PHARMACOLOGY AND BEHAVIOR GENETICS OF HEROIN DEPENDENCE IN MICE

by

Gad Klein

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

2008

This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

1/28/08	Benjamin Kest, Ph.D.
Date	Chair of Examining Committee
1/28/08	Joseph Glick
Date	Executive Officer

Richard Bodnar, Ph.D.

Robert Ranaldi, Ph.D.

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

PHARMACOLOGY AND BEHAVIOR GENETICS OF HEROIN DEPENDENCE IN MICE

by

Gad E. Klein

Advisor: Professor Benjamin Kest, Ph.D.

Exposure to opioid drugs can lead to a dependent state that is manifested by physical symptoms during naloxone-precipitated withdrawal (NPW). Because heroin pharmacology is similar to morphine, it has been assumed that they utilize common neural substrates. This dissertation reports several studies that test this assumption. The specific aim of the first study was to examine jumping frequency as a valid measure of NPW from heroin in mice. The data show a positive dose-response relationship between acute and chronic heroin doses and NPW jumping, and reveal the interval yielding maximal responding. These doses and drug intervals were used in all subsequent studies.

The second study assessed the contribution of opioid (δ_1, δ_2 & κ) and excitatory amino acid (NMDA and AMPA) receptors to NPW jumping frequency in heroin-dependent mice. We found that δ_1 & δ_2-antagonists attenuated both acute and chronic heroin dependence, while the κ antagonist increased chronic heroin, but had no effect on acute heroin, dependence. The NMDA and AMPA receptor antagonists MK-801 and LY293558 appear to impact heroin dependence as well. Acute heroin dependence was not affected by single injections of either MK-801 or LY293558, but continuous infusion or chronic injection of these antagonists was effective in reducing acute and chronic

heroin dependence. The data thus indicate that the attenuation of heroin dependence in acute and chronic paradigms likely results from neuronal adaptations from long term pretreatment with this class of drugs.

In a third study, we assessed the contribution of genotype on heroin withdrawal magnitude by surveying 6 inbred mouse strains for NPW jumping after acute and chronic heroin injection. The data demonstrate that the magnitude of NPW jumping frequencies in inbred mice following acute and chronic heroin treatment is associated with genetic variability. The data also show that acute and chronic heroin dependence share common genes. The data also reveal a strong genetic correlation between acute and chronic heroin and acute and chronic morphine dependence, indicating that common physiological substrates underlie dependence to these two opioids.

The fourth study studied the ability of antisense oligonucleotides (AS ODNs) targeting various exons from the MOR-1 to alter heroin and morphine dependence. AS ODNs directed against exon-1 of the μ-receptor attenuated heroin and morphine dependence, but had no impact on heroin analgesia. These data suggest that heroin and morphine dependence are mediated by splice variants containing exon-1, but that heroin analgesia may be mediated by a separate variant of the μ-receptor. Overall, these studies characterize heroin dependence in mice, and reveal that morphine and heroin dependence are not identical processes.

The completion of this dissertation and of my graduate career has been a long time in coming. My labmates and friends have helped me through by being study partners and time wasting partners. Getting through classes, doctoral exams, clinical externships, repeated mouse injections, and rodent allergies would have been a lot harder and a lot less fun without them. My advisors have guided me from being someone who didn't really know what science meant to someone who knows how to think scientifically, and for that I will forever be grateful.

My siblings have been supportive and proud throughout the whole process even when they couldn't quite answer the question, "So what exactly does your brother/brother in-law do?" I believe that the answer usually varied between, "he plays with mice hopped up on heroin" and "he tests peoples brains to make sure they think properly", which I guess wasn't all that inaccurate. My parents and in-laws watched my children on numerous occasions and without them and their constant encouragement there is no way this dissertation would have been completed. Their pride in my accomplishment is reflected in the fact that they are demanding on pain of death that I attend my graduation ceremony and get hooded just so they can "shep" some nachas.

My children, Ezra and Noam, have made this journey a bit more complicated, but a lot more fun and meaningful. The memory of a 2-year-old Ezra grabbing an anesthetized mouse off the lab bench when I turned my head and pointing out its ears, nose, eyes and tail will stay with me forever. I love you guys.

Gabi, you have been with me since the beginning of this thing and have been nothing but supportive even when our schedules were hectic, I was out late studying or doing research and our lives were changing from semester to semester. Although it seemed like we would never make it to this point, here we are and stronger than ever. I love you and dedicate this dissertation to you and to our beautiful family.

Table of Contents

List of Figures

List of Tables

Glossary of Abbreviated Terms

MOR - μ opioid receptor
Oprm - μ opioid receptor gene
Oprk1 - κ-opioid receptor gene
EAA - excitatory amino acid
NPW - naloxone precipitated withdrawal
KO - knockout
NMDA - N-methyl-D-aspartate
AMPA - alpha-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid
mGluR - metabotropic glutamate receptors
AS ODN - antisense oligodeoxynucleotide
s.c. - subcutaneous
i.t. – intrathecal
i.c.v. - intracerebroventricular
AC - adenylyl cyclase
cAMP - cyclic adenosine monophosphate
LAAM - levo-alpha acetyl methadol
M6G - morphine-6-beta glucuronide
6-MAM - 6-monoacetylmorphine
β-FNA - beta-funaltrexamine
DAMGO - [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin
NTI - naltrindole
5'-NTII- naltrindole 5' isothiocyanate
BNTX - 7-benzylidenenaltrexone
NTB - naltriben
nor-BNI - nor-binaltorphimine
GABA - Gamma-aminobutyric acid
CaMKII - Ca^{2+}/calmodulin-dependent protein kinase II
ED_{50} - effective dose 50
cM - centiMorgan
MS - mismatch
% M.P.E. - % maximum possible effect
QTL - quantitative trait loci

Chapter 1.

Introduction

In 1806 Wilhelm Sertürner isolated morphine, one of the active components of the opium poppy. Morphine enjoyed widespread use as an analgesic and it was known to cause both psychological and physical dependence. Attempts to create morphine-like drugs with no dependence liability led to the synthesis of numerous compounds such as heroin and oxycodone. These drugs shared structural similarities with morphine and their use triggered morphine-like actions including the development of physical dependence (Inturrisi, 2002).

Subsequently, the endogenous opioid compounds termed enkephalins, endorphins, and dynorphins were discovered (Hughes et al., 1975; Goldstein & Cox, 1977; Goldstein et al., 1979). Both β-endorphin and enkephalin were shown to have morphine-like effects (Hughes, 1975). Different types of opioid receptors were proposed, and both behavioral and pharmacological evidence indicated that there were three major receptor types termed mu (μ), delta (δ), and kappa (κ). Although the endogenous ligands bound with some affinity to all three receptor types, it was found that these ligands displayed certain receptor preferences. The enkephalins preferred δ, the dynorphins preferred κ, and β-endorphins preferred μ (Terenius, 2000). It was also found that many of the synthesized morphine-like compounds bound preferentially to the μ-receptor and mediated most of their effects through this receptor type (Kosterlitz, 1985; Pasternak, 2001). Due to their preference for the μ-receptor these drugs became known as μ-opioids. All three receptor subtypes were soon cloned (Evans et al., 1992; Kieffer et al., 1992; Chen et al.,1993; Minami et al.,1993) and it was found that deletion of the gene coding

for the μ-receptor led to an almost complete loss of morphine's actions in-vivo including analgesia and physical dependence (Matthes et al., 1996). Mu receptor knockout mice also displayed lack of heroin induced analgesia, locomotor stimulation, and reward (Contarino et al., 2002; Kitanaka et al., 1998). It thus became clear that the μ-opioids morphine and heroin exert their effects primarily through the μ-receptor.

Physical dependence to opioids is a phenomenon in which the central nervous system undergoes physiological changes in response to opioid drugs, especially those preferring the μ-subtype such as morphine and heroin. However, this process usually remains hidden until it is "unmasked" by cessation of drug treatment or precipitation of withdrawal by receptor blockade with an antagonist. The withdrawal process is often an expression of physical behaviors or disturbances that occurs following elimination of the μ-opioid from the nervous system, or at least from its association with the opioid receptor (Koob et al., 1992). The presence of physical dependence to the opioid drug is reflected behaviorally by this withdrawal syndrome (Kest et al., 2001) and the magnitude of the withdrawal is considered a measure of the severity of physical dependence. The magnitude and types of physical behaviors manifested depend upon numerous factors. These factors include, but are not limited to opioid receptor subtype involvement (Tsuji et al., 2000; Abdelhamid et al., 1991), type of μ-opioid administered (Wiley & Downs, 1979), as well as method of administration and duration of exposure to the drug (El-Kadi & Sharif, 1994; DeLander et al., 1984). Although it will be discussed in greater detail later on, it should be noted that this withdrawal syndrome is manifest following both chronic and acute exposure to μ-opioid drugs, and that there is evidence that they may be mediated by somewhat different mechanisms (Marshall & Weinstock, 1971).

The desire to alleviate, or to avoid altogether, this aversive state of physical withdrawal is thought to trigger the continued abuse of μ-opioids and/or relapse after cessation of drug use (Koob & Le Moal, 2001). Research has focused on the role of non-mu opioid receptors and excitatory amino acid (EAA) receptors that might modulate mu-mediated dependence (Trujillo & Akil, 1991; McLemore et al. 1997). Research has also focused on the genetic influences on dependence and withdrawal partly due to the recognition that for some individuals, opioid use leads to minimal or no dependence, while others are highly susceptible. Studies with inbred (isogenic) mouse strains have clearly demonstrated genotype-dependent effects on withdrawal severity (Kest et al., 2002a; Liang et al., 2006). As inbred mouse strains display similar phenotypes and are genetically virtually identical to each other, but vary from other inbred strains, understanding the relationship between these differences may help in elucidating individual the individual responses to dependence and withdrawal. Thus, there have been some attempts to identify the contribution of genetic background to inter-individual differences. However, the vast majority of literature studying physical dependence and naloxone precipitated withdrawal centers around morphine and not heroin. These studies are aided by the use of naloxone to precipitate withdrawal (NPW) at the experimenters choosing, and the use of the subsequent jumping frequencies as a reliable index that is easily and rapidly obtained. Although both morphine and heroin are u-preferring opioids, there is reason to suspect that they may act via distinct mechanisms to cause dependence. For example, morphine deficient CXBK mice which show an attenuated response to morphine in analgesic assays retain their analgesic sensitivity to heroin analgesia (Rossi et al., 1996). Additionally, the selective antagonist 3-O-methylnaltrexone, antagonized

the analgesic effects of heroin at doses ineffective against morphine (Brown et al., 1997; Walker et al., 1999). These studies implicate pharmacological differences between morphine and heroin. Thus, it is possible that these two mu-agonists mediate dependence through distinct mechanisms. Since there is a paucity of experimental studies of heroin dependence and withdrawal, it is not possible to determine whether heroin dependence is mediated by mechanisms similar to morphine.

The specific aims of the studies comprising the dissertation characterize the contributions of various opioid and EAA receptor subtypes as well as the influence of genotype underlying naloxone precipitated withdrawal from heroin. Antisense studies exploring specific exons of the μ-opioid receptor gene are also utilized to assess the possible differential role of various μ-receptor splice variants in heroin dependence.

The **first aim** will be to assess whether naloxone precipitated withdrawal jumping frequency is, as has been demonstrated for morphine (El-Kadi & Sharif, 1994; Way et al., 1969), a valid measure for studying heroin dependence, and the optimal protocols of this technique. Thus, we first generated a time course for the evaluation of naloxone precipitated withdrawal following exposure to heroin. Once a time course had been established it was necessary to establish optimal experimental doses of both heroin and naloxone in order to properly examine the phenomenon of naloxone precipitated withdrawal jumping in heroin dependent mice. Therefore, groups of male outbred (CD-1) mice were compared for naloxone-precipitated withdrawal jumping following various doses of both heroin and naloxone. As before, both acute and chronic exposure paradigms were examined as the optimal dose for both heroin and naloxone under the two conditions may be distinct. Having established the validity of jumping frequencies

as an index of withdrawal magnitude, the **second aim** of this dissertation will be to assess the contribution of non-mu opioid receptor subtypes and EAA receptors to the development of both acute and chronic heroin dependence by testing their effect on jumping during NPW from heroin. Specifically, naive groups of male outbred (CD-1) mice were treated with a δ_1-opioid, δ_2-opioid, κ-opioid, NMDA, or AMPA receptor antagonist concurrently with acute or chronic heroin treatment.

The **third aim** of this dissertation will examine the influence of genetic background on acute and chronic heroin dependence. Previous studies utilizing strain surveys in mice have demonstrated significant genetic variance between inbred strains in the development of morphine dependence (Kest et al. 2002a). Inbred mouse strains, which are often established by at least 20 generations of sibling mating, are homozygous at every allele and thus genetically identical (Han et al., 2004). Although some inbred strains are more closely related to other strains, overall each individual inbred mouse strain is genetically unique. Thus, distinct behavioral differences between the strains can assist in elucidating the genetic mechanism underlying the behavior. Here we utilize inbred strains in order to examine the contribution of genotype to heroin dependence. Then, since we are also utilizing many of the same strains of mice used in a prior inbred strain survey examining morphine dependence, the correlation of the current data with the strain sensitivity in the morphine study will indicate whether there are shared genetic substrates between morphine and heroin dependence. Finally, the use of antisense oligodeoxynucleotides (AS ODNs) targeting specific exons of the mu receptor has previously shown that morphine and heroin analgesia utilize different μ-receptor isoforms (Rossi et al., 1996). Thus, the final and **fourth aim** of this dissertation will assess the

contribution of specific μ-receptor splice variants to heroin withdrawal. In this study, AS ODNs will be administered to CD-1 mice subject to either morphine or heroin dependence.

In order to provide a context for the current series of studies, the following next section will provide a review of the relevant literature and previous findings. The topics covered will include 1) acute and chronic opiate dependence; 2) withdrawal as a measure of physical dependence; 3) the contribution of opioid receptor subtypes to opioid dependence; 4) the contributions of various EAA receptor subtypes to opioid dependence; 5) behavioral genetics of opioid dependence; 6) summary of research; 7) and a rationale for present studies.

Background

1. Introduction to Dependence on μ-opioids

a) Acute & Chronic Dependence

Physical dependence and withdrawal from μ-opioids can be induced by several different methods. There are both acute and chronic models of physical dependence. In the acute model, animals are injected with a single dose of opioid, and then with a single dose a few hours later (McLemore et al., 1997). Injections of μ-agonist and antagonist can be administered subcutaneously (s.c.), spinally (i.t), or intracerebroventricularly (i.c.v.) (Bell & Adler, 1988; Granados-Soto et al., 2000; Easterling & Holtzman, 2000) indicating that both spinal and supraspinal opioid receptors are involved in this phenomenon. Acute models of dependence allow for punctuate assessment of dependence and can give us insight into the initial adaptive changes that occur in opioid dependence.

The distinguishing feature between models of acute and chronic physical dependence is the duration of agonist exposure. There are a few methods through which chronic dependence can be induced. One model is that of ascending doses of multiple injections over a number of days, followed by an acute injection of an antagonist to precipitate withdrawal (Craft et al., 1999). However, this model has limitations as individuals may experience short-term spontaneous withdrawal between the successive agonist injections. This issue can be solved by the use of osmotic mini-pumps or by implantation of agonist slow release pellets which can provide a continuous source of agonist over the course of days (Kest et al., 2002a; Georges et al., 2006). The pellets and mini-pumps can then be removed prior to an acute injection of an antagonist and

precipitation of withdrawal. While it has been noted that the various routes of administration differentially influence the development of dependence, Kest et al. (2002a) has shown that there is a high degree of genetic similarity in the withdrawal response to mice made dependent through the use of osmotic mini-pumps and both multiple and acute injections. This genetic similarity between chronic and acute dependence seems to underlie the animal's general susceptibility to physical dependence rather than the mechanism through which these forms of physical dependence actually develop (see next section).

The withdrawal syndrome seen following acute dependence is less severe and sometimes of a different quality than what is seen after chronic dependence (Blasig et al., 1973; Smits, 1975), which implies that the two phenomena may be mediated by distinct processes (Yamamoto et al., 1978; Nehmad et al., 1982). Still other researchers provide evidence that acute and chronic dependence differ in quantity and severity, but not quality suggesting that similar substrates underlie both processes (McLemore et al., 1997; Wiley & Downs, 1979). It was demonstrated that common genetic substrates underlie physical dependence to morphine following either acute or chronic treatment (Kest et al., 2002a). Additionally, the withdrawal syndromes in animals following acute and chronic dependence are similar and include teeth chattering, ptosis and jumping (Kest et al., 2001). Often however, a higher dose of antagonist is needed to precipitate the full range of withdrawal behaviors in acute dependence, which seems to indicate that the severity of withdrawal signs are positively related to the dose of drug used. While some acute dependence studies have demonstrated observable withdrawal signs following spontaneous withdrawal (Harris & Gewirtz, 2004; Liu & Schulteis, 2004) the degree of

dependence after a single administration usually necessitates an antagonist precipitated withdrawal in order for the animal to express behavioral symptoms. This is not the case in chronic dependence where observable signs are usually present following spontaneous withdrawal, although they may take a while to become evident (Kowalczyk et al., 2004).

The biochemical processes underlying acute and chronic morphine exposure are thought to be differentially mediated. For example, an AMPA antagonist was found to block the development of acute, but not chronic dependence in mice (McLemore et al., 1997), and NMDA receptor antagonists block acute, but not chronic dependence in neonatal rats (Zhu & Barr, 2000; Jones et al., 2002). In mice, the mRNA of c-fos, fos-B, c-jun and jun-B was upregulated slightly following acute administration of morphine, but not after chronic exposure to μ-opioids for six days (Hayward et al., 1990; Couceyro & Douglass, 1995; Ammon-Treiber, 2005). Duman et al. (1988) found that acute morphine exposure decrease adenylyl cyclase (AC) levels, and chronic exposure raises AC levels, while similar results were found for cyclic adenosine monophosphate (cAMP) levels in the rat locus coeruleus (Nestler & Tallman, 1988).

Similarities between acute and chronic dependence are seen in the resemblance of withdrawal signs and the fact that severity of withdrawal signs are related to drug dose (Marshall & Weinstock, 1971), while their differences lay primarily in the magnitude of the withdrawal signs and the underlying biochemical substrates. Thus, the collective data imply that while both methods are valid tools for studying the phenomenon of physical dependence to opioids, they are distinct processes that should be examined separately.

2. Withdrawal as a measure of physical dependence

a) Withdrawal behaviors

Physical dependence on μ-opioids is an unwanted side effect of drug administration that is manifested by a characteristic withdrawal syndrome of multiple aversive behavioral and physiological signs in a wide variety of species. In essence the presence of physical dependence to the opioid drug is reflected behaviorally by this withdrawal syndrome (Kest et al., 2001) and the magnitude of the withdrawal is considered a measure of the severity of physical dependence. As will be explained in further detail below, withdrawal is typically observed following abrupt termination of morphine intake or precipitated by administration of a narcotic antagonist such as naloxone. Precipitated withdrawal jumping, a significant aspect of the withdrawal syndrome, is currently the most reliable way of assessing the phenomenon of dependence in mice (Kest et al., 2001; El-Kadi & Sharif, 1994; Marshall & Weinstock, 1971). While there are a few biological markers associated with dependence and the withdrawal syndrome (e.g. upregulation of transcription factors, excitatory/inhibitory neuronal responses), these markers are not easily measured and don't necessarily correlate with the length or dose of opioid exposure or show a dose-response relationship with the precipitating opioid antagonist. Additionally, a significant number of studies examining these various cellular and molecular responses have been performed in vitro, but have yet to be demonstrated in vivo (Lane-Ladd et al., 1997). In contrast, the aversive nature of the withdrawal syndrome precipitated by naloxone, as indexed by withdrawal jumping frequency in rodents, has recently been demonstrated to be a motivational factor for the continued administration of heroin and is thought to play a significant role in long-term

heroin abuse and relapse behavior (Kenny et al., 2006). Therefore, methods to mitigate aspects of this syndrome, as explored in this dissertation may contribute to abuse rehabilitation and continued drug abstinence.

In animals, μ-agonist withdrawal is characterized by a syndrome of consistent and measurable physical signs. Symptoms include escape behaviors and teeth chattering (Bozarth & Wise, 1984), wet-dog shakes (Craft et al., 1999), writhing (Bell & Adler, 1988), locomotion (van der Laan & de Groot, 1988), diarrhea, ptosis, and tremor (Gellert & Holtzman, 1978), hypothermia (Belknap, 1989), decreased food intake (El-Kadi & Sharif, 1994; Bell & Adler, 1988), and jumping (El-Kadi & Sharif, 1994).

In humans precipitated withdrawal is followed by symptoms that can include nausea and vomiting, profuse sweating, marked yawning, extreme fatigue, aches and pains, diarrhea and a painful dysphoric state that resembles influenza (Farrell, 1994; Koob et al., 1992). Spontaneous withdrawal symptoms are milder and include drug craving in the early stages, followed by anxiety, agitation, increased sweating, aching, increased bowel motility and a similar painful dysphoric state that resembles influenza (Farrell, 1994; Koob et al., 1992).

b) Jumping behavior as the most reliable index of withdrawal in animals

The jumping response, characterized by all four paws removed from the cage surface, is frequently used in animals as an indicator of naloxone precipitated withdrawal (NPW). A comparison of naloxone dose-response curves for jumping and other withdrawal behaviors indicates that as the dose of naloxone increases, the magnitude of jumping behavior also increases (Marshall & Weinstock, 1971; Smits, 1975). This correlation only held true for the jumping response. Additionally, the frequency of NPW

jumping following chronic morphine injection shows a positive relationship with increases in morphine or naloxone dose (Kest et al., 2001) and duration of morphine exposure (Kest et al., 2001; El-Kadi & Sharif, 1994). A comparison of morphine time-effect curves for jumping frequency and locomotor activity indicate that withdrawal jumping responses are not an artifact of the stimulatory effects of morphine (Smits, 1975).

