, R., Niphakis, M.J., Cravatt, B., and Lichtman, A.H. Dual FAAH/MAGL inhibition attenuates somatic signs of spontaneous morphine withdrawal in ICR mice. *Presented at International Cannabinoid Research Society 2012*
- Gamage, T.F., Thakur, G., Ross, R.A., Pertwee, R.G., and Lichtman, A.H. Evaluation of putative CB1 receptor negative allosteric modulators Org-27569 and PSNCBAM1 in whole animals. *Presented at the Carolina Cannabinoid Collaborative 2011*
- Gamage, T.F., Ross, R.A., Pertwee, R.G., and Lichtman, A.H. Investigation into the behavioral effects of the purported CB1 negative allosteric modulator Org-27569. *Presented at International Cannabinoid Research Society 2011*
- Gamage, T.F., Burston, J.J., Pertwee, R.G., Ross, R.A., Wiley, J.L., Long, J.Z., Cravatt, B.F., and Lichtman, A.H. Effects of the MAGL inhibitor, JZL184, and the CB1 negative allosteric modulator, Org-27569, on sweet caloric consumption in male C57BL6 mice. *Presented at the Carolina Cannabinoid Collaborative 2010*
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- Gamage, T.F., Wiley, J.L., Lichtman, A.H., Warner, J.A., King, L.S., Grainger, D.B., and Vann, R.E. Absence of Fatty Acid Amide Hydrolase Facilitates Acquisition of Anandamide's Discriminative Stimulus. *Presented at Society for Stimulus Properties of Drugs 2007*
- Gamage, T.F., Vann, R.E., and Damaj, M.I. Does the NACHR $\alpha 5$ subtype play a role in nicotine's discriminative stimulus? *Presented at Virginia Academy of Science, 2007*

Gamage, T.F., Warner, J.A., Taylor, N.L., Vann, R.E., Lichtman, A.H., Martin, B.R., and Wiley, J.L., Characterization of the Discriminative Stimulus Effects of Anandamide. *Presented at Society for Stimulus Properties of Drugs 2006*

Gamage, T.F., Vann, R.E., Warner, J.A., Marshall, E.M., Taylor, N.L., and Wiley, J.L., The effects of cannabidiol, a constituent of marijuana, on the discriminative stimulus and conditioned place aversion effects of Δ^9 -THC. *Presented Virginia Academy of Science, 2006*

Awards and Honors

- Honorable mention, Best Presentation at VAS 2013
- Honorable mention, Best Poster Presentation at SSPD 2006
- NIDA Training Grant Award (2T32DA007027-36)– 2010-2012
- International Cannabinoid Research Society Travel Award – 2009, 2011, 2012
- Carolina Cannabinoid Collaborative Travel Award – 2010
- Amgen Summer Research Scholarship at University of California San Diego - 2007
- Award for Excellence in the Presentation of Research at the UCSD Summer Research Conference

Behavioral Techniques

- Stereotaxic surgery
- Mouse and rat i.p., s.c., i.c.v. p.o., routes of administration
- MED-PC programming
- Any-Maze
- Conditioned place preference and avoidance
- Operant behavior
- Light/dark box, elevated plus maze, novelty-induced hypophagia
- Scoring of somatic withdrawal signs

Teaching and Professional Experience

- Editor of Medical Sciences Division, Virginia Academy of Science, 2009-2012
- Invited Commentator on Cannabis and Cannabinoids, WVCW – April 20, 2011
- Guest Lecturer
 - PSYC401: Biological Psychology, 2012
 - Lectures on schizophrenia and antipsychotics
 - PHTX400: Drugs and their action, 2010-2012
 - Lectures on antipsychotics, antiparkinson agents, cannabinoids, nicotine, antidepressants and mood stabilizers
- Teaching Assistant, Department of Psychology, VCU, 2007-2008
 - Learning and Cognition, Theories of Personality, History of Psychology
- SGA Representative, Pharmacology and Toxicology Student Organization, 2010
- Mentor for Joseph Crowley, undergraduate independent research, 2012-2013
- Mentor for William Farmer, summer high school student, 2009

Research Interests

- Endocannabinoid modulation of reward and decision making
- Affective aspects of drug withdrawal

- Opioid/Cannabinoid interactions
- Motivation and choice behavior in drug addiction
- Plasticity in drug addiction and depression
- Allosteric modulation of cannabinoid receptors

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Society for Stimulus Properties of Drugs, 2006-2008