It was also noted that NPW jumping following acute administration of narcotic agents known to have high dependence liability such as morphine, heroin and Levo-Alpha Acetyl Methadol (LAAM) was more frequent and more consistent than the NPW jumping seen after mixed agonist-antagonist drugs such as pentazocine, cyclazocine and buprenorphine (Wiley & Downs, 1979). Finally, in a study assessing NPW jumping using numerous different μ-agonists including morphine, levorphanol, meperidine, methadone and codeine, the incidence of withdrawal jumping corresponded with the ability of these drugs to induce physical dependence in humans (Nakamura et al., 1983; Iorio et al., 1975).

c) Spontaneous withdrawal

Spontaneous withdrawal responses are observed after cessation of chronic exposure to, or following an acute administration of, a μ-opioid agonist (Kishioka et al., 1996; Langerman et al., 2001). Withdrawal behaviors such as jumping (Huffman et al., 1985), decreased food and water intake (Langerman et al., 2001), weight loss (Kishioka et al., 1996), decreased locomotor activity (van der Laan & de Groot, 1988) and hypothermia (Kest et al., unpublished results) have been observed as early as 6 hours and as late as 4 days after morphine treatment in rodents. Spontaneous withdrawal behaviors

have been found to be dose-dependent, as greater cumulative doses of morphine have been associated with greater changes in the magnitude of withdrawal behaviors (Langerman et al., 2001). Research has indicated that these withdrawal behaviors are relatively slow to develop and are often so mild in comparison to precipitated withdrawal as to be imperceptible. However, we have found that mice undergoing spontaneous withdrawal from higher doses of morphine will exhibit withdrawal signs within hours that are significantly different from baseline and are comparable to those seen during NPW (Kowalczyk et al., 2004). Although NPW is currently the preferred method used to study physical dependence, a reliable model of spontaneous withdrawal would be remarkably useful for the study of clinical drug treatment approaches. This is because unless administration of an antagonist is required to counteract life-threatening cases of acute overdose most human opiate addicts go through spontaneous withdrawal after cessation of drug use.

d) Naloxone precipitated withdrawal (NPW)

Physical withdrawal responses following chronic exposure to a μ-opioid are frequently precipitated by injection of an opiate antagonist such as naloxone. Naloxone is a wide-spectrum opioid antagonist that has a high affinity for the μ-receptor (Gackenheimer et al., 2005), and dose-dependently elicits robust withdrawal responses such as jumping, weight loss, diarrhea and hypothermia in μ-opioid dependent animals (Way et al., 1969; Belknap, 1989; El-Kadi & Sharif, 1994). However, other rodent studies have demonstrated that chronic exposure to delta or kappa agonists followed by administration of naloxone can precipitate minimal to moderate withdrawal behaviors (although not jumping; Cowan et al., 1988**;** Maldonado et al., 1990). This implies that

direct stimulation of the other opioid subtypes can also result in dependence and withdrawal. Interestingly, naloxone precipitated withdrawal signs (jumping, sniffing, teeth chattering, ptosis) following chronic morphine administration were completely abolished in μ-receptor gene "knockout" mice (Matthes et al., 1996). The authors point out that there was a lack of withdrawal response in dependent "knockout" mice even when using high doses of morphine and naloxone able to interact directly with the other opioid receptor subtypes. They conclude that in the absence of the μ-receptor, the δ and κ receptors play no role in dependence. Thus, in mice expressing the μ-receptor, the other opioid receptors likely impact μ-mediated dependence through receptor-receptor interaction and second messenger systems rather than through direct modulation. It is plausible that the withdrawal behaviors noted following δ and κ-agonist administration (Cowan et al., 1988; Maldonado et al., 1990) are a result of these subtypes interacting with the μ-receptor or due to overlapping second messenger systems. While more research is needed to fully clarify this issue, it seems clear that the behavioral responses following NPW are mediated primarily through the μ-opioid receptor.

Peak withdrawal in mice is observed for a punctuate 15-minute period when naloxone is administered 3 hours after the last treatment (El-Kadi & Sharif, 1994; Way et al., 1969). Naloxone precipitated withdrawal is often the preferred mode for the assessment of dependence because the behaviors can be easily and reliably observed, and quickly assessed (El-Kadi & Sharif, 1994) (see above.).

As with spontaneous withdrawal, μ-agonist treatment dose and chronicity affect the magnitude of withdrawal. Marshall & Weinstock (1971) treated mice with s.c. injections of various morphine doses for different durations with the maximum duration

of treatment being ten days. They found that increased NPW jumping was correlated with larger doses and longer treatment duration. These finding were supported in an acute paradigm as well as mice pretreated with higher doses of morphine displayed significantly more NPW jumping behavior (Smits, 1975). Mice treated with higher doses of other μ-agonists such as heroin, LAAM and methadone also jumped more than mice treated with lower doses of these same drugs (Wiley & Downs et al., 1979). As mentioned above, some spontaneous withdrawal behaviors have been found in at least one study to be dose-dependent (Langerman et al., 2001), however these behaviors are relatively slow to develop and can be milder than those seen in precipitated withdrawal. The frequency of NPW jumping following chronic and acute morphine injection shows a positive relationship with increases in morphine or naloxone dose (Kest et al., 2001; Kosersky et al., 1974) and duration of morphine exposure in chronic paradigms (Kest et al., 2001; El-Kadi & Sharif, 1994). The jumping response is easily measurable and in various μ-agonists has been demonstrated to correspond with the ability of these drugs to induce physical dependence in humans (Nakamura et al., 1983; Iorio et al., 1975). For these reasons we have chosen to utilize a naloxone precipitated jumping response as the dependent measure in our studies. Currently, we cannot generalize data from our studies utilizing precipitated withdrawal to spontaneous withdrawal from heroin or morphine as there are no data correlating the two phenomenons. However, recent data from our lab indicates that mice undergoing spontaneous withdrawal from higher doses of morphine will exhibit withdrawal signs, including jumping, that are significantly different from baseline and are comparable to those seen during NPW (Kowalczyk et al., 2004; Papaleo

& Contarino, 2006). This data is likely the subject of a future dissertation from our laboratory.

It is well documented that μ-opioids disinhibit dopaminergic neurons in various areas of the cortex through inhibition of Gamma-aminobutyric acid (GABA) interneurons (Johnson & North, 1992; Alger & Nicoll, 1980). This is relevant to NPW as research indicates that high doses of naloxone are capable of antagonizing the GABA interneurons that are disinhibited by μ-opioids (Gumulka et al., 1979). Thus, it is possible that that at least part of the naloxone precipitated hyperactive jumping response is a result of GABA receptor blockade following exposure to morphine (Dingledine et al., 1978).

3. The Contribution of Opioid Receptors to Opioid Dependence

a) Mu opioid receptor (MOR)

Mu receptors are located throughout the central nervous system (CNS), and particularly in regions that have been found to contribute to opiate withdrawal (DeLander et al., 1984; Arvidsson et al. 1995a). It has been shown that the binding of morphine to the μ-receptor is the initial step in the development of physical dependence. Intrathecal administration of beta-funaltrexamine (beta-FNA), a long acting irreversible μ-antagonist, prior to morphine administration significantly reduced, but did not abolish the development of physical dependence in rats (DeLander et al., 1984). Supraspinal chronic administration of beta-FNA with concurrent morphine administration also profoundly reduced the development of dependence in rats in a dose dependent manner (Aceto et al., 1986). More recent data has demonstrated that pretreatment with antibodies directed against the μ-receptor significantly attenuates NPW signs in morphine dependent mice

(Sanchez-Blazquez et al., 1996). The same result was obtained when AS ODNs were directed against the μ-receptor (Sanchez-Blazquez et al., 1997). Finally, the development of physical dependence is abolished in mice lacking the μ-opioid receptor gene (Oprm) (Matthes et al., 1996; Sora et al., 2001). This data indicates that both spinal and supraspinal μ-receptors contribute to morphine withdrawal and that the μ-receptor is a mandatory component in the development of dependence to μ-agonists such as morphine and heroin.

b) MOR splice variants and antisense technology

Opioids acting at the μ opioid receptor, despite their apparently similar pharmacology, can differ in their side effects profiles. Additionally, patients who become tolerant to one μ-opioid drug often show incomplete tolerance to a second μ-opioid drug, a phenomenon known as incomplete cross-tolerance. Furthermore, although morphine is generally an ineffective analgesic in the μ-opioid receptor deficient CXBK mouse (Pick et al. 1993), the analgesic response to heroin and the morphine metabolite morphine-6-beta glucuronide (M6G) is not compromised (Rossi et al. 1996). These findings led researchers to consider the possibility that not all μ-opioids utilize identical μ-opioid receptor mechanisms and that there may be more than one μ-receptor splice variant (Pasternak, 2004).

Initial reports of μ-receptor subtypes were based on pharmacological studies that provided evidence for two distinct μ-receptors termed μ_1 and μ_2 (Wolozin & Pasternak, 1981). Following the cloning of the Oprm gene encoding the μ-receptor (Wang et al., 1993) it became possible to utilize what is known as antisense technology to study the possibility of alternative splicing of the Oprm gene in more detail. While “knockout”

mice allow for the study of the impact of a particular gene, the antisense technique makes it possible to study individual exons and thus draw conclusions regarding the impact of possible alternate splicing of that gene. This method is often referred to as a "knockdown" technique. This "knockdown" is accomplished when a DNA sequence prevents translation of a complementary mRNA that carries the code for the target gene. This complementary DNA sequence of oligodeoxynucleotides that is introduced into the animal hybridizes with the sense strand, or the mRNA sequence coding for the gene, and the technique is therefore known as anti-sense.

Antisense studies blocking specific exons of the μ-receptor soon supported the hypothesis of Oprm alternative splicing for various μ-receptor mediated functions such as analgesia (Rossi et al., 1995), dependence (Sanchez-Blazquez et al., 1997), feeding and regulatory challenges (Hadjimarkou et al., 2002), receptor internalization (Koch et al., 2001), and ligand binding, (Choi et al., 2006). Many different splice variants of the μ-receptor were subsequently identified and labeled alphabetically from MOR1A through MOR1O (Bare et al., 1994; Zimprich et al., 1995; Pan et al. 1999; Pan et al., 2000; Pan et al. 2001, Kvam et al., 2004; Pan et al., 2005). Based on this research the murine Oprm gene alone contains at least 19 known exons and can be alternatively spliced into at least 28 μ-receptor variants. To date, only one prior study has examined the role of a single MOR exon (exon-1) on morphine dependence (Sanchez-Blazquez et al., 1997). There are currently, no studies utilizing antisense strategies to explore the role of different exons, and thus possible alternative splicing, in heroin dependence.

c) MOR splice variants and physical dependence

While the relevance of the many splice variants to dependence is still unclear there is some evidence that at least a few of these variants play a role in morphine dependence. A study that used AS ODNs directed against exon 1 of the Oprm gene found attenuated withdrawal jumping in morphine dependent mice (Sanchez-Blazquez et al., 1997), suggesting the possibility of splice variant specific involvement in morphine dependence. Additionally, the CXBK mouse, a recombinant inbred strain, was found to be deficient in the MOR1B splice variant mRNA expression in the brain (Narita et al., 2003). The MOR-1B splice variant is composed of exons 1,2,3, and 5 of the Oprm gene, whereas the original MOR-1 splice variant is composed of exons 1,2,3, and 4. This strain has also been found to display reduced sensitivity to the dependent effects of chronic morphine administration (Suzuki & Misawa, 1990; Suzuki et al., 1992b), perhaps indicating a role for the MOR1-B splice variant in morphine dependence.

The impact of μ-receptor endocytosis or internalization, and thus receptor desensitization, on opioid dependence has been hypothesized (Nestler, 2004a). It is thought that these processes impact receptor signaling and thus the multiple downstream mechanisms involved in the development of physical dependence to μ-opioids (Finn & Whistler, 2001; Bailey & Connor, 2005; Williams et al., 2001). There is evidence that some of the proposed μ-receptor splice variants differ both in their internalization and desensitization rates, as well as in the μ-agonists that are capable of triggering these processes. Intracerebroventricular administration of both DAMGO and morphine were capable of internalizing the MOR-1C splice variant while MOR-1 was internalized only by morphine administration (Abbadie & Pasternak, 2001). Koch et al. (1998) found that

DAMGO mediated internalization and resensitization was significantly faster in the MOR-1B variant than in MOR-1. The same group also found that DAMGO treatment caused a rapid internalization and desensitization of the MOR-1C, MOR-1D and MOR-1E variants, while morphine triggered a rapid internalization of only MOR-1D and MOR-1E. Morphine's ability to internalize MOR-1 and MOR-1C was significantly slower (Koch et al., 2001). While further study and elucidation of these mechanisms are certainly necessary, this data suggests a further role for μ-receptor splice variants in dependence and a possible explanation for the varied dependence liabilities of different μ-agonists.

d) Delta opioid receptor (DOR)

The delta (δ) receptor shares a significant structural homology to the mu receptor (George et al., 2000) and has similar anatomical distribution in many brain areas (Arvidsson, 1995b). It has also been shown that morphine interacts with δ receptors in vivo and plays a role in morphine physical dependence and withdrawal. Indeed, concomitant administration of the δ antagonist naltrindole (NTI) with morphine significantly reduces the development of naloxone precipitated withdrawal behaviors (Abdelhamid 1991). It should be noted that NTI is a non-selective antagonist that is two times more sensitive to δ than to μ receptors and it is therefore possible that lack of selectivity played a role in the diminished withdrawal response. However, a more recent study showed that administration of antisense oligonucleotides against the δ receptor similarly reduces morphine dependence in mice when compared to a mismatch control (Suzuki et al. 1997a).

There is now strong pharmacological evidence that there are at least two δ-receptor subtypes termed δ_1 and δ_2 (Zaki et al., 1996). Initial evidence seemed to implicate δ_2 receptors in morphine dependence. Abdelhamid (1991) blocked only δ_2 receptors with a δ_2 specific antagonist naltrindole 5' isothiocyanate (5'-NTII) and compared them to groups of mice that were administered NTI. While both groups increased the naloxone ED50, the dose of naloxone required to precipitate withdrawal after 5'-NTII exposure was significantly greater than the dose required after NTI administration. Subsequent experiments demonstrated that the selective δ_2 receptor antagonist 5'-NTII suppressed naloxone precipitated jumping and diarrhea in morphine dependent mice, but a δ_1 selective antagonist (D-Ala2,-Leu5, Cys6) had no impact on the naloxone precipitated behaviors (Miyamoto, 1994). This was true for both acute and chronic morphine dependence (Miyamoto, 1993a; Miyamoto, 1993b). Antisense probes against the δ_2 receptor were also found to attenuate morphine dependence (Kest et al., 1996; Sanchez-Blazquez, 1997).

Interestingly, Suzuki et al. (1997b) found that pretreatment with 7-benzylidenenaltrexone (BNTX) a selective δ_1 antagonist significantly suppressed naloxone induced weight loss in morphine dependent mice. However this suppression was not as strong as that effected by pretreatment with either 5'-NTII or naltriben (NTB), both δ_2 specific antagonists. Additionally, in the same experiment, both NTB and 5'-NTII reduced naloxone precipitated jumping and body shakes while BNTX pretreatment had no effect on these withdrawal behaviors. This evidence implies that the δ_1 receptor may also be involved in opioid dependence albeit in a lesser, perhaps more modulatory, role.

A recent study (Nitsche, 2002) shows that δ-opioid receptor knockout mice, which have no detectable δ_1 or δ_2 binding, retain naltrexone precipitated withdrawal behaviors following chronic morphine treatment. The authors suggest that this argues against the proposed role for the involvement of δ receptors, especially δ_2, in dependence. They propose that the discrepancy in results from previous studies may be explained by high doses and long term treatments with both NTI and 5'-NTII, or by the overestimated selectivity of these compounds. While this study provides strong evidence that the δ receptor has no *required* role in the development of morphine dependence, it is likely that the δ receptor does indeed play a role in the development of dependence in mice where the receptor protein is actually present. Additionally, the ability of antisense probes against the δ-receptor to attenuate morphine dependence (Sanchez-Blazquez, 1997) cannot be explained by excess dosage or lack of specificity.

In addition to the evidence just cited that supports an in vivo interaction between the μ and δ receptors, it has been suggested that the modulation of opioid dependence by δ-receptors may, in fact, result from an actual physical complex between the two receptors (Daniels, 2005). Recent evidence indicates that μ-opioid receptors can form hetero-oligomers with δ-opioid receptors (George et al., 2000; Gomes et al., 2000). The physical association of the two receptors forms a novel entity with unique binding and signaling properties (Gomes et al., 2000). Indeed the μ/δ receptor complex had a lower affinity for both μ (DAMGO) and δ (DPDPE) specific agonists than did each receptor separately. Further, DPDPE induced internalization was almost completely abolished, while DAMGO induced receptor internalization was unaffected (George et al., 2000).

In light of the strong evidence that δ-antagonists can attenuate opioid dependence and the more recent discovery of the μ/δ complex, significant research has been focused on finding therapeutic combinations of μ-agonists and δ-antagonists and, perhaps more importantly, ligands that possess mixed μ-agonist/δ-antagonist profiles. Schiller et al. (1999) were the first to report on a ligand with μ-agonist/δ-antagonist properties. As predicted this ligand (DIPP-NH(2)[Psi]) produced analgesia in the rat tail-flick assay, but did not show evidence of dependence after chronic use. Although the previously mentioned studies were all performed in vitro, it has now been conclusively demonstrated that μ and δ-receptors physically interact in vivo and that bivalent ligands that target this complex produce analgesia with an almost absent physical dependence liability (Daniels, 2005). This approach may yet produce therapeutically available advanced analgesics with low dependence liability for the treatment of pain. (See Table 1)

e) Kappa opioid receptor (KOR)

Kappa (κ) receptors are the third class of opioid receptors. The role of this class of opioid receptors in opioid dependence is more equivocal. This is in part due to evidence indicating that activation of the κ-receptor opposes some μ-receptor mediated actions (Pan, 1998) and from the relative lack of κ-receptor subtype specific antagonists (Connor & Kitchen, 2006). Administration of nor-binaltorphimine (nor-BNI), a selective κ-receptor antagonist, along with morphine potentiated naloxone precipitated weight loss in these mice (Suzuki et al., 1992b). Additionally, morphine dependent mice given intrathecal injections of nor-BNI displayed increased withdrawal behaviors when compared to controls (Cui et al., 2000). Thus it appears that antagonism of the κ-receptor

Table 1. Summary of research investigating effects of δ-opioid antagonists on acute and chronic morphine dependence

***Acute* μ-Opioid Administration and δ-Opioid Drugs**					
Author	**μ-opioid Treatment**	**Drug Name & Type**	**Drug Administration**	**Withdrawal Measure**	**Effect of δ-Opioid on Withdrawal**
Abdelhamid et al. 1991	Morphine	Naltrindole (general δ ant)	Just prior to morphine	Jumping	Decrease
Abdelhamid et al. 1991	Morphine	5'-NTII (δ_2 ant)	Just prior to morphine	Jumping	Decrease
Miyamoto et al.1993a	Morphine	Naltriben (δ_2 ant)	Just prior to morphine	Jumping	Decrease
Kest et al. 1996	Morphine	DOR-1 receptor antisense	3 days prior to morphine[1]	Jumping	Decrease
***Chronic* μ-Opioid Administration and δ-Opioid Drugs**					
Author	**μ-opioid Treatment**	**δ-Opioid Treatment**	**δ-Opioid Administration**	**Withdrawal Measure**	**Effect of δ-Opioid on Withdrawal**
Abdelhamid et al. 1991	Morphine	5'-NTII (δ_2 ant)	Concurrent w/ morphine	Jumping	Decrease
Abdelhamid et al. 1991	Morphine	Naltrindole (δ ant)	Concurrent w/[2] morphine	Jumping	Decrease
Miyamoto et al.1993b	Morphine	Naltriben (δ_2 ant)	Prior to[3] or concurrent[2] w/ morphine	Jumping/diarrhea	None
		5'-NTII (δ_2 ant)	Prior to and concurrent w/ morphine	Jumping/diarrhea	Decrease
Miyamoto et al.1994	Morphine	DAla2,Leu5,Cys6 enkephalin	Concurrent w/ morphine	Jumping/diarrhea	None
Suzuki et al. 1997	Morphine	Naltrindole (general δ ant)	Concurrent w/ morphine	Jumping, weight-loss, shakes	Decrease
		Naltriben (δ_2 ant)	Concurrent w/ morphine	Jumping, weight-loss, shakes	Decrease
		5'-NTII (δ_2 ant)	Concurrent w/ morphine	Jumping, weight-loss, shakes	Decrease
		BNTX (δ_1 ant)	Concurrent w/ morphine	Jumping, shakes	None
				Weight-loss	Slight decrease
Sanchez-Blazquez 1997	Morphine	δ receptor antisense	Prior to and concurrent w/morphine	Jumping	Decrease

[1] - Treatment with AS ODNs against DOR-1 once per day for 3 days prior to single morphine injection on Day 4.

[2] - Antagonist was administered prior to every morphine injection during chronic morphine treatment.

[3] - A single antagonist dose was administered immediately prior to initial morphine injection. No further antagonist injections were made during chronic morphine treatment.

system enhances the withdrawal response in mice dependent on μ-agonists. However, due to the fact that nor-BNI is not selective for the κ subtypes, it is unclear from these studies what role these subtypes might play in dependence.

Although three subtypes of the κ-receptor were proposed, current data supports the existence of two subtypes of the κ receptor which have been identified through pharmacological studies of κ specific ligands. The first of these (κ_1) is U69593 sensitive and the second (κ_2) is bremazocine sensitive. In some studies U50,488H (U50), a selective κ_1 agonist, did not suppress physical dependence to morphine (Fukagawa et al., 1989; Tsuji et al., 2000), while TRK-820, a κ-agonist that binds to both κ_1 and $\kappa_2$receptors, did suppress NPW signs in morphine dependent animals (Tsuji et al., 2000). Dynorphin A, an endogenous κ-agonist that binds to both subtypes, also suppressed NPW signs in morphine dependent mice (Takemori et al., 1992). Thus the evidence seems to show that antagonism of the κ-receptor potentiates morphine withdrawal, while κ_2-subype agonist activation, both endogenously and exogenously, seems to counteract this effect.

It has been proposed that agonist activation of κ_2-receptors opposes μ-receptor mediated dependence. Research indicates that μ-opioids disinhibit dopaminergic neurons in various areas of the cortex through inhibition of Gamma-aminobutyric acid (GABA) interneurons (Johnson & North, 1992; Alger & Nicoll, 1980). During chronic μ-opioid administration it is this disinhibition that is responsible for the excitation of dopaminergic neurons thought to play a role in the development of dependence (Bonci & Williams, 1997). Presynaptic κ-receptors on these same dopaminergic neurons inhibit dopamine release thus counteracting the disinhibition caused by the μ-receptor mediated process.

This process may at least partially explain the apparent opposing effects of κ- and μ-agonists in the development of dependence (Nestler, 2001; Pan 1998).

However, others have found that administration of U50 does attenuate morphine dependence both spinally (Cui et al., 2000) and supraspinally (Tao et al., 1994). Additionally, Simonin et al. (1998) found that κ_1 knockout mice showed significantly less dependence to morphine than did controls. This evidence has led some to postulate that the κ_1-receptor participates in the long-term changes that lead to the development of μ-agonist mediated dependence (Narita et al., 2001). Unfortunately, due to the dearth of κ-receptor subtype specific antagonists and the inability to clone any of the κ-receptors aside from κ_1 (Connor & Kitchen, 2006), the role of this receptor in dependence remains somewhat ambiguous. (See Table 2)

f) ORL-1 Receptors

Opioid receptor-like-1 (ORL-1) receptors are structurally similar to kappa-3 opioid receptors and bind with an endogenous ligand orphanian FQ/nociceptin (OFQ/n) that is specific to this receptor (Mollereau et al., 1994; Pan et al., 1996; Rossi et al., 1997; Gaveriaux-Ruff & Kieffer, 1999). Kotlinska et al. (2000) reported that when administered to morphine dependent rats, an acute injection of OFQ/n prior to NPW dose-dependently reduced withdrawal behaviors, and that high doses almost abolished withdrawal responses. However, OFQ/n had no effect on morphine-naïve animals or morphine pretreated animals in the absence of naloxone. Kest et al., (2001) demonstrated that absence of the OFQ/n peptide in knockout mice resulted in an increased magnitude of NPW jumping after chronic morphine administration when compared with heterozygous knockout or wildtype mice. The observed potentiation of the withdrawal response in

Table 2. Summary of research investigating effects of κ-opioid antagonists on chronic morphine dependence

Chronic μ-Opioid Administration and κ-Opioid Drugs					
Author	**M-opioid Treatment**	**Drug Name & Type**	**Drug Administration**	**Withdrawal Measure**	**Effect of Drug on Withdrawal**
Suzuki et al. 1992	Morphine	Nor-binaltorphimine (κ ant)	Concurrent w/ morphine[1]	shakes, weight-loss	Increased
Cui et al. 2000	Morphine	nor-BNI (κ ant)	Prior to NPW[2]	Shakes	Increased
		U-50 (κ_1 ago)	Prior to NPW	Shakes	Decreased
Tsuji et al. 2000	Morphine	TRK-820 (κ ago)	Concurrent w/ morphine	Jumping, shakes, weight-loss	Decreased
		U-50 (κ_1 ago)	Concurrent w/ morphine	Jumping, shakes, weight-loss	None
Takemori et al. 1992	Morphine	Dynorphin A (κ ago)	Prior to NPW	Jumping	Decreased

[1] - Antagonist doses were administered immediately prior to every morphine injection during chronic morphine treatment.

[2] - A single antagonist dose was administered immediately prior to NPW.

these mice may be attributed to a diminished inhibitory influence of ORL-1 receptors in the absence of its endogenous ligand, OFQ/n. Overall, the data indicate that the OFQ/n peptide and activation of the ORL-1 receptor has a modulatory role in morphine dependence and withdrawal.

4. **Contribution of EAA Receptor Subtypes to Opioid Dependence**

There is growing evidence that glutamatergic transmission plays a modulatory role in dependence to μ-opioids. Indeed, the release of extracellular glutamate is significantly upregulated in morphine dependent mice treated with naltrexone to precipitate withdrawal, indicating a direct biochemical connection between morphine withdrawal and glutamatergic signaling (Aghajanian et al., 1994). It has even been suggested that the morphine withdrawal syndrome can be understood as a state of glutamate hyperactivity in certain regions of the brain (Rasmussen, 2002) Glutamate transporters are known to reduce the levels of extracellular glutamate and so the effect of glutamate transporters on opioid dependence was investigated. It was found that DL-threo-h-benzyloxyaspartate, a glutamate transporter inhibitor, enhanced morphine withdrawal symptoms in rats while MS-153, a glutamate transporter activator, decreased withdrawal symptoms in morphine dependent mice (Sekiya et al., 2004; Nakagawa et al., 2001).

Not only are neurotransmitter levels involved in withdrawal, but the levels of transporters themselves seem to change during dependence and withdrawal. The mRNA expression of GLT-1, a glutamate transporter, was decreased in the thalamus and striatum of morphine dependent mice and elevated in the striatum following NPW (Ozawa et al., 2001), although the elevation is likely in response to the overexpression of glutamate

during withdrawal (Nakagawa & Satoh, 2004). Finally, gene transfer of the GLT-1 gene into the locus coeruleus prior to morphine pellet implantation caused an overexpression of GLT-1 and a significant attenuation of NPW signs (Ozawa et al., 2001). Taken together these studies provide evidence that glutamatergic transmission is involved in morphine dependence.

a) NMDA receptors

It is well known that N-methyl-D-aspartate (NMDA) receptors are involved in synaptic plasticity especially in terms of long term potentiation and learning. It was therefore postulated that NMDA receptors might play a role in the long term adaptive changes that occur during opioid dependence. Initial evidence seemed to support this hypothesis as co-infusion of MK-801, an NDMA antagonist, and morphine attenuated the NPW response in mice (Trujillo & Akil, 1991). Additionally, mice who received chronic infusion of morphine and only a single injection of MK-801 prior to naloxone did not show an attenuation of the NPW response (Trujillo & Akil, 1991). These results imply a role for NMDA receptors in the long-term neuronal adaptations that occur in dependent animals. McLemore et al. (1997) noted that 18-hour chronic infusion of two different NDMA antagonists prior to a single injection of morphine did block acute morphine dependence. Again, this indicates that chronic antagonism of the NMDA receptor system can significantly impact the development of dependence while acute antagonism of the NMDA receptor system does not seem to play a significant role in the development of dependence to μ-opioids.

More recent molecular evidence supports the pharmacological data. Antisense ODNs directed against the NR1 subunit of the NMDA receptor significantly attenuates withdrawal signs in mice (Zhu & Ho, 1998). Additionally, NMDA receptor NR2A subunit knockout mice display significantly reduced NPW signs (Miyamoto et al., 2004) and the rescue of NR2A protein by electroporation into the nucleus accumbens (NAcc) of these knockout mice significantly reversed the loss of withdrawal behaviors (Inoue et al., 2003).

The mechanism through which NMDA receptors impact μ-opioid mediated physical dependence has not been fully elucidated, but one theory implicates the ability of the NMDA receptor to modulate intracellular levels of Ca^{2+}. Hamdy et al. (2004) found that co-administration of MK-801 and morphine prevented the NPW induced increase of CaMKII phosphorylation and the NPW induced increase of c-Fos protein expression in the cortex. The authors postulate that the attenuation of dependence is related to the ability of MK-801 to block the entrance of Ca^{2+} into the cell. This Ca^{2+} blockade reduces the amount of CaMKII phosphorylation which decreases the phosphorylation of the transcription factor CREB. This results in a significantly reduced c-Fos expression during NPW. Interestingly, these same authors noted that acute administration of MK-801 prior to NPW in morphine dependent mice did not attenuate the NPW induced phosphorylation of CaMKII or c-Fos. This supports the previously mentioned behavioral data that only chronic NMDA antagonism plays a role in the cellular adaptations involved in dependence and withdrawal.

Due to the ability of NMDA receptors to attenuate dependence there has been research into the clinical application of NMDA receptor antagonists in the treatment of

opiate addiction. Unfortunately, many of the researched antagonists (e.g. MK-801, ketamine) induce psychotomimetic adverse effects. However, there is evidence that NMDA antagonists such as memantine (Bisaga et al., 2001) and dextromethorphan (Manning et al., 1996) can attenuate physical dependence to opioids and investigations into their clinical utility are underway. (See Table 3)

b) **AMPA receptors**

The alpha-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) receptors are a group of ionotropic glutamate receptors. The role that they play in μ-opioid dependence however, is less clear. Initial evidence indicated that neither chronic i.c.v. treatment (Fundytus & Coderre, 1994) nor continuous subcutaneous infusion (McLemore et al., 1997) of AMPA specific antagonists were able to attenuate chronic morphine dependence in mice. However, there is evidence that pretreatment with DNQX, a competitive AMPA antagonist inhibited withdrawal jumping in mice (Cappendijk, et al., 1993). Similarly, it was found that selective AMPA antagonists LY293558 and LY300168 attenuate both withdrawal induced activation of LC Rasmussen & Vandergriff, 2003). McLemore et al. (1997) found that continuous subcutaneous infusion of LY293558 prior to a single injection of morphine blocks acute dependence as well.

Further evidence of AMPA receptor involvement arises from studies involving various subunits of the AMPA receptor. An autoradiographic study of AMPA receptor binding indicated that following spontaneous withdrawal from chronic morphine administration AMPA receptor binding was increased in the rat cortex, caudate-putamen and hippocampus (Jang et al., 2000). Additionally, AMPA-type glutamate receptor-A

Table 3. Summary of research investigating effects of NMDA antagonists on acute and chronic morphine dependence

***Acute* μ-Opioid Administration and NMDA Receptor Drugs**					
Author	**μ-opioid Treatment**	**Drug Name & Type**	**Drug Administration**	**Withdrawal Measure**	**Effect of Drug on Withdrawal**
McLemore et al. 1997	Morphine	LY235959 (NMDA ant)	Continuous infusion prior to single Morphine inj.	Jumping	Decreased
		MK-801 (NMDA ant)			
***Chronic* μ-Opioid Administration and NMDA Receptor Drugs**					
Author	**μ-opioid Treatment**	**Drug Name & Type**	**Drug Administration**	**Withdrawal Measure**	**Effect of Drug on Withdrawal**
Hamdy et al. 2004	Morphine	MK-801 (NMDA ant)	Continuous infusion concurrent w/ Morphine	Jumping, teeth chattering, tremor	Decreased
			Prior to NPW[1]	Jumping, teeth chattering, tremor	None
Bisaga et al. 2001	Morphine (human study)	Memantine (NMDA ant)	6 hours prior to NPW[2]	Clinical Institute for Narcotic Withdrawal Scale	Decreased
Trujillo & Akil 1991	Morphine	MK-801 (NMDA ant)	Concurrent w/ morphine[3]	Jumping	Decreased
			Prior to NPW	Jumping	None
Zho & Ho, 1998	Morphine	NMDA subunit NR1 antisense	Concurrent w/ morphine	Jumping, rearing, teeth chattering	Decreased

[1] - A single antagonist injection was administered, immediately prior to NPW.
[2] - A single antagonist injection was administered six hours prior to NPW.
[3]- Antagonist was administered prior to every morphine injection during chronic morphine treatment.

subunit knockout mice demonstrated attenuated NPW signs after chronic morphine treatment (Vekovischeva et al., 2001).

NMDA receptors are often modulated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Wang et al., 2005). Research indicates that this protein kinase plays a significant role in μ-opioid dependence as well. Treatment with the CaMKII antagonist K93 immediately prior to NPW significantly inhibited withdrawal signs in dependent mice (Tang et al., 2006). It is certainly possible that CaMKII modulation of AMPA receptors impacts the behavioral signs seen during chronic treatment and withdrawal. (See Table 4)

5. Behavioral genetics of opioid dependence

Techniques such as transgenic ("knock-out" & "knock-in") mice can be powerful for understanding the genetic basis of morphine dependency, and can often produce powerful effects on withdrawal. However, the a priori selection of genetic targets based on prior pharmacological studies yields findings that are largely confirmatory and are unfortunately also of little value in identifying previously unknown genes that contribute to variability in withdrawal magnitude (Kest et al., 2004). These techniques may also suffer from other confounds such as developmental "compensatory" effects of the altered gene. Inbred mouse strains, which are often established by at least 20 generations of sibling mating, are homozygous at every allele and thus genetically identical (Han et al., 2004). Although some inbred strains are more closely related to other strains, overall each individual inbred mouse strain is genetically unique. Thus, distinct behavioral differences between the strains can assist in elucidating the genetic mechanism underlying the behavior. These studies can also assist in identifying specific strains with

Table 4. Summary of research investigating effects of AMPA antagonists on acute and chronic morphine dependence

***Acute* μ-Opioid Administration and AMPA Receptor Drugs**					
Author	**M-opioid Treatment**	**Drug Name & Type**	**Drug Administration**	**Withdrawal Measure**	**Effect of Drug on Withdrawal**
McLemore et al. 1997	Morphine	LY293558 (AMPA ant)	Continuous infusion prior to single Morphine inj.	Jumping	None
***Chronic* μ-Opioid Administration and AMPA Receptor Drugs**					
Author	**M-opioid Treatment**	**Drug Name & Type**	**Drug Administration**	**Withdrawal Measure**	**Effect of Drug on Withdrawal**
Vekovischeva et al., 2001	Morphine	AMPA subunit KO Mice	N/A	Jumping, digging, shakes	Decreased
McLemore et al. 1997	Morphine	LY293558 (AMPA ant)	Continuous infusion concurrent w/ Morphine	Jumping	Decreased
Cappendijk et al. 1993	Morphine	DNQX (AMPA ant)	Prior to NPW[1]	Jumping	Decreased
Rasmussen et al. 1996	Morphine	LY293558 (AMPA ant)	Prior to NPW	Shakes, writhes, diarrhea	Decreased
				firing of LC neurons	Decreased

N/A – Not available (no antagonist was used in knock-out mice devoid of receptors

[1] - A single antagonist injection was administered, immediately prior to NPW.

widely divergent behavioral responses, which can then be utilized in linkage mapping studies to identify possible trait relevant genes mediating the behavior (Kest et al., 2002a).

Genetic differences have been shown to impact NPW behaviors. Hoffman et al. (1998) assessed NPW in three different strains of rats made chronically dependence on morphine. He reported that although the Spontaneously-Hypertensive, Wistar-Kyoto, and Sprague Dawley strains all displayed relatively comparable numbers of overall withdrawal responses, significant differences in the types of behaviors were observed from each group. Specifically, Sprague-Dawley rats demonstrated more wet-dog shakes, while Wistar-Kyoto rats exhibited more ptosis and diarrhea, and Spontaneously-Hypertensive animals displayed more abdominal stretches. A comparison of NPW responses in the Lewis and Fischer 344 rat strains found that Lewis rats exhibited greater jumping and locomotive behaviors, while the Fischer 344 rats displayed greater weight loss and irritability. Genetic differences in withdrawal have also been noted in inbred mouse strains. Brase et al. (1977) compared the naloxone ED_{50} in six mouse strains exposed to chronic morphine treatment. It was found that the C57BL/6 mice were approximately 13 times more sensitive to NPW than the DBA/2J and A/J strains, which exhibited the least sensitivity. Another study noted that the outbred CD-1 mouse strain displayed significantly greater naloxone precipitated jumping magnitude following chronic morphine exposure than either the C3H/He or DBA/2 inbred mouse strains, while the DBA strain jumped significantly more than the C3H/He strain (Suzuki et al., 1991).

More recent inbred strain survey studies have examined NPW in a larger number of inbred strains in order to identify highly sensitive and insensitive strains (Kest et al.,

2002a; Liang et al., 2006) and have looked at both acute and chronic dependence (Kest et al., 2002a). Both studies found significant interstrain variability in the genetic contribution to morphine dependence. Two strains, 129P3 and SWR, merited significant attention as they were either completely refractory to or highly sensitive to NPW jumping, respectively (Kest et al., 2002a). A linkage mapping study of chronic morphine dependence using the 129P3 and C57BL/6 progenitor strains has identified a 28 cM-wide region of Chromosome 1 (32–60 cM; peak at 51 cM), accounting for 20% of the overall variance for NPW jumping between these two strains (Kest et al., 2004). A second study noted two strains, SM and 129Sv were resistant, while one strain, B10.D2-H2/oSNJ was highly sensitive to NPW jumping (Liang et al., 2006). No further linkage studies have been done utilizing these particular behaviorally divergent strains. A significant correlation between the inbred strains for acute and chronic morphine dependence was also found. Since the demonstration of genetic correlation of two heritable traits among isogenic strains can be used as evidence of the existence of pleiotropic genes with a common influence on both traits, it implies a common genetic mechanism for the two phenomena (Hegmann & Possidente, 1981). While much progress has been made in recent years utilizing this strain survey approach, one area which has not been studied in significant detail is the genetic contribution to dependence on other opioid drugs.

6. Summary

Physical dependence is a result of both chronic and acute stimulation of μ-opioid receptors by an agonist and presents behaviorally as a withdrawal syndrome following dissociation of the opioid drug from the receptor. It is clear that binding of μ-opiates such as morphine and heroin to μ-opioid receptors is a prerequisite for the development

of physical dependence to these drugs (Matthes et al., 1996). The acute and chronic interaction of opiate drugs with this receptor has been implicated in the development of dependence, although alternative splice variants of the μ-receptor itself may differentially impact this process (Rossi et al., 1995 & 1996). Both δ and κ-opioid receptors appear to play a modulatory role in morphine-dependence, albeit in reverse roles. Whereas administration of δ-receptor antagonists attenuates morphine dependence, κ-blockade appears to increase NPW signs and according to some data can even precipitate morphine withdrawal on its own (Abdelhamid et al., 1991; Miyamoto, 1994;
Suzuki et al., 1997). Finally, studies utilizing inbred mice demonstrate significant genetic influence on morphine dependence, and highlight the significant genetic variability underlying this process.

Behaviorally there is a clear syndrome marked by various somatic symptoms that is evident following cessation of drug treatment or antagonist precipitated withdrawal. In mice NPW jumping is considered the most reliable assessment of this behavioral syndrome and is the best measure of the magnitude of dependence in mice (Kest et al., 2001; El-Kadi & Sharif, 1994; Marshall & Weinstock, 1971). Often it is the desire to alleviate, or to avoid altogether, this physically aversive state of withdrawal that triggers continued abuse of μ-opioids and/or relapse after cessation of drug use (Koob & Le Moal, 2001). While this particular aspect of withdrawal may not play as significant a role in the clinical and relatively well controlled use of analgesics such as morphine, it certainly is critical when discussing opioid drugs of abuse such as heroin. In fact, recent data has demonstrated that cues predicting the onset of heroin withdrawal provoke heroin self-stimulation in dependent rats. It is postulated that the awareness of the imminent,

physically aversive withdrawal syndrome plays a key role in this self-stimulation behavior and that this same knowledge plays a critical role in provoking craving and relapse in human heroin addicts (Kenny et al., 2006).

As is abundantly clear from the review above, the vast majority of literature studying physical dependence and naloxone precipitated withdrawal centers around morphine and not heroin. Given the conspicuous paucity of experimental studies of heroin dependence and withdrawal, it is not known whether heroin dependence is mediated by similar mechanisms. Finally, the growing body of literature suggesting use of naloxone to treat heroin overdose and addiction (Sporer & Kral, 2007), as well as the widespread and growing illicit abuse of heroin in general (NIDA, 2005) suggests that heroin dependence and withdrawal are phenomenon that deserve significant attention.

7. Rationale for the Specific Aims of the Present Dissertation

Specific Aim 1: Assessment of naloxone precipitated withdrawal jumping as a reliable and sensitive index of heroin dependence and the establishment of optimal heroin withdrawal protocols. Research in the area of acute and chronic morphine dependence is usually facilitated by precipitating withdrawal through injection of the wide-spectrum opioid receptor antagonist naloxone. Among NPW signs in mice, jumping frequency is widely considered the most sensitive and reliable index of withdrawal intensity and is by far the most commonly used (El-Kadi and Sharif, 1994; Ritzmann, 1981; Smits, 1975; Saelens et al. 1971; Way et al. 1969; Miyamoto and Takemori, 1993; Kest et al., 2004; Kest et al., 2002a). Several lines of evidence lend validity to this measure of morphine dependence (Smits, 1975; Marshall & Weinstock,

1971) and for a variety of opioids, jumping frequencies in mice during NPW correlate well with their known physical dependence liability in man (Saelens et al. 1971). Thus, the **first specific aim** of this dissertation is to assess the sensitivity of NPW jumping as a measure of heroin dependence and to establish optimal protocols for its study. Accordingly, we obtained time- and dose- response data by varying injection intervals, as well as heroin and naloxone dose following both acute and chronic heroin administration. Due to heroin's ability to rapidly cross the blood brain barrier (Oldendorf et al., 1972) we would expect the optimal heroin- naloxone interval would be shorter than that seen for morphine. At this time we cannot generate a hypothesis as to the most effective doses of either heroin or naloxone. Heroin is a potent drug with unknown effects on naloxone precipitated withdrawal jumping, and thus we might expect that jumping frequencies will be higher than those following morphine exposure.

Specific Aim 2: Evaluation of the contribution of opioid and excitatory amino acids (EAA) receptors to heroin dependence. Morphine preferentially binds and exerts its physiological effects, including analgesia and dependence, at the μ opioid receptor subtype (Matthes et al., 1996; Berrendero et al., 2002). Nonetheless, other opioid receptor subtypes have a modulatory role. For example, antisense oligonucleotides targeting the δ receptor decrease NPW precipitated jumping (Suzuki et al. 1997a). Antagonist studies have indicated that there may be a differential effect depending on the δ-subtype targeted. δ_2-receptor antagonists attenuate most of the classic NPW signs including jumping, body shakes and weight loss, while the δ_1 subtype decreases NPW body weight loss, but not the other withdrawal signs (Suzuki et al. 1997b). The role of

kappa opioid receptors is more equivocal. Mice with a disruption in the κ-opioid receptor gene (Oprd1) appear to have an attenuated NPW syndrome following chronic morphine administration (Simonin et al. 1998). However, other studies involving κ antagonists have shown a potentiating effect on NPW signs (Suzuki et al. 1992a), and dynorphin, an endogenous κ preferring agonist, has been reported to inhibit morphine withdrawal symptoms (Suzuki et al. 1992b).

EAA receptors also make a critical contribution to morphine dependence. For example, blockade of AMPA receptors have been shown to decrease NPW jumping and other opioid withdrawal signs (McLemore et al. 1997;Vandergriff and Rasmussen 1999; Noda and Nabeshima 2004). NMDA receptors have proven to play a very important role in the development of dependence and the presence of NPW signs such as jumping, diarrhea, and ptosis, in rodents (Noda and Nabeshima 2004) and withdrawal symptoms such as nausea/vomiting, restlessness, abdominal pain, and muscle aches in humans (Bisaga et al. 2001). Although there are mu receptor antagonists that can be used to block activity at the mu receptor, doing so would also block the development of dependence and render useless any attempt to explore the modulatory effect of this receptor on dependence. Therefore the **second specific aim** of this dissertation is to examine the role of δ and κ opioid receptors and the excitatory amino acid receptors for both acute and chronic heroin dependence. We hypothesize that these receptor systems will play a similar, but not identical role in heroin dependence compared to morphine dependence. As evidence indicates that heroin may activate different splice variants of the mu opioid receptor (Rossi et al., 1995) it is possible that these isoforms interact with the kappa, delta and EAA receptor systems differently than does morphine.

Specific Aim 3: Assessment of the contribution of genotype to heroin withdrawal. A comprehensive analysis of the genetic contribution to morphine withdrawal has demonstrated significant inter-strain variability in mice (Kest et al. 2002a; Liang et al., 2006). As genetic variability appears to influence morphine withdrawal jumping behavior, it is likely that the jumping behavior elicited during withdrawal from heroin is also under the influence of genetic differences. This same study also confirmed that acute and chronic morphine dependence share common genetic substrates (Kest et al., 2002a). Research indicates that heroin pharmacodynamics differs from those of morphine, and thus acute and chronic heroin dependence may not share similar genetic substrates as did acute and chronic morphine dependence. As a significant strain correlation between two phenotypes implies common genetic mechanism we induced both acute and chronic heroin dependence in six different mouse strains previously displaying a wide range of sensitivities to morphine dependence. This enabled us to assess the genetic contribution to heroin dependence in general and acute and chronic heroin dependence in particular. We then correlated their NPW jumping frequencies to those obtained from the same mouse strains undergoing NPW from both acute and chronic morphine dependence (Kest et al. 2002a). This allows for a correlation between the inter-strain genetic variability in heroin and morphine withdrawal. Therefore the **third specific aim** of this dissertation is to examine the genetic contribution to both acute and chronic heroin withdrawal through the use of inbred mouse strains and to compare these findings to morphine withdrawal in order to advance our understanding of the similarities and differences between these two μ-opioid drugs. We hypothesize that the genetics of acute and chronic heroin will be

highly correlated as they are in morphine dependence (Kest et al., 2002). Additionally, we expect that the correlation between chronic heroin and chronic morphine exposure will be similar as heroin is metabolized into morphine especially in chronic paradigms where there is ample time for significant morphine levels to develop. However we believe that there may be a difference between acute heroin and acute morphine as in this paradigm there is not enough time for heroin's effects to be mediated primarily by morphine and thus the genetic contribution may be significantly different.

Specific Aim 4: Assessment of the contribution of μ-opioid receptor splice variants to both heroin and morphine dependence. Antisense mapping of the μ-opioid receptor gene has added significantly to existing pharmacological studies investigating opioid subtypes, and has provided considerable evidence for the existence of opioid receptor splice variants that may differentially mediate various opioid controlled processes such as analgesia and respiratory depression (Rossi et al. 1995a; Romberg et al. 2003). There is also evidence that different slice variants contribute to the process of opioid dependence and withdrawal. A study using AS ODNs directed against exon 1 of the Oprm gene found attenuated withdrawal jumping in morphine dependent mice (Sanchez-Blazquez et al., 1997), suggesting the possibility of splice variant specific involvement in morphine dependence. Interestingly, Oprm splice variants such as MOR-1B, MOR-1C, MOR-1D, and MOR-1E have been found to differ in their internalization and desensitization rates (Abbadie & Pasternak, 2001; Koch et al., 1998), processes hypothesized to impact intracellular mechanisms involved in dependence (Nestler 2004a). There are currently no studies reporting on the possible differential role of various Oprm exonic regions on

heron dependence. Therefore, the **fourth specific aim** of this dissertation is to explore the dependence profile of the original MOR-1 variant of the μ-receptor by investigating the role of exons 1, 2, 3 & 4 in the development physical dependence following chronic heroin administration. Since morphine and heroin analgesia are mediated by distinct splice variants (Rossi et al., 1996; Brown et al., 1997), mice rendered dependence to morphine were also tested here to assess whether these two opioids also differ in the splice variants underlying their dependence effects. At this point we cannot properly hypothesize as to which targeted exons may play a role in either heroin or morphine dependence. The current study is exploratory and will hopefully elucidate the various splice variants involved in morphine and heroin dependence.

CHAPTER 2.

General Methods

1) Subjects

Outbred male adult CD-1 albino mice (minimum 6 weeks old) (Charles River, Kingston, NY) were used throughout except for the strain survey (specific aim #3) where the following inbred strains were used: 129P3, A, C3H/He, C57BL/6, SWR and BALB/c mice (all Jackson Laboratory, Bar Harbor, ME). Every dose of every condition was comprised of at least 6 mice ($n \geq 6$) , and each mouse was used only once. All mice were housed four to a cage with the same sex/strain mates upon arrival to the Queens College Animal Facility. Mice were allowed free access to food (Purina chow) and water in a temperature controlled (22º C) environment maintained on a 12:12 h light/dark cycle (lights on at 07:00 h). All testing was performed between 8 AM and 5 PM following an acclimation period of at least one week after arrival to the vivarium at Queens College.

2) Drugs and Delivery Methods

The following drugs were dissolved in a 0.9% physiological saline vehicle and injected subcutaneously: morphine sulfate, 3,6,-diacetylmorphine sulfate (heroin hydrochloride), the κ_1 opioid receptor antagonist BNTX, the δ_2 opioid receptor antagonist NTB, the δ_1 opioid antagonist nor-BNI (all gifts of NIDA, Rockville, MD), naloxone hydrochloride (Sigma-Aldrich, St. Louis, MO), and the respective NMDA and AMPA receptor antagonists MK-801 (Sigma-Aldrich, St Louis, MO) and LY293558 (gift of Eli Lily Co., Indianapolis, IN). For the paradigms involving chronic s.c. injection of heroin hydrochloride, the drug was always administered at 09:00 h with additional daily injections during chronic treatment at 13:00 and 17:00 h.

Drugs administered via i.c.v. injection included morphine sulfate, 3,6,-diacetylmorphine sulfate (heroin hydrochloride), and antisense oligonucleotide probes (see following section). Intracerebroventricular injections were performed as previously described (Rossi et al., 1995; Hadjimarkou et al., 2004). In brief, anesthetized mice received a midline incision along the sagittal structure to allow for visualization of the sutures. A freehand microinjection of a drug solution was administered directly through the skull into the lateral ventricle at 0.5 mm posterior to the bregma suture and 1-1.5 mm lateral to the midline suture. The solution was injected in a volume of 5µl over the course of at least 30 seconds, using a Hamilton microsyringe with a 27-gauge needle that extended 2 mm below the surface of the skull.

Osmotic pumps (Model 2001; Alza Pharmaceuticals, Mountain View, CA) with a flow rate of 1µl/h were used for continuous subcutaneous heroin administration. These pumps were implanted through a small dorsal midline incision in mice under oxygen/isoflourane inhalant anesthesia, which was then closed with stainless steel surgical staples. Continuous subcutaneous administration was also accomplished through the use of morphine sulfate pellets (various doses) or placebo control pellets wrapped in nylon mesh that were subcutaneously implanted into the nape of the neck under identical surgical procedures. When necessary, pumps and pellets were removed and/or replaced under anesthesia.

3) Antisense Oligonucleotide Probes

AS ODN sequences targeting exons 1, 2, 3, and 4 (Sigma Genosys) were prepared as previously described (Rossi et al. 1995b) (Figure 1) and were diluted in 0.9% normal saline at a concentration of 1μg/μl. AS (5 ug) was administered by i.c.v. injection (see prior section for details) using an injection volume of 5μl. To assess the specificity of the antisense probes, two controls were used. The first is a normal saline control and the second is a mismatch (MS) probe in which the order of two pairs of nucleotide bases is reversed (Table 5).

4) Surgical Procedures

For all surgical procedures including osmotic pump implantation, pellet implantation, and i.c.v injections mice were exposed to isoflourane/oxygen inhalant anesthesia for approximately 2-3 minutes until full anesthesia was observed. At this point the surgical procedure was started and performed under constant exposure to the isoflourane.

5) Naloxone precipitated withdrawal

The jumping response was used to assess the magnitude of withdrawal in morphine or heroin dependent mice. All mice were individually placed into a clear Plexiglas cylinder (25 x 11 cm) and allowed ~15 minutes to acclimate to their surroundings. Mice were then injected with naloxone hydrochloride and immediately placed back into their cylinders. Jumping frequency was then directly observed over a 15-minute period. A jump is characterized by elevation of all mouse paws from the

Figure 1. Schematic map of AS ODNs directed against different exons of the MOR-1.

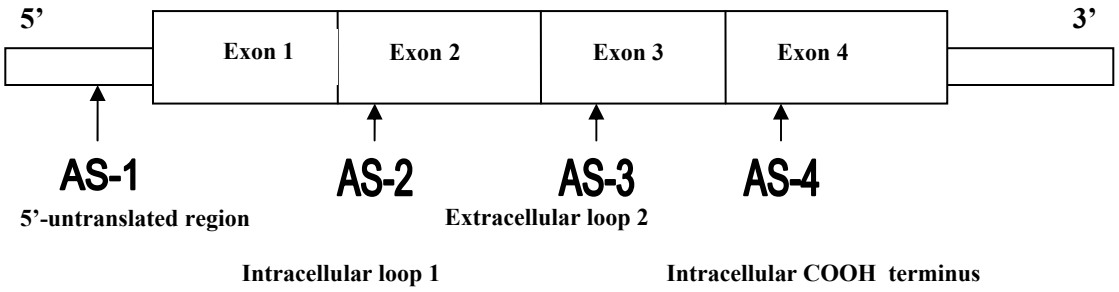


Table 5. Sequence of opioid AS ODNs targeting exons 1-4 of the mouse MOR-1.

Probe	**ODN Sequence (5'- 3')**	**Position**	**Bases**
Exon 1 AS	CGC CCC AGC CTC TTC CTC T	5'-Untranslated region	195-213
Exon 2 AS	TTG GTG GCA GTC TTC ATT TTG G	Intracelullar loop 1	572-593
Exon 3 AS	CCA CCA GCA CCA TCC GGG	Extracellular loop 2	1118-1135
Exon 4 AS	CAG GAA ACC AGA GCC TCC CA	Intracellular COOH terminus	1558-1577
Mismatch	CGC CCC **GA**C CTC TTC C**CT** T		

Note: Bold/underlined characters denote differences between AS and mismatch ODNs.

horizontal surface. The average number of jumps over the 15-minute period is used as the measure of dependence for all groups of mice. A 15-minute period was chosen to achieve optimal peak effects of naloxone, as the jumping declines thereafter (Way et al., 1969). Withdrawal was always precipitated between 4 and 8 h into the light cycle (lights on at 06:00 h). Other precipitated-withdrawal behaviors such as diarrhea, locomotion, wet-dog shakes, ptosis, and grooming are not often reliably observed in mice and are not dose-dependent, and therefore were not tallied. All behavioral measures were observed by the experimenter and at least one individual uninformed about the experimental conditions. Prior to involvement in the experiment, the uninformed rater was be trained to identify jumping behaviors. The uninformed rater and the primary investigator each assessed the jumping behavior of different mice on the same days. Attempts were made to counterbalance the groups of mice that were tested by the examiner on each day. The raw data from each of the examiners for each group of mice were combined upon termination of testing, and the average number of jumps for each group was calculated.

6) Nociceptive assay

Nociception was assessed using the tail-withdrawal test, which was chosen due to its stability in the context of repeated testing (Elliot et al., 1995; Kest et al., 2002; Nemmani et al., 2004). In this assay of acute thermal nociception, the mouse is gently restrained and the distal half of the tail is immersed in water maintained at 50.0º C by an immersion heater/circulator. Latency to reflexive withdrawal of the tail is recorded twice to the nearest 0.1 sec, with each determination separated by 30 sec. The two

determinations are then averaged. A cutoff latency of 20 seconds is employed to prevent the possibility of tissue damage.

7) Statistical Methods

Withdrawal jumping frequencies were analyzed using independent t-tests (Experiments #1 & #2), a one way ANOVA (Experiments #1, #2, and #4), a two way ANOVA (Experiment #3) followed post hoc by Fisher's LSD (Protected t-Tests) (Experiments #1, #2, and #3, and #4).

Tail withdrawal latencies in the antisense oligonucleotide studies were analyzed using a two-way (AS ODN X day) ANOVA followed post hoc by the Neuman-Keuls test. Analgesia data is expressed as % maximum possible effect (% M.P.E.) using the formula [post-heroin latency – baseline latency] / [cut-off latency - baseline latency] x 100) as previously described (Nemmani, Grisel, Stowe, Smith-Carliss, & Mogil, 2004).

Narrow-sense trait heritability for heroin dependence in inbred strains (Experiment #3) was determined by comparing the between-strain variance to the total variance. Since animals are isogenic (i.e., genetically identical) within individual inbred strains, between-strain variance provides a measure of additive genetic ("allelic") variation (V_A), whereas within-strain variance ("error variance") represents environmental variability (V_E). An estimate of narrow-sense heritability (h^2) for each trait was obtained using the formula: $h^2 = V_A/(V_A + V_E)$ (Falconer and Mackay, 1996). Since the six strains were chosen randomly from among 11 strains previously tested for morphine dependence (Kest et al., 2002), these values are likely accurate estimates (Hegmann and Possidente, 1981). To assess genetic correlation between acute and

chronic heroin dependence in experiment #3, strains were also ranked from smallest to highest according to jumping frequencies and subject to Spearman's rank statistic (r_s), ensuring that correlation estimates were not unduly influenced by extreme scoring strains. The genetic correlation between the present heroin data and that obtained previously for morphine dependence (Kest et al., 2002) were calculated separately for acute and chronic paradigms. All correlations were subject to Bonferroni correction for multiple comparisons.

For all statistical tests criteria for significance was $p \leq 0.05$.

Chapter 3 – Experiments 1 - 4

Experiment 1: Assessment of naloxone precipitated withdrawal jumping as a reliable measure of heroin dependence in mice

1) Introduction

Manifested by a characteristic withdrawal syndrome of multiple aversive physical signs, opiate dependence remains a primary concern of physicians, leading them to under-medicate pain patients (Breivik, 2001). For narcotic drug abusing populations, the aversive withdrawal symptomology is regarded as a causative factor in their continued opioid use, engendering dangerous drug seeking behavior (Jasinski, 1977). Although physical dependence is commonly associated with chronic opioid intake, acute opioid treatment comprised of even a single exposure can cause dependence in a wide variety of species (McLemore et al. 1997; Smits, 1975; Jasinski, 1977; Kest et al. 2001). Despite their qualitatively similar withdrawal symptoms, there is behavioral and biochemical evidence that acute and chronic dependence are mediated by distinct mechanisms (Kest et al. 2001; McLemore et al. 1997; Nehmad et al. 1982).

Research into the mechanism of opioid dependence in rodents has been facilitated by precipitating withdrawal by injecting the wide-spectrum naloxone. Among NPW signs in mice, frequency of uncontrollable stereotypical jumping (i.e., a hyperactivity response) is widely considered the most sensitive and reliable index of withdrawal intensity and is by far the most commonly used (El-Kadi and Sharif, 1994; Smits, 1975; Saelens et al. 1971; Kest et al., 2002). For a variety of opioids, jumping frequencies in

mice during NPW correlate well with their known physical dependence liability in man (Saelens et al. 1971).

In contrast to morphine, very little is known regarding heroin dependence. In order to facilitate such study we investigated whether jumping frequency could serve as a reliable measure of naloxone-precipitated withdrawal from heroin after its acute and chronic administration. While both opioids preferentially bind and activate μ-opioid receptors (Watson et al., 1996; Selley et al., 2001), heroin, which is rapidly converted to 6-monoacetylmorphine (6-MAM) in vivo (Inturrisi et al. 1983), crosses the blood brain barrier significantly faster and more effectively than morphine (Oldendorf et al. 1972). We therefore also attempt to establish protocols for naloxone precipitated heroin withdrawal by evaluating the latencies between opioid and naloxone injection required for optimal response.

2) Materials and Methods

Subjects. Adult male CD-1 mice were used in this study. Every dose of every condition was comprised of at least 6 mice, and each mouse was used only once. (See General Methods section for information regarding housing and drug preparation.)

Drugs. Heroin hydrochloride and naloxone hydrochloride were injected s.c. as previously described in the General Methods section.

Naloxone Precipitated Withdrawal. Heroin withdrawal was assessed for 15-minutes as previously described in the General Methods section.

Time-Response Studies. In the acute heroin dependence protocol, mice were injected once with heroin (50 mg/kg). To study chronic dependence, heroin does of 10,

20, and 40 mg/kg were injected t.i.d. on Days 1, 2, and 3, respectively. A final heroin injection (40 mg/kg) was made before NPW on day 4. At the completion of heroin treatment, mice were injected with a single bolus dose of naloxone (50 mg/kg) and jumping frequencies were tallied at various hourly intervals. The heroin and naloxone doses chosen are those previously shown to elicit maximal jumping responses in the study of acute and chronic morphine dependence in mice (El-Kadi and Sharif, 1994; Kest et al., 2002). Heroin control mice were injected with saline instead of naloxone and naloxone control mice were injected with saline instead of heroin at the heroin-naloxone interval eliciting the greatest jumping frequency.

Dose-Response Studies

Heroin. The relationship between heroin dose and NPW jumping was determined for acute and chronic dependence paradigms. For acute dependence, mice were injected with a single heroin dose (2-50 mg/kg) followed by naloxone (50 mg/kg). We used a heroin-naloxone interval (2 h) determined from the above time-response studies to yield maximal jumping responses following acute heroin injection.

For chronic dependence studies, heroin doses of 5, 10, and 20 or 10, 20, and 40 mg/kg were injected into separate groups of mice t.i.d. on Days 1, 2, and 3 respectively. On Day 4, mice received a final single 40 mg/kg heroin injection followed by a naloxone (50 mg/kg) injection using an interval determined from the above time-response studies to yield maximal jumping responses following chronic heroin treatment (1 h).

Heroin control mice in both acute and chronic treatment paradigms were injected with saline instead of naloxone using the heroin dose eliciting the greatest jumping frequency.

Naloxone. The relationship between naloxone dose and NPW jumping was determined for acute and chronic heroin dependence paradigms. For acute dependence, mice were injected once with heroin (50.0 mg/kg). For the chronic treatment condition, mice were injected with 5, 10, and 20 mg/kg of heroin on days 1, 2, and 3 respectively, followed by a final heroin injection of 20 mg/kg on day 4. A range of naloxone doses (0.3-50.0 mg/kg) were injected after the end of heroin treatment using a heroin-naloxone interval shown in the time-response studies to elicit maximal jumping frequencies (acute: 2 h; chronic: 1 h).

Naloxone control mice in both acute and chronic treatment paradigms were injected with saline instead of naloxone using the heroin dose eliciting the greatest jumping frequency.

Data Analysis. Withdrawal jumping frequencies between treatment groups were analyzed using a one-way ANOVA. An individual t-test was used to compare the saline-naloxone and heroin saline groups in order to determine any difference between these two control groups. All analyses were followed post hoc by Fisher's LSD (Protected t-Tests) (See General Methods section).

3) Results

Time-response Studies

Acute Heroin Injection. Jumping frequencies after injecting the acute heroin bolus dose was maximal when naloxone was injected 2 h later (Figure 2A). Thus, this heroin-naloxone interval was used to test heroin and naloxone control mice. Since the mean jumping frequencies of the two control groups were not significantly different

(heroin controls: 1.3 jumps; naloxone controls: 2.3 jumps), they were pooled into a single control group. Relative to control values, significant NPW jumping was observed when naloxone was injected 1, 2, or 3 h after heroin. Nonetheless, values obtained at 2 h were significantly greater than those obtained at 1 or 3 h. Jumping frequencies obtained at heroin-naloxone intervals of 0.5 and 5 h were not significantly different than those obtained in controls.

Chronic Heroin Injection. For chronic heroin treatment, maximal jumping responses were obtained at a heroin-naloxone interval of 1 h (Figure 2B), and therefore both heroin and naloxone control groups were tested at that time. Again, since the mean jumping frequencies of the two control groups were virtually identical (heroin controls: 2.6 jumps; naloxone controls: 2.2 jumps), they were pooled. Significant jumping relative to control mice was also observed at heroin-naloxone intervals of 2 and 3 h, but not 0.5 h. There was no significant difference between jumping values obtained at 1, 2, and 3 h.

Dose-Response Studies

Acute Heroin Doses. There was a positive relationship between heroin dose and NPW jumping, with the acute 50 mg/kg heroin dose causing the greatest mean jumping frequency (Figure 3A). Accordingly, mice in the heroin control group were injected with the identical heroin dose (but without subsequent naloxone). Although all heroin doses increased jumping frequency relative to heroin controls, these differences were significant only after acute heroin injections of 10 and 50, but not 2, mg/kg. Although there was no significant difference between mice treated with the 10 and 50 mg/kg heroin doses, their frequency values were significantly greater (between 45%-50%) than that obtained after 2 mg/kg heroin.

Figure 2A. Time-response study of naloxone precipitated withdrawal from acute heroin treatment. Mice were injected with a single heroin dose (50 mg/kg). Withdrawal was precipitated by injecting naloxone (50 mg/kg) at various intervals afterwards. Control mice were subject to the identical treatment protocols but received saline injections in place of either heroin or naloxone and tested at the heroin-naloxone interval yielding maximal frequencies. Significant differences from control values (* $p < 0.05$; ** $p < 0.01$) are indicated.

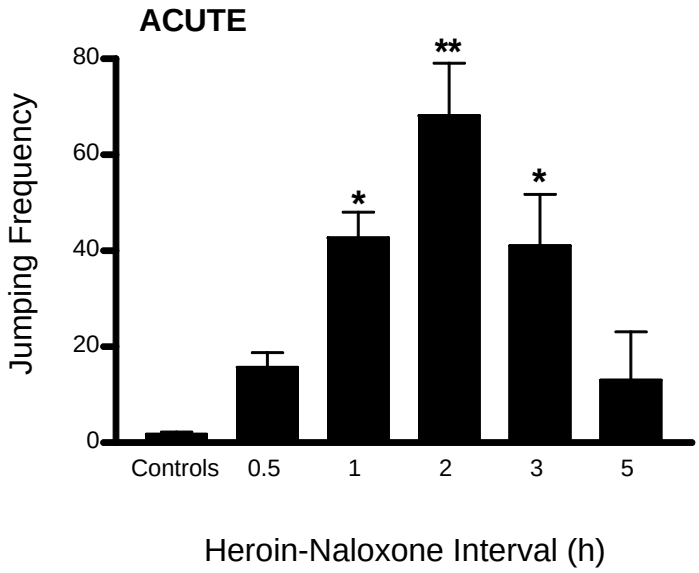

ACUTE
Jumping Frequency
80
60
40
20
0
**
*
*
Controls
0.5
1
2
3
5
Heroin-Naloxone Interval (h)

Figure 2B. Time-response study of naloxone precipitated withdrawal from chronic heroin treatment. Mice were injected t.i.d. for three days with escalating heroin doses (10, 20, and 40 mg/kg on treatment Days 1, 2, and 3, respectively) and a final 40 mg/kg heroin dose on Day 4. Withdrawal was precipitated by injecting naloxone (50 mg/kg) at various intervals after heroin treatment was completed. Control mice were subject to the identical treatment protocols but some mice received saline injections in place of heroin and others in place of naloxone and tested at the heroin-naloxone interval yielding maximal frequencies. Significant differences from control values (* $p < 0.05$; ** $p < 0.01$) are indicated.

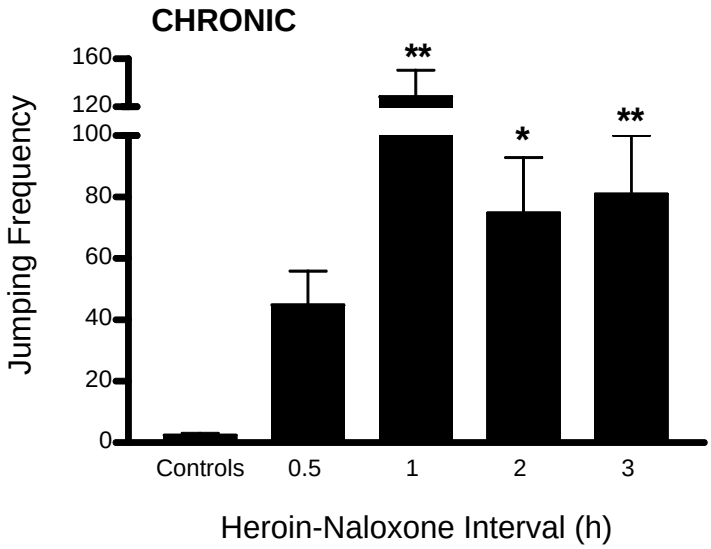
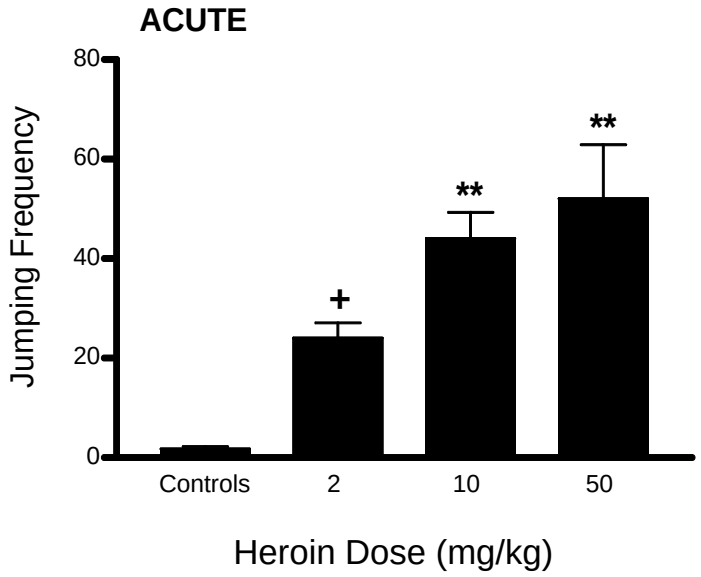

CHRONIC
Jumping Frequency
160
120
100
80
60
40
20
0
**
*
**
Controls
0.5
1
2
3
Heroin-Naloxone Interval (h)

Figure 3A. Dose-response relationship between acute heroin doses and jumping frequency. Mice received a single injection of various heroin doses. Withdrawal was precipitated 2 h later using a single dose of naloxone (50 mg/kg). Heroin control mice were injected with saline instead of naloxone using the heroin dose eliciting the greatest jumping frequency. Significant differences from control group (** $p < 0.01$) and all other non-control groups (+) are indicated.


ACUTE
Jumping Frequency
80
60
40
20
0
+
**
**
Controls
2
10
50
Heroin Dose (mg/kg)

Chronic Heroin Doses. For chronic heroin treatment, maximal jumping responses were obtained after treatment with the higher dosing regimen of 10, 20, and 40 mg/kg t.i.d. on days 1, 2, and 3, respectively (Figure 3B). Relative to the heroin control group, who thus received the identical heroin dosing regimen but without subsequent NPW, both chronic heroin dose groups displayed significantly greater jumping frequencies. Although mice treated with the greater heroin doses jumped more (~30%) than those treated with the lower chronic doses, this difference was not significant.

Naloxone Doses: Acute Heroin Treatment. After an acute 50 mg/kg heroin injection, the 50 mg/kg naloxone dose elicited the greatest mean jumping frequency (Figure 4A) and so naloxone control mice were injected with this naloxone dose (without prior heroin treatment). Relative to these controls, all three naloxone doses (2, 10, and 50 mg/kg) elicited significant jumping, and there was no significant difference between the groups.

Naloxone Doses: Chronic Heroin Treatment. There was a clear positive relationship between naloxone dose and jumping frequency, with the largest naloxone dose (50 mg/kg) eliciting the greatest mean jumping frequency (Figure 4B) after chronic heroin treatment. Thus, naloxone control mice were injected with 50 mg/kg naloxone only. Relative to these controls, significant jumping was elicited after naloxone doses of 3 and 50, but not 0.3, mg/kg. Furthermore, each naloxone dose significantly increased jumping frequencies between 2- and 2.5- fold relative to the preceding lower dose.

Figure 3B. Dose-response relationship between chronic heroin doses and jumping frequency. Mice were injected t.i.d. for three days with escalating heroin doses (5, 10, and 20 or 10, 20, and 40 mg/kg on treatment Days 1, 2, and 3, respectively) and a final single heroin dose (40 mg/kg) on Day 4. Withdrawal was precipitated 1 hour later by injecting naloxone (50 mg/kg). Heroin control mice were injected with saline instead of naloxone after being injected with the heroin dose eliciting the greatest jumping frequency. Significant differences from control group (** $p < 0.01$) are indicated.

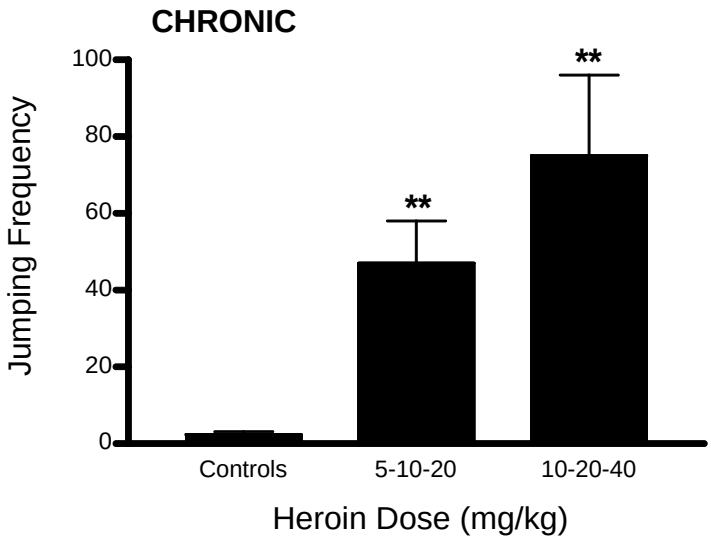

CHRONIC
Jumping Frequency
100
80
60
40
20
0
**
**
Controls
5-10-20
10-20-40
Heroin Dose (mg/kg)

Figure 4A. Dose-response relationship between naloxone doses after acute heroin treatment and jumping frequency. Mice were injected with a single heroin dose (50 mg/kg). Various naloxone doses were injected 2h later. Naloxone control mice were injected with saline instead of heroin followed by an injection of the naloxone dose eliciting the greatest jumping frequency. Significant differences from control group (* $p < 0.05$; ** $p < 0.01$) are indicated.

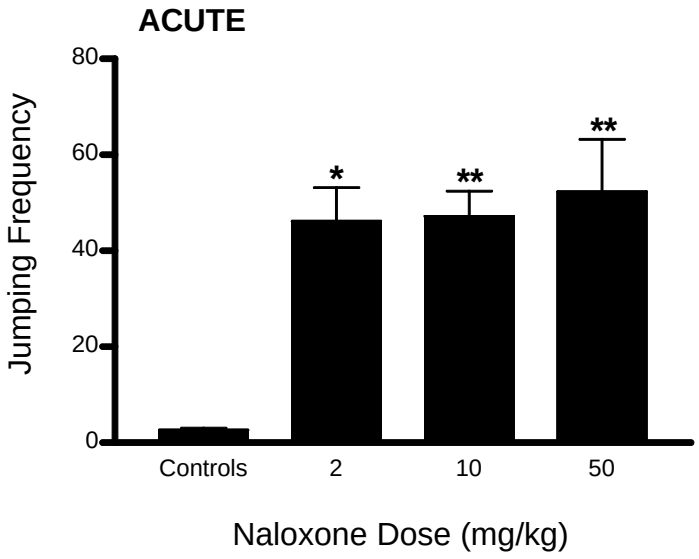
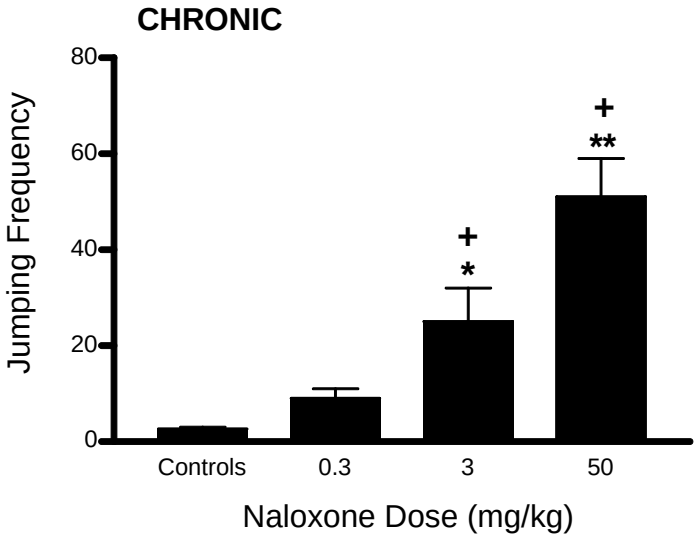

ACUTE
Jumping Frequency
80
60
40
20
0
*
**
**
Controls
2
10
50
Naloxone Dose (mg/kg)

Figure 4B. Dose-response relationship between naloxone doses after chronic heroin treatment and jumping frequency. Mice were injected t.i.d. for three days with escalating heroin doses (5, 10, and 20 mg/kg on treatment Days 1, 2, and 3, respectively) and a final single heroin dose (40 mg/kg) on Day 4. Naloxone doses were injected 1 h after the final heroin dose. Naloxone control mice were injected with saline instead of heroin followed by an injection of the naloxone dose eliciting the greatest jumping frequency. Significant differences from control group (* $p < 0.05$; ** $p < 0.01$) and the lower preceding dose (+) are indicated.


CHRONIC
Jumping Frequency
80
60
40
20
0
+
**
+
*
Controls
0.3
3
50
Naloxone Dose (mg/kg)

4) Discussion

Previous studies have demonstrated a positive dose-response relationship between morphine dose and subsequent jumping frequencies during NPW (Kest et al., 2001; Wiley & Downs, 1979; Marshall & Weinstock, 1969). On the basis of these and other studies, NPW jumping frequency is widely considered a reliable and sensitive index of morphine withdrawal severity and, accordingly, morphine dependence. The present study sought to determine whether there is a similar relationship between heroin treatment and NPW jumping responses. Since there has yet to be a demonstration of a similar relationship between morphine dose and other observable measures typical of opioid withdrawal such as ptosis, diarrhea, wet-dog shakes, or rearing, these were not studied here.

The data show a positive dose-response relationship between either acute or chronic heroin doses and NPW jumping frequencies. Although the increased frequency between a particular dose and the one preceding or following was not always significant, this may simply reflect the doses chosen for study. That is, the trend in the data indicate that eliminating some doses and/or testing additional higher or lower doses than those presently employed would have likely resulted in additional significant differences between doses. In order to minimize the total number of mice studied and lethality associated with large opioid bolus doses, these additional doses were not tested. Furthermore, chronic heroin treatment elicited jumping frequencies of greater magnitude than acute heroin bolus doses, even when none of the doses used in a particular chronic paradigm were greater than the largest acute dose tested. In contrast to the experimental mice treated with heroin followed by naloxone, jumping frequencies tallied in heroin

control mice were minimal (mean: 2-4 jumps/15 min). In fact, these already low mean values are overestimates as the overwhelming majority of subjects were non-responders, regardless of the heroin injection protocol or dose used. Collectively, these data indicate that the responses of experimental mice were a consequence of their heroin dependency, and that, like morphine dependence, jumping frequency is a reliable and sensitive index of heroin withdrawal magnitude in mice.

Previous studies have also reported a positive dose-response relationship between naloxone doses used to precipitate withdrawal after acute and chronic morphine treatment and jumping frequency (Kest et al., 2001; El-Kadi and Sharif, 1994; Smits 1975). Here, increasing the naloxone dose also caused significant concomitant increases in jumping frequencies following chronic, but not acute, heroin administration. At present we have no explanation as to why increasing naloxone dose after an acute heroin injection did not concomitantly increase jumping frequencies, particularly since such increases are evident after acute morphine injection (Kest et al., 2001), and the present acute heroin protocols utilized highly similar experimental methodologies including the range of naloxone doses tested. Although the interval between morphine and naloxone injection of 3 h in that study was slightly longer than the 2 h heroin-naloxone interval used here, this difference is unlikely to be critical since in both studies jumping frequencies were tallied immediately after naloxone injection and there is no reason to suspect that differences in naloxone pharmacokinetics between morphine and heroin treated mice that would cause differences in naloxone bioavailability or efficacy.

The time-response data indicate that maximal NPW responding is obtained after acute and chronic heroin treatment using a heroin-naloxone interval of 2 and 1 h,

respectively. It is possible that prolonged heroin exposure during chronic treatment caused pharmacodynamic changes at opioid receptors themselves or recruited additional and/or distinct adaptive changes in any of several neural adaptations that underlie dependence subsequent to their activation. Additional studies are needed to distinguish between these possibilities. Despite their differences, the heroin-naloxone interval yielding maximal responses after acute (2 h) and chronic (1h) heroin are still smaller than those demonstrated for acute (3h) and chronic (3h) morphine treatment (El-Kadi and Sharif, 1994; Wiley & Downs, 1979). It seems logical to us that ability of heroin to more rapidly cross the blood-brain barrier and penetrate the brain relative to morphine (Oldendorf, 1972) may underlie this difference.

Experiment 2: The Contribution of Opioid Receptors and Excitatory Amino Acid Receptors to Heroin Dependence in Mice

1) Introduction

Although all opioids have some dependence liability, our current understanding of opioid dependence is based predominantly on studies using morphine (Nestler, 1994). Although morphine dependence has been shown to be a consequence of activity at the μ type (Matthes et al., 1996; Berrendero et al., 2002), δ and κ receptors have a modulatory role. The δ receptor type has been further resolved into subtypes (Quock et al., 1999), each with a specific contribution to morphine dependence. For example, blocking the δ_2-subtype with NTB or 5'-NTII attenuated the jumping, body shakes and weight loss accompanying NPW after chronic morphine treatment. In contrast, δ_1-receptor antagonists including BNTX were either without effect on any withdrawal measure or blocked the withdrawal-induced changes in body weight only (Miyamoto et al., 1994; Suzuki et al. 1997b). AS ODNs targeting δ_2-receptors similarly attenuated NPW jumping after acute and chronic morphine treatment (Kest et al., 1996; Suzuki et al. 1997a).

The role of kappa opioid receptors is more equivocal. Whereas mice with a disruption in the κ-opioid receptor gene appear to have attenuated withdrawal signs including NPW jumping following chronic morphine administration (Simonin et al. 1998), administration of the κ-receptor antagonist nor-BNI increased NPW weight loss in mice (Suzuki et al., 1992). Additionally, administration of dynorphin A (1-13), an opioid peptide with greater relative affinity for κ than μ and δ receptors (Chavkin et al., 1982), immediately prior to NPW significantly suppressed the withdrawal jumping response in

mice made chronically dependent on morphine (Takemori et al., 1992). The contribution of κ-opioid receptors to acute morphine dependence has not been reported.

By activating NMDA and AMPA receptors, excitatory amino acids such as glutamate and aspartate also make a critical contribution to morphine dependence. For example, continuous infusion or repeated injection of MK-801 attenuated NPW jumping frequencies in mice rendered dependent after acute or chronic morphine administration, respectively (McLemore et al. 1997; Trujillo & Akil, 1991; Verma & Kulkarni, 1995; Gonzalez et al., 1997). These data collectively indicate a role for NMDA receptors in mechanisms contributing to both acute and chronic morphine dependence. The contribution of AMPA receptors to morphine dependence has been much less studied, but a contribution to morphine dependence is nonetheless indicated. Chronic infusion of the selective antagonist LY293558 attenuated jumping frequencies in mice subject to acute morphine dependence (McLemore et al. 1997). Although the same LY293558 infusion dose was ineffective in mice chronically treated with morphine, "knock-out" mice lacking the AMPA GluR-A subunit show reduced jumping frequencies under these conditions (Vekovischeva et al., 2001).

It has been commonly thought that heroin, which is rapidly converted to 6-MAM and then morphine in vivo (Inturrisi et al. 1983), would logically have a pharmacological profile similar to that of morphine. Indeed, both opioids preferentially bind and activate μ-opioid receptors (Watson et al., 1996; Selley et al., 2001). However, recent data indicate mechanistic differences between morphine and heroin with respect to analgesia processes (Ikeda et al., 1999; Pick et al. 1993; Rossi et al. 1996; Rossi et al. 1995). Additionally, studies using AS ODNs to target different exons of the μ-opioid receptor

gene have demonstrated that different splice variants mediate morphine and heroin analgesia (Rossi et al. 1995). Since there are very few studies of heroin dependence, it is unknown whether the mechanisms underlying heroin and morphine dependence are somewhat distinct. Specifically, it is possible that opioid and EAA receptors make different contributions to heroin and morphine dependence. There is no currently no data that can address this possibility.

Thus, the aim of the present study is to assess the contribution of δ and κ-opioid, as well as NMDA and AMPA types of EAA receptors to heroin dependence by using selective antagonists previously used in studies with morphine.

2) Materials and Methods

Subjects. Adult male CD-1 mice were used in this study. Every dose of every condition was comprised of at least 6 mice, and each mouse was used only once. (See General Methods section for information regarding housing and drug preparation.)

Drugs. Heroin hydrochloride, naloxone hydrochloride, BNTX, naltriben, and nor-BNI, MK-801 and LY293558 were administered by s.c. injection as previously described in the general methods section. MK-801 and LY293558 only, were also administered by continuous infusion via osmotic pumps.

Naloxone Precipitated Withdrawal. Heroin withdrawal was assessed for 15-minutes as previously described in the General Methods sections.

Opioid and EAA Receptor Antagonist Studies. The acute and chronic heroin dependence paradigms used in these studies were those shown in the above time- and dose- response studies to elicit maximal NPW jumping frequencies.

Acute Dependence. Groups of mice were injected with a single bolus dose of heroin (50 mg/kg) followed 2 h later by naloxone (50 mg/kg). Some groups were injected 30 minutes prior to heroin with either BNTX (0.5 mg/kg), naltriben (1 mg/kg), MK-801 (0.05 mg/kg), or LY293558 (5 mg/kg). The BNTX and naltriben doses used are those previously reported as effective in the morphine dependence literature (Suzuki, 1997b). MK-801 and LY293558 doses were based on our pilot studies and were the maximal allowable dose not causing severe motor impairment and/or lethality. Since McLemore et al. (1997) have shown that MK-801 and LY293558 can attenuate acute morphine dependence after their continuous subcutaneous infusion, separate groups received these drugs using the identical delivery protocols described in that study. Specifically, pumps infusing cumulative daily doses of 1 and 60 mg/kg containing MK-801 and LY293558 were implanted 16 hours prior to heroin. nor-BNI (10 mg/kg) was injected 8 hours prior to heroin, corresponding to its maximal blockade of κ-receptors (Endoh et al., 1992). An antagonist control group was injected with saline instead of an antagonist.

Chronic dependence. Heroin doses of 10, 20, and 40 mg/kg were injected into separate groups of mice t.i.d. on days 1, 2, and 3, respectively. A final 40 mg/kg heroin dose injection on Day 4 was followed 1 h later by naloxone (50 mg/kg). NTBX, naltriben, MK-801, and LY 293558 doses identical to those used for the acute heroin study immediately above were injected 30 minutes prior to each heroin injection. We also injected the identical nor-BNI dose. Since this drug is an irreversible antagonist and a single injection provides κ-opioid receptor blockade for up to 96 hours (Endoh et al.,

1992), it was injected 8 h prior to the first heroin injection on days 1 and 3 only. Antagonist controls were injected with saline instead of antagonist.

E) Data analysis. Jumping frequencies between groups were subject to an independent *t*-test or one-way ANOVA. Fisher's LSD (Protected t-Tests) tests were used to make post-hoc comparisons with control groups. (see General Methods section).

4) Results

Opioid and EAA Receptor Antagonists

Acute Heroin Injection. The respective δ_1 and δ_2 opioid receptor antagonists BNTX and naltriben, but not the κ antagonist nor-BNI, significantly reduced mean heroin NPW jumping frequencies by ~35%-50% relative to antagonist controls (Fig. 5A). Although neither the respective NMDA nor AMPA receptor antagonists MK-801 or LY293558 altered jumping frequencies after their acute s.c. injection, both drugs caused marked and significant frequency reductions of between 60%-80% when they were delivered by continuous infusion.

Chronic Heroin Injection. With the exception of nor-BNI, all antagonists significantly reduced mean heroin NPW jumping frequencies by about 50 - 60% relative to antagonist controls (Fig. 5B). In contrast, nor-BNI increased jumping relative to controls by ~70%.

Figure 5A. Effect of opioid and excitatory amino acid receptor blockade on withdrawal jumping after acute heroin treatment. Mice were injected with a single heroin dose (50 mg/kg). All mice were also injected with one of the following antagonists (see text for doses and injection schedule): BNTX, NTB, nor-BNI (δ_1, δ_2, and κ opioid receptor antagonists, respectively), MK-801 and LY293558 (NMDA and AMPA receptor antagonists, respectively). MK-801 and LY293558 were also delivered by continuous infusion.. Withdrawal was precipitated by naloxone (50 mg/kg) injection 2 h after heroin injection. Control mice in were injected with saline instead of an opioid or EAA antagonist. Significant differences from control group are indicated (* p <0.05).

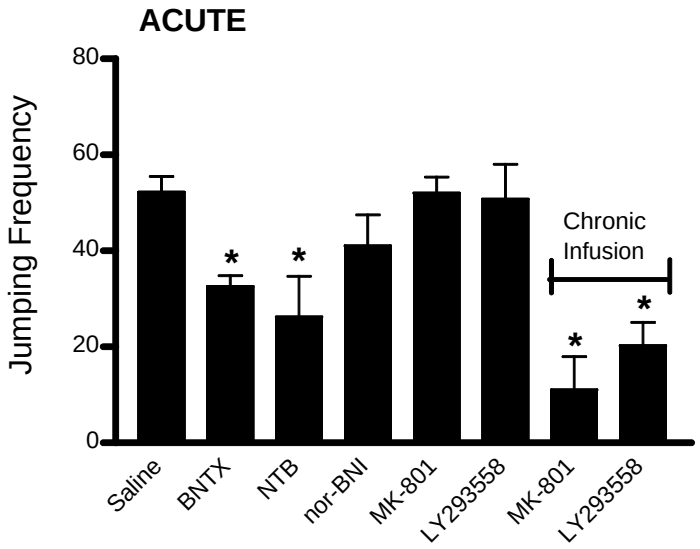
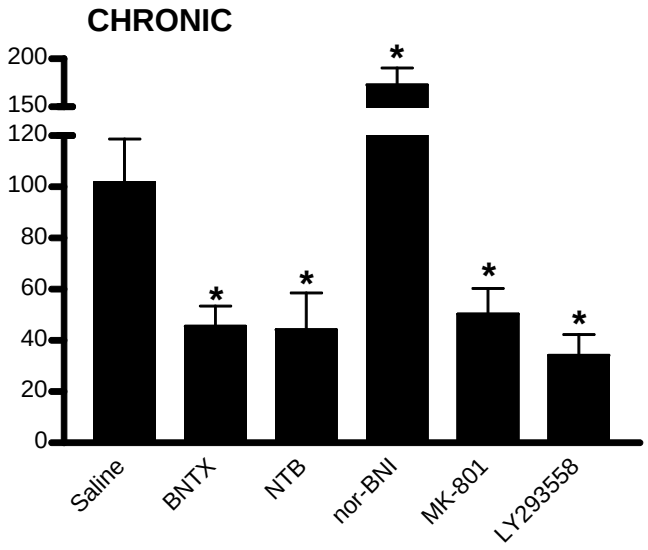

ACUTE
Jumping Frequency
80
60
40
20
0
Chronic
Infusion
*
*
*
*
Saline
BNTX
NTB
nor-BNI
MK-801
LY293558
MK-801
LY293558

Figure 5B. Effect of opioid and excitatory amino acid receptor blockade on withdrawal jumping after chronic heroin treatment. Mice were injected t.i.d. for three days with escalating heroin doses (10, 20, and 40 mg/kg on treatment Days 1, 2, and 3, respectively) and a single final 40 mg/kg heroin dose on Day 4. All mice were also injected with one of the following antagonists (see text for antagonist doses and injection schedule): BNTX, NTB, nor-BNI (δ_1, δ_2, and κ opioid receptor antagonists, respectively), MK-801 and LY293558 (NMDA and AMPA receptor antagonists, respectively. Withdrawal was precipitated by naloxone (50 mg/kg) injected 1 h after the last heroin injection. Control mice were injected with saline instead of an opioid or EAA antagonist. Significant differences from control group are indicated (* $p < 0.05$).


CHRONIC
200
150
120
100
80
60
40
20
0
*
*
*
*
*
Saline
BNTX
NTB
nor-BNI
MK-801
LY293558

5) Discussion

In the present study, the respective δ_1 and δ_2 opioid receptor antagonists BNTX and NTB significantly reduced NPW jumping frequencies relative to controls after both acute and chronic heroin injection, suggesting that activity at either δ-receptor subtype enables heroin dependence. Although our findings with NTB parallel the results from studies of acute and chronic morphine dependence, the ability of BNTX to reduce jumping frequencies do not. That is, BNTX doses identical or even greater than that used here were ineffective in reducing NPW jumping in mice after chronic morphine treatment (Suzuki et al., 1997b). These BNTX data are supported by the finding that the δ_1 receptor selective antagonist DALCE is also ineffective in reducing NPW jumping responses after chronic morphine treatment (Miyamoto et al., 1994). Thus, the prevailing evidence indicates that δ_1 receptors do not contribute to morphine dependence and, with the present data, suggest that the δ-opioid receptor modulation of morphine and heroin dependence is not identical. Converging lines of evidence indicate a close association of μ and δ binding sites in an opioid receptor complex, and this association is thought to underlie the ability of δ_2 opioid receptors to attenuate morphine dependence (George et al, 2000; Gomes et al., 2000; Daniels et al., 2005). We suspect that the ability of NTB to attenuate NPW jumping after heroin, which also acts primarily at the μ-receptor (Liu et al., 2003), results from the same mechanism. However, since there are no similar studies reporting an anatomical association or functional interaction between μ and δ_1 receptors, the basis by which δ_1 receptors modulate heroin dependence is not obvious to us. Furthermore, any such mechanism would have to be distinct from that mediating morphine dependence.

In contrast to δ-opioid receptor antagonists, the selective and irreversible κ-opioid receptor antagonist nor-BNI was without effect on NPW jumping frequencies after acute heroin injection. We do not believe this finding resulted from using an insufficiently large nor-BNI dose since this dose has been shown to provide effective κ-opioid receptor blockade on several behavioral measures (Narita et al., 1990), including chronic morphine dependence (Suzuki et al., 1992). Indeed, here, injecting the identical nor-BNI dose only twice over 4 days significantly increasing jumping frequencies in mice injected repeatedly and chronically with heroin over 4 days. Instead, we believe that κ-opioid receptors act exclusively to restrict NPW jumping after chronic but not acute heroin treatment. This suggests that distinct mechanisms underlie acute and chronic opioid dependence, an assertion previously advocated regarding acute and chronic morphine dependence (Kest et al., 2001; Yamamoto et al., 1978; Nehmad et al., 1982). Interestingly, the identical nor-BNI dose tested here significantly increased weight loss in mice and rats consequent to NPW after chronic morphine treatment (Suzuki et al., 1992). Similarly, weight loss, "wet-dog" shakes, and teeth chattering were also increased after intrathecal nor-BNI injection in rats chronically treated with morphine (Cui et al., 2000). These findings parallel the increased jumping caused by nor-BNI in our mice chronically injected with heroin. How κ-opioid receptor blockade increases NPW symptoms is not well understood (but see Suzuki et al., 1992).

NMDA receptor activity is critically important to neuronal plasticity, resulting in adaptive changes after morphine exposure that results in dependence (Trujillo & Akil, 1991; Inoue et al., 2003). Indeed, MK-801 has been reported to attenuate NPW symptoms when delivered prior to acute and chronic morphine injections (McLemore et

al., 1997; Trujillo & Akil, 1991; Verma & Kulkarni, 1995). Here, MK-801 was always injected 30 min prior to heroin in both the acute and chronic heroin treatment protocols, presumably before neuronal adaptive processes have been initiated. After an acute heroin injection, we indeed found that NMDA receptor blockade reduced jumping frequencies, but only when MK-801 was delivered via continuous infusion starting 16 h before heroin and not when it was injected as an acute bolus dose 30 min before. It is unlikely that the acute MK-801 bolus dose was not sufficiently large since the same dose was effective against chronic dependence when injected prior to each heroin injection. These data suggest that the ability of MK-801 to attenuate heroin dependence might not result from the blockade of NMDA receptors per se, but from physiological and/or neuronal adaptations resulting from prolonged MK-801 treatment. For example, chronic MK-801 administration or NMDA receptor blockade can reduce the density of the glutamate binding site within the NMDA receptor (Beart & Lodge, 1990; Manallack et al., 1989), which would presumably down-regulate NMDA receptor activity and the consequent biochemical cascade that underlies dependence., and alter $GABA_A$ and dopamine D2 receptors that contribute to opioid dependence (Lannes et al., 1995; Micheletti et al., 1992). This supposition is also consistent with the finding that mice injected daily with MK-801 followed by saline for 8 consecutive days show attenuated NPW jumping and weight loss in response to an acute morphine injection on Day 9 (Koyuncuoglu et al., 1999), and warrants further study.

Like NMDA receptors, AMPA receptors mediate long-term central nervous system changes (Bettler and Mulle, 1995; Shahi and Baudry, 1993). Not surprisingly then, these receptors contribute to morphine dependence (Vandergriff & Rasmussen,

1999; McLemore et al., 1997) and, as demonstrated here, they apparently do so with respect to heroin dependence as well. We observed that LY293558 delivery by continuous infusion - but not acute bolus dose injection - prior to heroin reduced acute heroin NPW, and injecting a bolus dose of LY293558 t.i.d. over 4 days also attenuated jumping frequencies in the chronic heroin treatment protocol. The finding that continuous LY293558 infusion reduces acute heroin dependence parallels the finding that it attenuates NPW jumping under identical acute morphine dosing and delivery protocols (McLemore et al., 1997).

Although pharmacological, electrophysiological and molecular cloning studies have demonstrated that EAA receptors are functionally and constitutively distinct (Bettler and Mulle, 1995; Watkins, 1994), our findings on the effect of LY293558 on heroin dependence was identical to our present finding with MK-801. Accordingly, we suggest that, like MK-801, it is possible that only prolonged LY293558 treatment will reduce NPW jumping. Unlike for chronic MK-801 treatment and NMDA receptor blockade, however, we are not aware of any studies reporting that chronic LY293558 administration or AMPA receptor blockade causes adaptive changes that might obviously impact heroin or opioid dependence. It is important to note that AMPA receptors have a highly overlapping anatomical distribution with NMDA receptors and are thought to assist in their activation by providing the postsynaptic depolarization necessary to remove the voltage-sensitive Mg^{++} blockade of NMDA receptors (Bliss and Collingridge, 1993; Patel and McCulloch, 1995). Since MK-801 and LY293558 were effective only under the identical specific delivery protocols, we can not rule out the possibility that blockade of AMPA receptors here by LY293558 reduced heroin dependence only indirectly, *via* an

AMPA receptor-mediated reduction in NMDA receptor activity. Mechanism notwithstanding, the present data indicate that activation of the EAA receptors NMDA or AMPA during acute and chronic heroin treatment enables heroin dependence.

Experiment 3: Strain survey for acute and chronic heroin dependence

1) Introduction

The severity of opioid dependence has been reported in human populations to be subject to inter-individual variation. That is, whereas even minimal prior opioid exposure can cause dependence in some individuals, others are resistant to dependence despite chronic intake (Pasternak, 2004; Ikeda et al., 2005). Studies with human and rodent subjects have demonstrated the significant contribution of genotype to the variability in withdrawal magnitude (Kest et al., 2002; Han et al., 2004; Ikeda et al., 2005). For example, rat and mouse strains differ in both their sensitivity to undergo withdrawal per se and in the incidence of the subsequent withdrawal symptoms (Hoffman et al., 1998; Brase et al., 1977; Liang et al., 2006). Withdrawal jumping frequency is also subject to substantial inter-strain variability (Brase et al. 1977; Kest et al., 2002; Suzuki et al., 1991). In a survey of 11 inbred (isogenic) mouse strains, substantial heritability estimates were obtained for both acute and chronic morphine dependence, and mean jumping frequency differences as great as 100-fold were obtained in the most divergent responders (Kest et al., 2002a).

Studies with different rat and mouse strains are not only informative for demonstrating the contribution of genetic background to opioid dependence liability, but also identifies strains with highly divergent responses that can serve as progenitors in subsequent linkage analysis studies to identify the genes contributing to inter-individual variation in dependence severity. This has indeed been the case with morphine dependence (Kest et al., 2004; Liang et al., 2006). However, despite the substantial abuse

of heroin, there are no rodent studies assessing the possible contribution of genotype to physical dependence induced by heroin. And, as mentioned previously, although morphine is an extensively used and prototypical opioid, there are important differences between heroin and morphine that render tenuous common generalizations between them.

To determine whether, like for morphine, the magnitude of heroin dependence is dependent on genotype, we compared NPW jumping frequencies of 6 inbred mouse strains injected with heroin. Since similar but not identical substrates underlie acute and chronic dependence to opioids (McLemore et al., 1997; Wiley & Downs, 1979) including heroin (Klein et al., 2007), separate groups of mice were subject to acute and chronic heroin injection protocols. The demonstration of a genetic correlation between two heritable traits among isogenic strains can be used as evidence of the existence of pleiotropic genes with a common influence on both traits (Hegmann and Possidente, 1981). If the traits have genes in common, then one can strongly infer their common physiology. Thus, NPW jumping frequencies obtained after acute and chronic heroin treatment were correlated. In addition, the inbred mouse strains tested here were previously rendered dependent to morphine and surveyed for NPW jumping using protocols virtually identical to those utilized in the present study (Kest et al., 2002). Thus, the strain data obtained here also affords the opportunity to assess the genetic correlation between heroin and morphine dependence.

2) Materials and Methods

Subjects. Male mice of 6 different inbred strains were used in this study. At least 6 mice of each strain was used and each mouse was used only once. (See General Methods section for strain types and specific care guidelines.)

Drugs. Heroin hydrochloride and naloxone hydrochloride were injected s.c. as previously described in the General Methods section.

Naloxone Precipitated Withdrawal. Heroin withdrawal was assessed for 15-minutes as previously described in the General Methods sections.

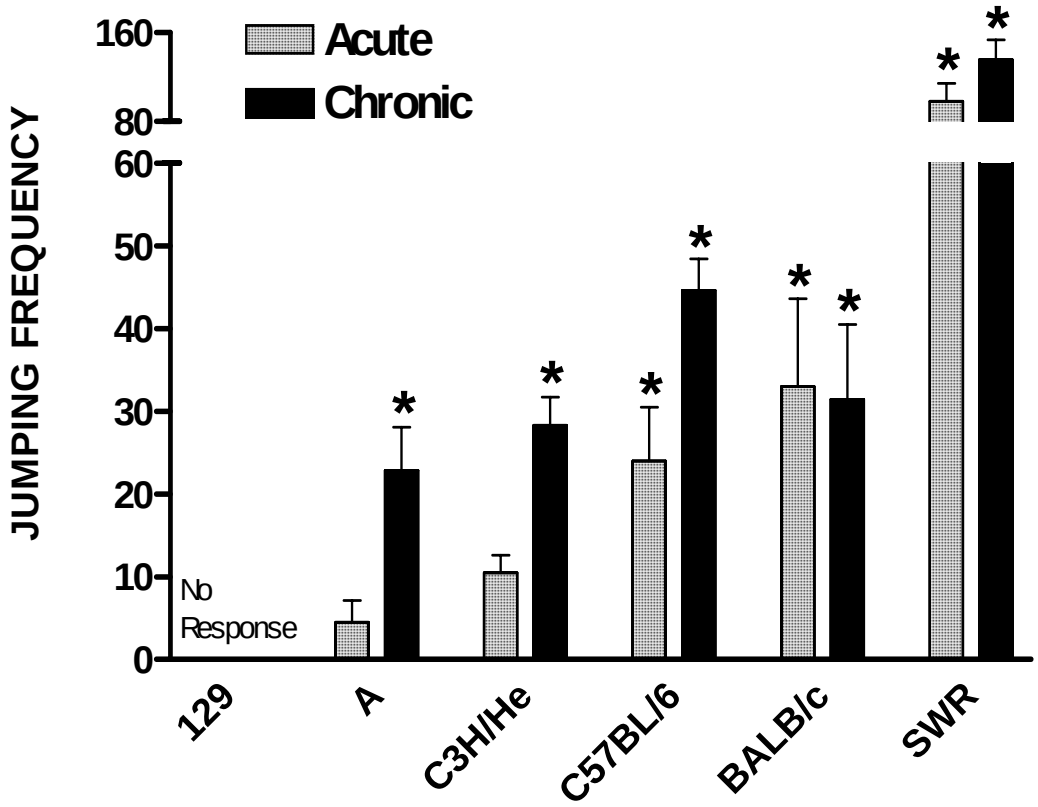
Heroin Treatment. Acute dependence was induced by a single subcutaneous 50 mg/kg heroin injection followed by a single naloxone dose (50 mg/kg) 2 h later. In the chronic dependence condition, heroin was injected t.i.d. (09:00 h, 13:00 h and 17:00 h) for three days using a dosing schedule of 5, 10, and 20 mg/kg on Days 1, 2, and 3, respectively. On Day 4, a final 20 mg/kg heroin dose was injected, followed by a single naloxone dose (50 mg/kg) 1 h later. Separate groups of mice from all strains acted as the control groups and were injected with saline instead of heroin, and then with naloxone according to the same schedule as the experimental groups.

Data Analysis. Separate two-way ANOVAs (strain X condition) were used to compare jumping frequencies in the acute and chronic dependence paradigms. (See General Methods section for details of correlational analyses.)

3) Results

There were significant main effects of strain, treatment, and their interaction (all $p < 0.001$) on jumping frequencies after both acute and chronic heroin treatment. As illustrated in Figure 6A, a wide range of strain frequency means values were obtained after each heroin treatment protocol. Specifically, whereas 129P3 mice did not respond after any heroin treatment, jumping frequencies as high as 98 and 136 were observed in SWR mice after acute and chronic heroin treatment, respectively. There were no significant strain differences in saline-treated controls (data not shown).

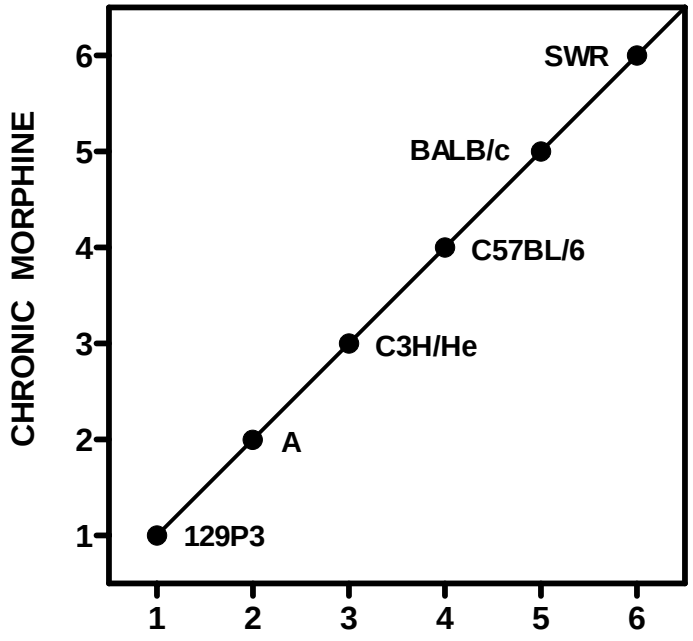
Figure 6A. Naloxone-precipitated withdrawal jumping frequencies in 6 inbred mouse strains after acute and chronic heroin treatment. Mice in the acute paradigm were injected with a single heroin dose (50 mg/kg) followed by a naloxone (50 mg/kg) injection 2 h later. Mice in the chronic dependence paradigm were injected t.i.d. for three days with escalating heroin doses (10, 20, and 40 mg/kg on treatment Days 1, 2, and 3, respectively) and a single final 40 mg/kg heroin dose on Day 4. Withdrawal was precipitated by naloxone (50 mg/kg) injection 1 h after the last heroin injection. Control mice of each strain (data not shown) were injected with saline instead of heroin and then naloxone according to the same schedule as the experimental groups. Significant differences from the control group of each respective strain are indicated (* $p < 0.05$).

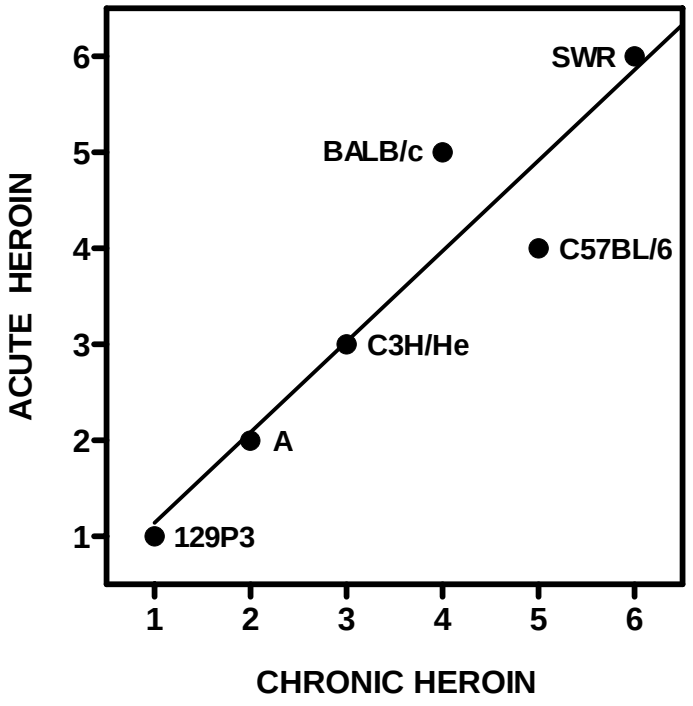

JUMPING FREQUENCY
160
80
60
50
40
30
20
10
0
Acute
Chronic
No
Response
129
A
C3H/He
C57BL/6
BALB/c
SWR
*

Narrow-sense heritability of acute and chronic heroin dependence was estimated as $h^2 = 0.98$ and $h^2 = 0.91$, respectively. These estimates are likely biased upward because of the extreme outlier status of the SWR strain. Even if this strain is excluded, however, heritability estimates remain substantially high, at $h^2 = 0.96$ and 0.86, respectively.

The scatterplot matrix in Figure 6B shows the regression of strain ranks for mean jumping frequencies after acute and chronic heroin and morphine treatment. The visual impressions of a correlation between responses are statistically confirmed by the r_s correlation coefficients provided in Table 7. All pairwise correlation coefficients were significant after Bonferroni correction for multiple comparisons, and indicate a high degree of genetic correlation.

Figure 6B. Genetic correlation of acute and chronic heroin and morphine dependence. Symbols represent inbred mouse strains arranged by their rank order of naloxone-precipitated withdrawal jumping frequency (1, lowest frequency; 6, highest frequency). Data are for the correlation between acute and chronic heroin injection (bottom left), between chronic heroin and chronic morphine injection (top left), and between acute heroin and morphine injection (bottom right). The corresponding Spearman rank coefficients (r_s = 0.94 & 1.0; Table 1) indicate a significant correlation for each pairwise comparison. Morphine strain data have been reported previously (Kest et al., 2002).


CHRONIC MORPHINE
6
5
4
3
2
1
SWR
BALB/c
C57BL/6
C3H/He
A
129P3
1
2
3
4
5
6


ACUTE HEROIN
6
5
4
3
2
1
SWR
BALB/c
C57BL/6
C3H/He
A
129P3
1
2
3
4
5
6
CHRONIC HEROIN

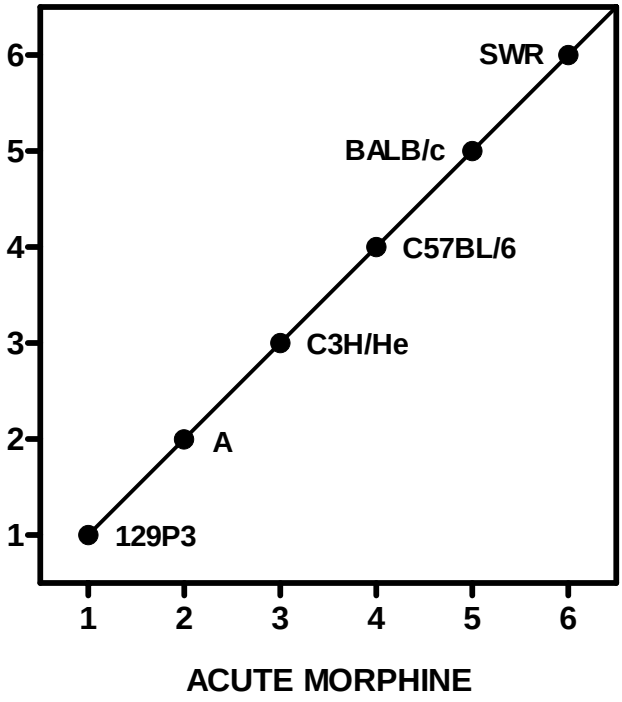

6
5
4
3
2
1
SWR
BALB/c
C57BL/6
C3H/He
A
129P3
1
2
3
4
5
6
ACUTE MORPHINE

Table 6. Spearman's rank correlation coefficients between inbred strains means on acute and chronic heroin dependence, acute heroin and morphine dependence, and chronic heroin and morphine dependence.

	Acute Heroin	Chronic Heroin
Chronic Heroin	0.94	--
Acute Morphine	1.00	--
Chronic Morphine	--	1.00

Mice in the acute heroin group were injected once (50 mg/kg) whereas those in the chronic heroin group were injected t.i.d. for three days (5, 10, and 20 mg/kg on days 1-3, respectively) and a single injection (20 mg/kg) on Day 4. Withdrawal in both cases was elicited by a single naloxone injection (50 mg/kg) at the completion of heroin treatment. Morphine data are from Kest et al. (2002). All correlation coefficients were significant ($P < 0.01$) after Bonferroni correction for multiple comparisons.

4) Discussion

Genetic variability. The present study surveyed NPW jumping frequencies in 6 inbred mouse strains after acute and chronic heroin treatment. All strains considered, the magnitude of dependence was greater following chronic relative to acute heroin administration, consistent with previous quantitative comparisons of withdrawal after acute and chronic opioid treatment in various species including humans (Eisenberg, 1982; Martin and Eades, 1964; Jasinski, 1977; Bickel et al. 1988; Heishman et al. 1989; Kest et al. 2001) including heroin (Klein et al., 2007). More relevant to the aims of this study, the data revealed an array of jumping frequency values and yielded relatively high h^2 estimates (> 0.86) even when an extreme responding (i.e., SWR) strain was excluded from analysis. These data demonstrate that the magnitude of acute and chronic heroin dependence is associated with genetic variability and a very highly heritable trait. The basis for these strain differences will almost certainly be many and varied, and depend on which two particular strains are being compared. That is, it is unlikely that a single mechanism will completely account for the quantitative distribution of jumping frequencies reported here. Although it is beyond the scope of the present report to speculate on the possible substrates underlying the withdrawal differences of each pairwise comparison, we do note that whereas heroin analgesia in some strains is mediated by the μ-opiate receptor subtype, it is mediated by the δ-opiate receptor subtype in others (Rady et al., 1991; Rady et al., 1994; Rady et al., 2000). These data indicate that genetic factors contribute to the opioid receptor selectivity of heroin in mice. Since dependence caused by μ-opiate receptor activity is more severe than that caused by δ-opiate receptor activity (Cowan et al., 1988; Maldonado et al., 1990), it is possible that

differences in withdrawal magnitude between some strains may result from differences in heroin opiate receptor type selectivity. This is an intriguing possibility that warrants study.

There was also a substantial range in mean frequency values between 129P3 and SWR, the most divergent strains in both treatment protocols. In addition to their respective ranking as least and most dependent strains, particularly noteworthy is the actual extent of their extreme sensitivities. Specifically, the present data identifies 129P3 mice as being absolutely refractory to jumping during heroin withdrawal. As noted above, only jumping frequency alone among withdrawal measures has been shown to have a positive dose-response relationship with withdrawal from opioids (Marshall & Weinstock, 1971; Smits, 1975; El-Kadi & Sharif, 1994; Kest et al., 2001) including heroin (Klein et al., 2007). Although 129P3 mice may thus be regarded as a genetic model of insensitivity to opioid dependence with potential heuristic value, it is certainly possible that evidence of dependence in this strain will be manifest when monitoring other NPW symptoms. In contrast, SWR were much more sensitive to NPW from heroin than any other strain, jumping ~3-fold more frequently than the strain with next highest tally after both acute and chronic heroin injection. Since this is the first survey of heroin withdrawal in inbred mice, we are thus unable to validate the observed pattern of withdrawal magnitude by comparison with other studies. Nonetheless, all mice studied here have been previously characterized for NPW from morphine, and were selected for inclusion in the present study to facilitate a comparison between heroin and morphine dependence, and is discussed below.

Genetic correlations. By using a sufficient number of randomly-chosen strains, the present study design should provide a valid estimate of the genetic correlation of acute and chronic dependence traits (Hegmann and Possidente, 2081). To ensure that correlation estimates were not unduly influenced by extreme scoring strains such as 129P3 and SWR, values subject to correlation were analyzed using Spearman's rank statistic. Based on these considerations, the covariation of naloxone-precipitated jumping among strains after acute and chronic heroin injection ($r = 0.94$) suggests the contribution of common genes and common (although not necessarily identical) physiological substrates. To date, we have indeed found a highly similar, but not identical, contribution of various opioid and excitatory amino acid receptors to heroin dependence (Klein et al., 2007). The remarkably high degree of genetic correlation between acute and chronic heroin dependence provides for the following additional implications. First, it attests to the resiliency of the putative mechanistic relationship between acute and chronic heroin dependence despite the inherent differences in treatment duration (a single injection vs. 3 daily injections for three days, respectively) and cumulative heroin dosing (50 mg/kg and 125 mg/kg, respectively). Second, it engenders confidence in our assessment of the interstrain variability in dependence after chronic heroin injection since the acute heroin treatment protocol is not exposed to the potentially confounding contribution of strain differences in contextual learning which has clearly been shown to affect dependence liability (Azorlosa et al. 1994; Deffner-Rappold et al. 1996). Third and lastly, the obtained significant correlation implies that NPW jumping frequencies after a single heroin exposure very likely reflect the development of physical dependence and are not artefactual motor responses, as has been proposed (Ritzmann, 1981).

The data also reveal a strong genetic correlation between acute heroin and morphine dependence, and between chronic heroin and morphine dependence. Heroin is rapidly converted to 6-MAM and then morphine in vivo (Inturrisi et al. 1983). Thus, it is possible that the impressive strain rank order correlation coefficients for withdrawal jumping frequencies after heroin and morphine treatment actually resulted from morphine activity in each case. However, 6-MAM levels are maintained long enough to be present for both the chronic (1h) and the acute (2h) heroin-naloxone intervals utilized in this paradigm. Thus, even if morphine does play some role in the observed jumping frequencies it does not fully explain the almost perfect statistical correlation. Collectively, the present correlation data indicate that the genetic liability to heroin dependence is independent of the magnitude or duration of heroin exposure, and that the same treatment strategy could be equally effective throughout. Given the impressive genetic association between heroin and morphine dependence, such interventions might be effective in treating dependence induced by other opioids as well, particularly those with a similar pharmacological profile as heroin.

Dependence- resistant and sensitive strains. In the present study, 129P3 and SWR mice were identified as dependence- resistant and sensitive strains after acute and chronic heroin administration. This characterization is consistent with their relative ranking among 11 inbred strains in a survey of acute and chronic morphine dependence (Kest et al., 2002a). In that study, 129P3 responses were minimal (<7 jumps/15 min). And whereas their jumping frequencies were among the lowest of all other strains, those of SWR mice were at minimum 2- to 2.5-fold greater. Among inbred strains, the SWR

mouse has not been the particular focus of many studies. Consequently, there are few if any descriptions in this strain of physiological systems, particularly those with relevance to dependence, which might differ from other mouse strains and afford insight into their relatively increased sensitivity to heroin and morphine dependence. We do believe that some comment regarding the dependence resistance of 129P3 mice is possible by referring to neurobiological characterizations of 129S6 mice (previously referred to as 129/SvEv), another 129 substrain. Such a comparison may be of heuristic value since they are highly related (although not identical; Simpson et al. 1997) genetically. There is evidence that 129S6 mice are deficient in GM1 ganglioside-regulated excitatory opioid function (Crain and Shen, 2000). GM1 ganglioside has been shown to block the translocation and activation of protein kinase C from cytosol to neuronal membranes, a biochemical pathway critical in the development of opioid dependence (Crain and Shen, 2000; Mayer et al. 1995). In another study, 129S6 mice were shown to possess deficiencies in the NMDA excitatory amino acid receptor system, and/or the biochemical cascade activating nitric oxide synthase consequent to its activation (Kolesnikov et al. 1998). The importance of the NMDA/ nitric oxide signaling system in dependence to opioids such as morphine (Elliott et al. 1995; Herman et al. 1995) and heroin (Klein et al., 2007) has been clearly demonstrated as well. Further studies are needed to determine if the compromised GM1 ganglioside and NMDA/nitric oxide signaling in the 129S6 mouse contribute to the ability of 129P3 mice to resist heroin dependence.

Experiment 4: Antisense Mapping of the MOR-1 Receptor for Morphine and Heroin Dependence.

1) Introduction

Decades of research have focused on designing an opioid analgesic agent that has the analgesic efficacy of morphine but is devoid of morphine's adverse effects. However, the mechanisms underlying the development of dependence are still poorly understood, which has impeded progress in this area.

Morphine's actions are mediated in the CNS by the μ-opioid receptor which is encoded by the Oprm. It has been shown that the binding of morphine to the μ-receptor is the initial step in the development of tolerance to and physical dependence. Studies show that animals pretreated both spinally (DeLander et al. 1984) and supraspinally (Aceto et al. 1986), with β-FNA, a μ-selective irreversible antagonist, did not develop physical dependence to morphine.

It has long been thought that heroin, which is rapidly converted to 6-MAM and then morphine in vivo (Inturrisi et al. 1983), would logically have a pharmacological profile similar to that of morphine. The greater rewarding properties of heroin were attributed to its ability to cross the blood brain barrier faster and more effectively than morphine (Oldendorf et al. 1972). Although both morphine and heroin act at μ-opioid receptors, recent pharmacological data indicate that their activities are dissociable. Additionally, these drugs can differ in their side effects profiles, and demonstrate incomplete analgesic cross-tolerance to each other. Furthermore, although morphine is generally an ineffective analgesic in the μ-opioid receptor deficient CXBK mouse (Pick

et al. 1993), analgesic response to heroin and the morphine metabolite morphine-6-beta glucuronide (M6G) is not compromised (Rossi et al. 1996). These findings led researchers to consider the possibility that not all μ-opioids utilize identical μ-opioid receptor mechanisms (Pasternak, 2003). Subsequent studies utilizing antisense oligodeoxynucleotides targeting various exonic regions of the mu opioid receptor, suggested a mechanism for these differences. For example, the i.c.v. injection of AS ODNs targeting exons 1 and 4, but not exons 2 or 3, of Oprm attenuated supraspinal morphine analgesia (Rossi et al. 1995a) demonstrating the specific contribution of the MOR-1 splice variants to morphine analgesia. In contrast, analgesia produced by heroin and M6G were attenuated by injection of an antisense probe targeting exons 2 and 3, but not exons 1 and 4, of the Oprm gene (Rossi et al. 1995a).

Antisense mapping of the Oprm also revealed that the same splice variants do not mediate all of morphine's effects. Supraspinal morphine analgesia is blocked by antisense probes against exons 1 and 4, while inhibition of gastrointestinal transit and spinal morphine analgesia are blocked only by the probe against exon 4 and not against exon 1 (Rossi et al. 1995a). A recent study found that morphine did not retain its respiratory depressant effects in exon 2 knockout mice (Romberg et al. 2003), implicating the involvement of a MOR splice variant in this morphine effect as well. Collectively, these studies show that morphine exerts its effects via some, but not all, exons of the Oprm.

The contribution of MOR splice variants to morphine and heroin dependence has not been reported. Therefore, the present study administered AS ODNs against mRNAs coding for exons 1, 2, 3 & 4 of the μ-receptor to investigate the profile of the original

MOR-1 variant of the μ-receptor in the development of physical dependence following chronic heroin and morphine administration. To confirm the successful delivery and efficacy of our AS ODN preparation and delivery protocols, morphine analgesia was also assessed.

2) Materials and Methods

Subjects. See General Methods section.

Drugs. Heroin hydrochloride and naloxone hydrochloride were injected s.c.. Antisense oligonucleotide sequences targeting exons 1, 2, 3, and 4 were prepared and injected as previously described in the General Methods section. The AS ODN probes utilized in this study are identical to those used by Rossi et al. (1997), and have thus been shown to be effective in causing specific reductions in morphine analgesia. Mismatch AS ODNs were also used as a control group as described in the General Methods section.

Treatment Protocol. Separate groups of mice were used to assess AS ODN effects on analgesia and dependence. This was done due to the fact that analgesia testing requires i.c.v. injection (see General Methods) of morphine or heroin during the antisense injection protocol. To do so in mice subsequently being made dependent by morphine pellets or heroin-filled pumps would introduce some possibly confounding variables. As summarized in Table 8, mice in both the analgesia and withdrawal protocols were administered an i.c.v. injection of AS ODN (5 μg) or an equal volume (5 μl) of saline on Days 1, 3, 5, and 7.

Morphine/Heroin Analgesia. To study the effects of MOR AS ODN probes on morphine analgesia, mice were assayed for nociception on Days 6 and 12 using the 50°C tail-withdrawal test (see General Methods section). Following assessment of baseline

Table 7. Antisense injection schedule for morphine and heroin dependence and analgesia paradigms

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
Analgesia	AS	X	AS	X	AS	*Test*	AS	X	X	X	X	*Test*
Withdrawal	AS	X	AS	X	AS	*	AS	X	X	X	X	*Test*
AS = Antisense oligonucleotides administered * = Morphine pellets/Heroin pumps implanted X = No treatment												

nociceptive sensitivity, mice were anesthetized and injected i.c.v. with morphine or heroin (1 μg/5 μl). Withdrawal latencies were reassessed 30 min later.

Morphine/Heroin Dependence. To study dependence, mice were subcutaneously implanted with a 25 mg morphine pellet, or a subcutaneously implanted with osmotic mini-pumps (see General Methods for details) filled with heroin sulfate on Day 6, corresponding with functional exon down regulation as indicated by our analgesia study (see results section). On Day 12, corresponding to the recovery of analgesia and apparent functional exon recovery, subjects received a single injection of naloxone (50 mg/kg, s.c.) and the magnitude of withdrawal from morphine was assessed.

E) Naloxone Precipitated Withdrawal. Morphine and heroin withdrawal was assessed for 15-minutes as previously described in the General Methods sections.

F) Data Analysis. Withdrawal latencies were analyzed using a two-way (AS ODN X day) ANOVA and frequency of withdrawal jumping was compared using one-way ANOVA, both followed post hoc with Neuman-Keuls test. For all comparisons, $p < 0.05$ was considered significant (see General Methods section).

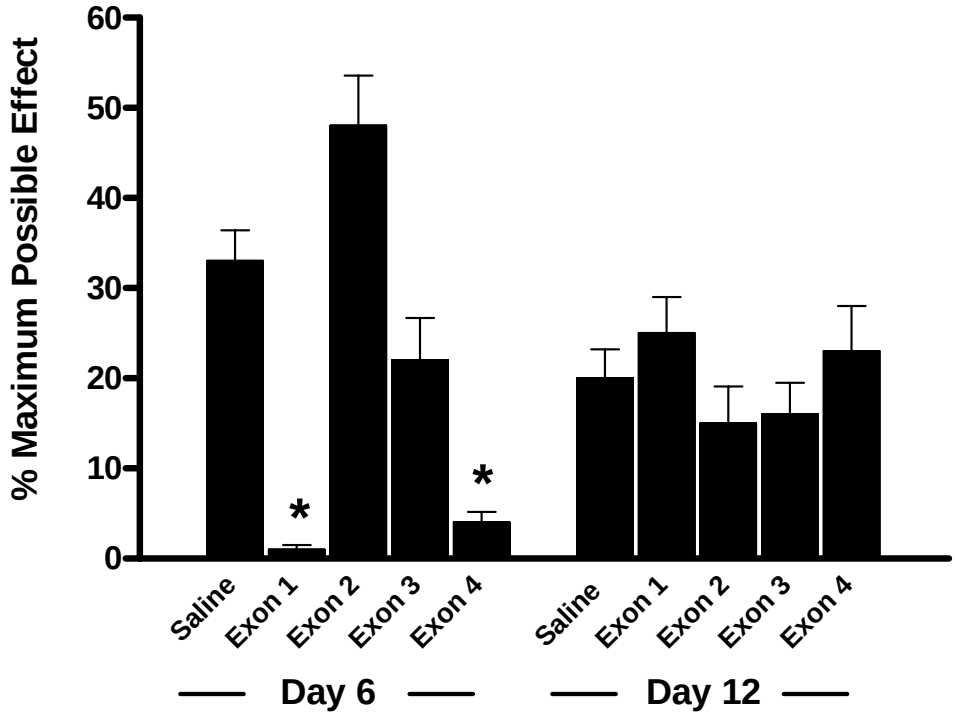
3. Results

Morphine Analgesia

Treatments with AS ODNs targeting exons 1 and 4, but not exons 2 or 3, of MOR significantly attenuate supraspinal morphine analgesia (Figure 7A). Morphine analgesia was significantly attenuated by exons 1 & 4 on Day 6 after antisense treatment, but not by exons 2 & 3 (Figure 7A). Morphine analgesia was evident in all four groups of mice by Day 12, indicating that the efficacy of exons 1 and 4 were restored five days after the final AS ODN injection on Day 7. Significant differences between baseline tail

withdrawal latency and latency following morphine administration indicates intact morphine analgesia in mice treated with AS against all four of the exons.

Figure 7A. Supraspinal morphine analgesia on Day 6 and Day 12 in mice treated with AS ODNS targeting Oprm exons 1, 2, 3 & 4. Control mice received saline instead of AS ODN treatment according to identical protocols used for experimental (AS ODN) groups (see text for treatment methods). Significant differences from saline control group is indicated (* $p < 0.05$).


% Maximum Possible Effect
60
50
40
30
20
10
0
*
*
Saline
Exon 1
Exon 2
Exon 3
Exon 4
Saline
Exon 1
Exon 2
Exon 3
Exon 4
Day 6
Day 12

Morphine Dependence

AS ODN targeting exon 1, but not those targeting exons 2, 3, and 4, significantly attenuated NPW jumping frequency on Day 12 (Figure 7B). Frequencies in mice treated with ODNs targeting exons 2, 3, and 4 did not differ from each other or from the two controls (saline injection, mismatch AS ODNs injection) groups**.** Data shows an approximate 50% reduction of withdrawal jumping in mice treated with AS ODN probes against exon 1. While we cannot directly correlate behavior change to levels of receptor mRNA reduction, previous studies have noted that AS ODN probes typically reduce receptor levels by 40 - 50% (Silva et al. 2003).

Heroin Analgesia

Treatments with AS ODNs targeting exons 2 and 3, but not exons 1 or 4, of MOR significantly attenuate supraspinal morphine analgesia (Figure 8A). Heroin analgesia was significantly attenuated by exons 2 & 3 on Day 6 after antisense treatment, but not by exons 1 & 4. Heroin analgesia was evident in all four groups of mice by Day 12, indicating that the efficacy of exons 2 and 3 were restored five days after the final AS ODN injection on Day 7 (Figure 8A). Significant differences between baseline tail withdrawal latency and latency following morphine administration indicates intact morphine analgesia in mice treated with AS ODNs against all four of the exons.

Heroin Dependence

AS ODN targeting exon 1, but not those targeting exons 2, 3, and 4, significantly attenuated NPW jumping frequency on Day 12 relative to saline injected, or AS ODN mismatch injected (control) mice (Figure 8B). Frequencies in mice treated with ODNs

Figure 7B. Naloxone-precipitated withdrawal in morphine dependent mice treated with AS ODNs targeting Oprm exons 1, 2, 3, & 4. Two separate control groups received either saline injection or mismatch AS ODN (see General Methods section) treatment according to identical protocols used for experimental (AS ODNs against exon 1, 2, 3, & 4) groups (see text for treatment methods). Significant differences from both saline and mismatch control groups are indicated (* $p < 0.05$).

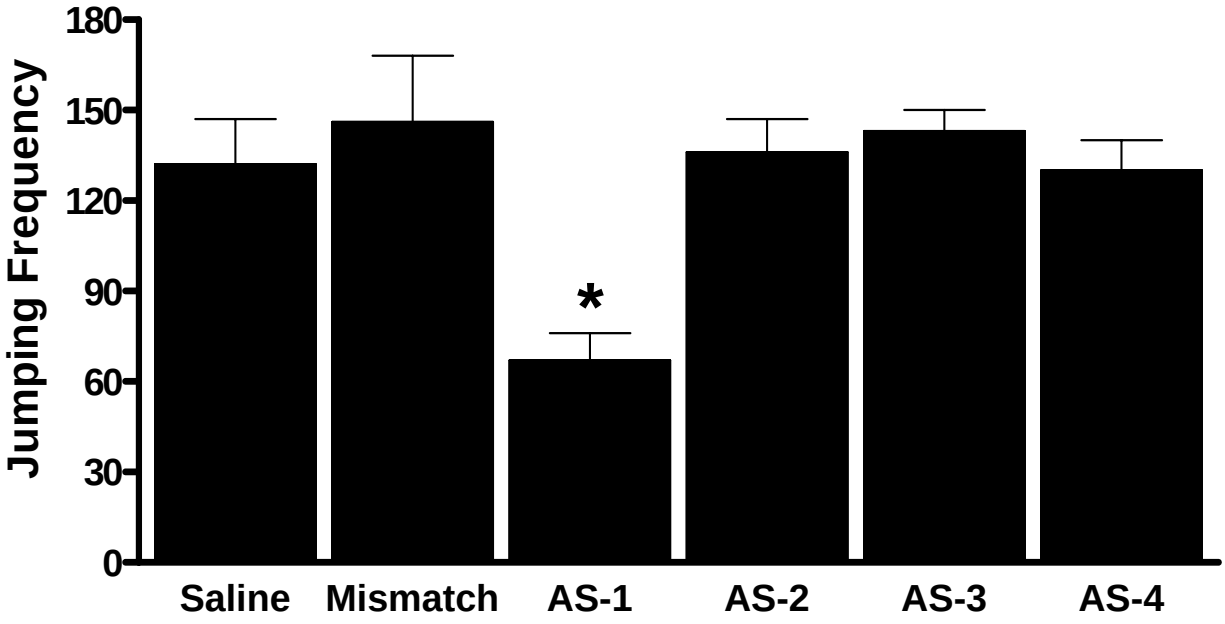

Jumping Frequency
180
150
120
90
60
30
0
*
Saline
Mismatch
AS-1
AS-2
AS-3
AS-4

Figure 8A. Supraspinal heroin analgesia in mice treated with AS ODNs targeting Oprm exons 1, 2, 3 & 4 on Day 6 and Day 12. Control mice received saline instead of AS ODN treatment according to identical protocols used for experimental (AS ODN) groups (see text for treatment methods). Significant differences from saline group are indicated (* $p < 0.05$).

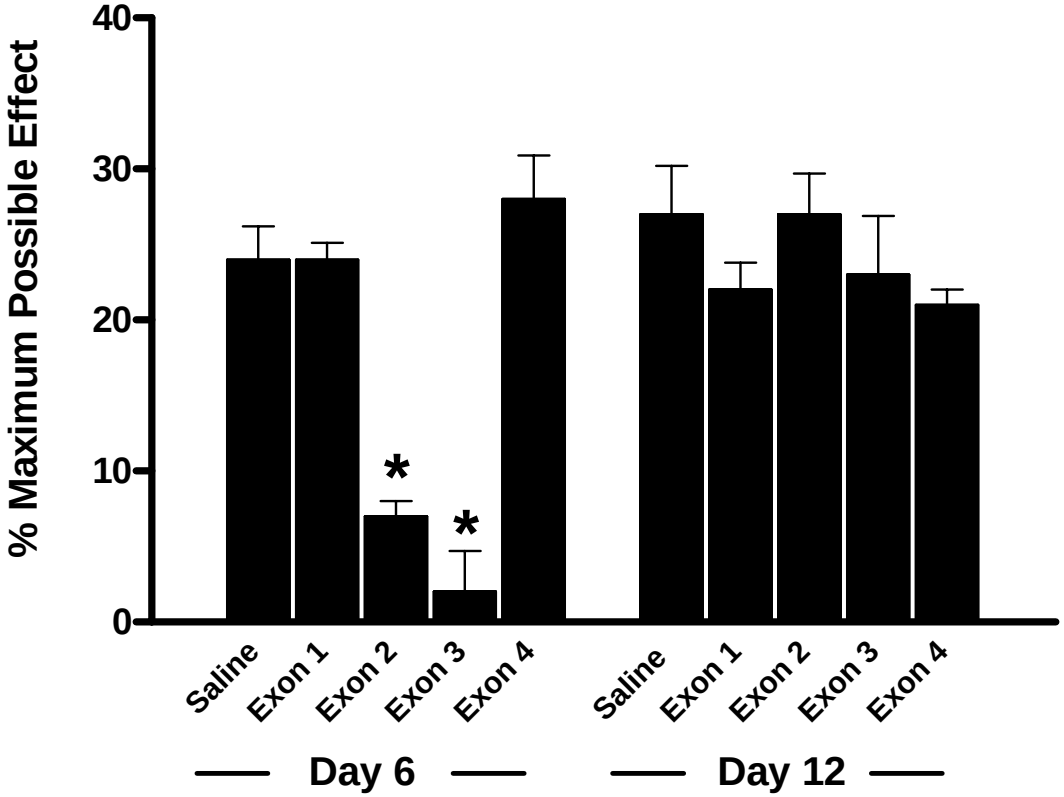
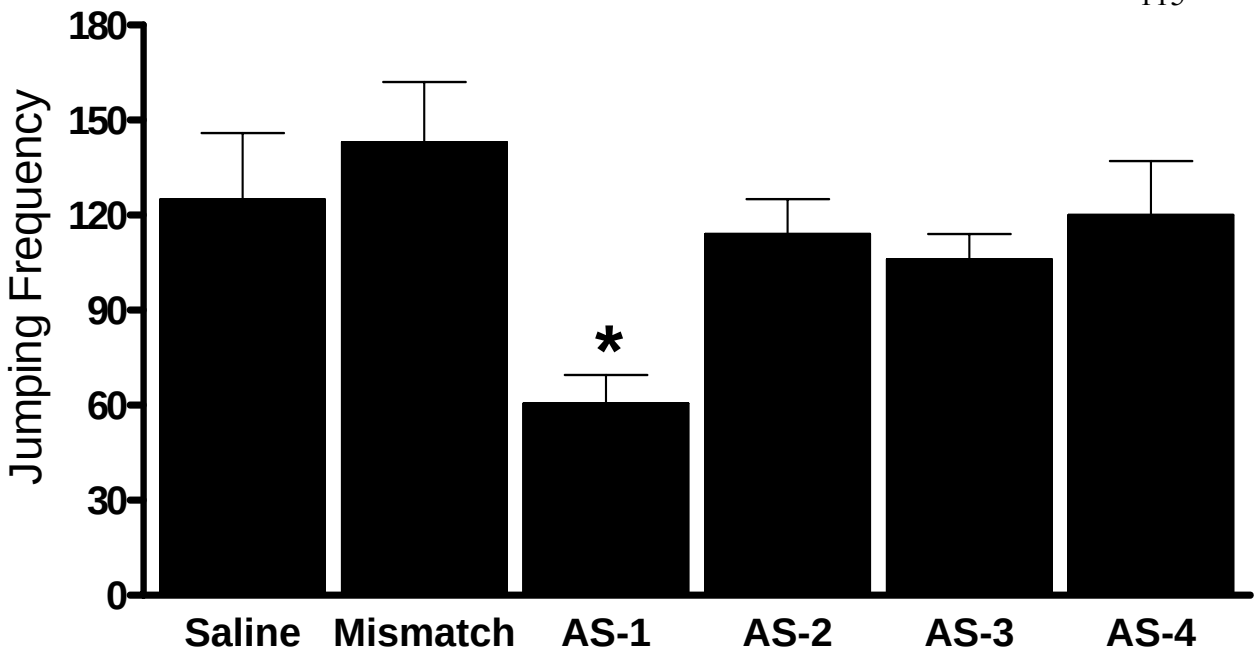

% Maximum Possible Effect
40
30
20
10
0
*
*
Saline
Exon 1
Exon 2
Exon 3
Exon 4
Saline
Exon 1
Exon 2
Exon 3
Exon 4
Day 6
Day 12

Figure 8B. Naloxone-precipitated withdrawal in heroin dependent mice treated with AS ODNs targeting Oprm exons 1, 2, 3, & 4. Two separate control groups received either saline injection or mismatch AS ODN (see General Methods section) treatment according to identical protocols used for experimental (AS ODNs against exon 1, 2, 3, & 4) groups (see text for treatment methods). Significant differences from both saline and mismatch control groups are indicated (* $p < 0.05$).


Jumping Frequency
180
150
120
90
60
30
0
*
Saline
Mismatch
AS-1
AS-2
AS-3
AS-4

targeting exons 2, 3, and 4 did not differ from each other or from the saline treated group**.** Data shows an approximate 50% reduction of withdrawal jumping in mice treated with AS ODN probes against exon 1. While we cannot directly correlate behavior change to levels of receptor mRNA reduction, previous studies have noted that AS ODN probes typically reduce receptor levels by 40 - 50% (Silva et al. 2003).

4. Discussion

Morphine dependence and analgesia. The present study used AS ODN probes to map the four exons (exons 1, 2, 3 & 4) that compose the original μ-opioid receptor, MOR-1, to investigate its role in morphine dependence. AS ODN directed against exon 1, but not 2, 3 or 4, of the Oprm gene significantly attenuated the development of physical dependence to morphine. These results confirm earlier findings that exon 1 is involved in morphine dependence (Sanchez-Blazquez et al.,1997), but are the first to rule out the involvement of the other MOR-1 exons. These results are similar to findings implicating exon 1 in morphine analgesia (Rossi et al. 1995a) and implicate a specific role for receptor isoforms containing exon 1 in morphine dependence as well as analgesia. The blockade of morphine analgesia on Day 6 and its subsequent restoration on Day 12 in mice treated with AS ODNs against exons 1 and 4 is consistent with previous findings (Rossi et al., 1996), and suggests that both naloxone and morphine had access to splice variants containing these exons during withdrawal testing. Therefore, the attenuation of NPW seen on Day 12 in mice treated with exon 1 AS ODNs most likely does not arise from a downregulation of the various exons at the time of NPW, but rather from decreased development of dependence during the dependence induction period. While it has long been postulated that opioid agonist binding to the μ-receptor is the

initial step in the development of morphine dependence and subsequent withdrawal (Matthes et al., 1996; Sora et al., 2001), these findings provide specific evidence that splice variants containing exon 1 are critical for this process.

The data also demonstrate that there is some overlap in the Oprm exons involved in mediating morphine analgesia and dependence (exon 1). However, whereas morphine dependence is not mediated by exon 4, analgesia is (Rossi et al., 1995; Klein et al., unpublished data). The present data thus support at a molecular level the previous demonstrations of a dissociation between morphine analgesia and dependence (Ling et al. 1984; Kaneto et al. 1985; Kest et al. 1998).

While numerous studies have demonstrated that alternate splice variants of the μ-receptor may mediate the pharmacologic actions of different μ drugs (Rossi et al., 1995, 1996, 1997; Schuller et al., 1999; Hadjimarkou et al., 2003), the ability of exon 4 to impact morphine analgesia, but not dependence also implies that splice variants may mediate different pharmacologic actions of the same μ-opioid. This fact is critical in the attempt to discover a single μ drug with strong analgesic properties and a low dependence liability. Unfortunately, while blockade of exon-1 attenuated the development of dependence it does not appear to be a viable target for the development of morphine-like analgesics with limited dependence liability. This is due to the fact that while our findings indicate that AS ODNs against exon-1 attenuates morphine dependence, they also confirm previous data indicating that AS ODNs against exon-1 attenuates morphine analgesia (Rossi et al. 1995b).

Heroin dependence and analgesia. Our data show that AS ODNs directed against exon 1, but not 2, 3 or 4, of the Oprm gene significantly attenuated the

development of physical dependence to heroin. These results imply that similar variants of the μ-receptor are involved in both morphine and heroin dependence. This is consistent with our previously presented data revealing a strong genetic correlation between heroin and morphine dependence, and imply that similar substrates are involved at the molecular level as well. As was mentioned (see Chapter 5), the current findings utilizing heroin could result from morphine activity as there is ample time over the course of several treatment days for the conversion of heroin to morphine and an increase in central morphine levels. However this possibility must be tempered with the growing data that even chronic heroin effects are not mediated solely by morphine (Rossi et al. 1996; Gilbert et al., 2004, Bao et al., 2007; Klein et al., 2007). The blockade of heroin analgesia on Day 6 and its subsequent restoration on Day 12 suggests that both naloxone and heroin had access to all splice variants during withdrawal testing. Thus, we suggest that AS ODNs against exon 1 reduced the number of exon 1 containing splice variants available to heroin, thus impacting the first step leading to the development of dependence.

It has been proposed that the analgesic properties of μ-opioids such as M6G, heroin and 6-MAM, a primary heroin metabolite, are mediated through different splice variants of the Oprm gene than are morphine's. Probes against exons 1 and 4 are effective against morphine analgesia, while AS ODN probes against exons 2 and 3 are effective against M6G (Rossi et al., 1997). Previous studies indicate that heroin and 6-MAM analgesia is impacted by all four exons of MOR-1, but to a significantly lesser extent by AS ODNs against exon 1 (Rossi et al, 1996), while our data indicate that exon 1 does not significantly impact heroin analgesia. It is possible that this discrepancy may be

due to methodological differences (radiant heat vs. hot water immersion) in the tail flick test. Another study using exon 1 knockout mice found that morphine analgesia is almost fully abolished in the homozygous knockouts while the analgesic response to heroin is retained in these mice, albeit at a slightly lowered potency (Schuller et al. 1999). This data implies that heroin analgesia and dependence may be mediated by separate splice variants of the μ-receptor. The clinical implications of these findings are that heroin-like agonists acting at specific splice variants of the μ-receptor may be able to elicit the desired (antinociceptive) effects of opiate treatment without the undesired (dependence and withdrawal) side effects. If this is indeed that case it bodes well for the development of heroin-like analgesics with limited dependence liability.

CHAPTER 4.

General Discussion

The overall aim of the present dissertation was to characterize at a behavioral level the phenomenon of heroin physical dependence in mice. A variety of approaches were utilized. First, studies were performed to validate NPW jumping as a measure of heroin withdrawal. Second, the contribution of opioid (δ, κ) and non-opioid (NMDA, AMPA) receptors in the development of heroin dependence was assayed using selective antagonists for each. A third study utilized isogenic inbred strains in order to investigate the contribution of genetic background to the variation in heroin NPW jumping frequencies. Finally, Oprm antisense probes were used in order to evaluate the possible impact of various MOR-1 splice variants on heroin dependence. Heroin has long been assumed to have a pharmacological profile virtually identical to that of the prototypical μ-opioid morphine because heroin is rapidly transformed into morphine in vivo (Oldendorf, 1972; Inturrisi et al., 1983). However, recent data indicates important differences in their mechanism of action (Rossi et al. 1996; Gilbert et al., 2004, Bao et al., 2007; Klein et al., 2007). Thus, the current research therefore sought to examine the assumptions about the similarity between heroin and morphine. As a result, the findings in the present studies were compared to data obtained in morphine-dependent rodent subjects (particularly mice).

The heroin/naloxone time-response data presented in Experiment 1 indicate that maximal NPW responding is obtained after acute and chronic heroin treatment using a heroin-naloxone interval of 2 and 1 h, respectively. This is a shorter interval that what has been demonstrated for acute (3h) and chronic (3h) morphine treatment (El-Kadi and

Sharif, 1994; Wiley & Downs, 1979). Heroin is rapidly converted to 6-MAM and then morphine in vivo (Inturrisi et al. 1983). Thus, it is possible that the jumping frequencies after heroin treatment actually resulted from morphine's actions and the shorter maximal heroin/naloxone latency arises solely due to its ability to more rapidly cross the blood-brain barrier. A role for morphine in chronic heroin dependence is an intriguing possibility since withdrawal is not precipitated soon after heroin administration and there is ample time over the several treatment days for the conversion of heroin to morphine and an increase in central morphine levels. This supposition must however be tempered with the caveat that, despite the conversion of heroin to morphine, the effects of chronic heroin treatment are not simply mediated by morphine. For example, chronic administration of these two opioids cause different neurochemical changes in brain μ-opiate receptor function (Bolger et al., 1988) and dependence that is differentially regulated by δ-opiate receptors (Suzuki et al., 1997b). There is also virtually no cross tolerance between morphine and either heroin or 6-MAM (Rossi et al., 1996). The possibility that morphine alone mediates dependence after either acute or chronic systemic heroin injection is also tenuous since the heroin-naloxone intervals utilized (1 h & 2 h) may not be of sufficient duration for morphine levels to appreciably accumulate. Specifically, 6-MAM levels are significantly increased while morphine levels are still relatively low during the first hour after heroin injection (Way et al., 1960; Way et al., 1965). By the time morphine levels are increased relative to 6-MAM 2h after heroin injection, mice are already undergoing NPW. It is not known how, or even whether, 6-MAM can induce dependence, but it is interesting to note that the pharmacological and exonic profile of 6-MAM in the production of analgesia is distinct from that of morphine

but identical to that of heroin (Rady et al., 1994; 1991, 2000; Brown et al., 1997; Schuller et al., 1999; Rossi et al., 1996). Further work is needed to address the possible contributions of 6-MAM and morphine to heroin dependence following acute and chronic heroin treatment. However, it is not unlikely that many of heroin's differences from morphine are due to its rapid metabolism to 6-MAM.

Future studies expanding on the data collected here could increase our understanding of the specific mechanisms involved in heroin dependence. For example, current and past (Suzuki et al., 1997) research shows that δ receptor antagonism is capable of attenuating the μ-opioid dependence induced by heroin and morphine, respectively, indicating a relationship between μ and δ receptor-mediated opioid systems. While there is evidence that μ and δ receptors interact physically by forming heterodimers or indirectly through common intracellular pathways (Gomes et al., 2000; Smith & Lee, 2003), the exact mechanism and nature of this interaction is not yet fully elucidated. Research has indicated that the two receptors act synergistically with regards to both DAMGO (Gomes et al., 2000; He & Lee, 1998) and heroin (Stevenson et al., 2005) analgesia. That is, small sub-clinical doses of δ-agonists potentiate μ-agonist analgesia beyond what would be expected based on the doses of each of the drugs used. Interestingly, research has indicated that this synergistic relationship does not extend to the reinforcing effects of heroin (Stevenson et al., 2005) or to tolerance (He & Lee, 1998). It would be interesting to determine whether δ-agonists are capable of potentiating heroin dependence, or if the synergistic relationship between the two systems is confined to analgesia.

The present strain survey (Experiment 3) data also identify two inbred strains (129P3 and SWR) with highly divergent NPW jumping frequencies during heroin withdrawal. A full genome scan of F2 mice derived from these strains can be used to identify QTLs, or chromosomal regions (and ultimately candidate genes) associated with heroin withdrawal severity. This approach has been successfully used to identify QTLs on Chromosomes 1, 5, and 10 that account for 43% of the total variance in NPW jumping frequencies in morphine dependent F_2 hybrid mice derived from C57BL/6 and 129P3 progenitors (Kest et al., 2004). As NPW jumping has been shown here to be a valid and sensitive measure of heroin dependence, the present identification of highly divergent strains such as 129P3 and SWR should facilitate success in identifying such QTLs. Once candidate genes are identified a variety of pharmacologic and behavioral studies can be utilized to identify particularly salient candidate genes that underlie the divergent behavioral responses to heroin dependence. The goal is that this will ultimately lead to better a better understanding of, and improved interventions for, heroin withdrawal.

With a few exceptions the currently identified μ-receptor splice variants all contain exon 1. Although this limits our ability to make a solid determination as to which splice variants may be playing a role in heroin dependence and analgesia, it does not mitigate the importance of these findings. It is possible that while heroin dependence is mediated primarily through one of the many splice variants that contain exon 1 and is therefore similar to morphine in this regard, heroin analgesia is mediated primarily by one of the few splice variants that do not contain exon 1. This could explain the relative retention of heroin analgesia and the lack of NPW following "knockdown" or "knockout" of this particular exon (Schuller et al., 1999). Interestingly, there is evidence of a

truncated, but functional human μ-receptor splice variant termed μ_3 that contains only exons 2, 3, & 4 (Cadet et al., 2003). This receptor binds to opioid alkaloids such as morphine and heroin and is currently the only identified human splice variant that does not contain exon 1 (Cadet et al., 2003). Although this variant does not appear to be present in brain tissue (Cadet, 2004), it provides evidence that μ-agonist binding to truncated receptors lacking exon 1 can result in functional effects and that targeting this exon is a viable option when researching future opioid analgesics.

The data reported here also shows the selective contribution of murine MOR exons in heroin dependence. However, only exons 1-4 were subject to study. At most recent count, there are 18 identified exons of this gene which can generate 25 recognized mu opioid receptor splice variants. Previous studies have noted that antisense "knockdown" of exons other than 1, 2, 3, & 4, as well as μ splice variants under the control of separate promoter regions can also differentially impact μ-agonists mediated effects such as analgesia and respiratory depression (Pan et al., 2000; Pan et al., unpublished data). Thus, future studies may wish to examine the impact of these other exons on heroin dependence through the use of AS ODN or KO techniques.

Although the findings in the current dissertation indicate that AS ODNs targeting exon 1 do not attenuate heroin analgesia, prior research has found that heroin analgesia is impacted by AS ODNs targeting exon 1 (Rossi et al., 1996; Schuller et al., 1999). It is important to note however that the magnitude of reduction in analgesia after exon 1 is weak, and markedly smaller than when exons 2 or 3 are targeted (Rossi et al., 1996; Schuller et al., 1999). One possible explanation for this discrepancy may be due to methodological differences. Although all studies used the tail flick test to measure

analgesia, the study of the present dissertation utilized hot water immersion while the prior studies utilized radiant heat. The radiant heat tail flick test directs the painful stimulus only at a small portion of the animal's tail and it has been demonstrated that the specific location of the tail that is stimulated can affect latency to withdrawal (Mogil et al., 2001). The hot water immersion task however, immerses nearly the entire tail in the painful stimulus. Thus, different nociceptive processes may be measured in the two different tasks. An additional difference of possible relevance is the method by which analgesia was determined. In the aforementioned studies (Rossi et al., 1996; Schuller et al., 1999), analgesia was defined as a doubling of baseline latencies and reported as the percentage of mice displaying heroin analgesia after exon 1 AS or control treatment. The current dissertation assessed analgesia as % MPE. This method sets the difference between the cutoff time of immersion in the hot water (20 seconds) and the animal's baseline latency as 100% effectiveness. The %MPE of heroin analgesia is then determined by assessing the post-treatment latencies in comparison to this value. Comparing the average %MPE values for each treatment group against a control group allowed us to determine whether the effectiveness of heroin analgesia in each treatment group changed following administration of the various AS ODNs. Thus, the current study was able to evaluate the extent to which AS ODNs against exon 1 attenuated heroin analgesia. While the prior studies identified the number of mice whose tail withdrawal latencies did or did not double following AS ODN treatment, the analysis does not permit a statement regarding the magnitude of analgesia and thus the presented data are not measuring the exact same phenomenon. These two factors might contribute to the discrepant results.

Although heroin is not approved for clinical use in the United States (US), other countries such as the United Kingdom (UK) do utilize heroin as a clinical analgesic (Wrench et al., 2007). The majority of studies assessing its clinical utility as an analgesic have found that heroin is at least as effective as morphine (Kendall & Latter, 2003). The current research indicates that AS ODNs targeting exon 1 attenuate heroin dependence in mice, while having no affect on heroin's analgesic potency. Even in the studies indicating that AS ODNs targeting exon 1 do impact heroin analgesia, the researchers found that its analgesic potency is only slightly decreased (Rossi et al., 1996; Schuller et al., 1999). Therefore, administering heroin in conjunction with AS ODNs targeting exon 1 may result in a powerful opioid analgesic with a significantly lowered dependence liability. Indeed drugs based on antisense technology have already been approved (The Vitravene Study Group, 2002). Practically, however, it is unlikely that that US will change its stance on clinical heroin use in the near future. This is because heroin has never been proven to be more effective that many of the other clinically used opiate drugs, including morphine (Drug Enforcement Administration, Drugs of Abuse, 2005) and heroin is also a highly addictive substance that would require significant resources to adequately control.

2. Clinical Implications

As illicit heroin use has increased in recent years it is important to gain a comprehensive understanding of the parameters that underlie and influence heroin dependence and withdrawal. This research has clinical implications and utility as it allows for further research into both the treatment of heroin abuse and the search for potent opioid analgesics with limited dependence liability. Research has indicated that the

desire to alleviate, or to avoid altogether, the physically aversive state of heroin withdrawal can trigger continued abuse of μ-opioids and/or relapse after cessation of drug use (Koob & Le Moal, 2001; Kenny et al., 2006). The current data indicate that heroin dependence and withdrawal may be attenuated by various methods. The ability of δ receptors to impact heroin dependence is important in light of recent research into ligands that possess mixed μ-agonist/δ-antagonist profiles, which have been shown to produce analgesia with attenuated tolerance (Schiller et al., 1999) and evidence that the δ and μ receptors interact in vivo (Daniels et al., 2005). The ability of δ_1 and δ_2 receptors to impact heroin dependence, as opposed to just δ_2 involvement as seen by morphine, further expands the possibility of utilizing these systems in the treatment of heroin abuse and relapse. Clinically relevant EAA receptor antagonists (e.g. memantine, NBQX) may also be useful for the treatment of heroin dependence and withdrawal, as other drugs of these classes show a clear ability to attenuate the development of heroin dependence in the current studies.

The dissociation between MOR exons underlying heroin dependence and heroin analgesia is noteworthy. It indicates that a single Oprm gene can generate splice variants of a receptor that differentially mediate the behavioral effects of a single μ agonist. Non-μ opioid drugs or mixed μ/non-μ agonists are able to provide analgesia with a somewhat lowered dependence liability. However, as μ agonists are the most potent analgesics used clinically, the ability to manufacture an agonist that retains its potent analgesia with lowered side effects of drug dependence is quite significant.

Specific clinical utility aside, as far as dependence liability and withdrawal is concerned heroin is remarkably similar to morphine. However, the current data indicate

that the contribution of various opioid and EAA receptors to heroin dependence is not identical to morphine dependence. Additionally, heroin presents with a different AS ODN profile than morphine. These differences makes it more likely that heroin is not solely a lipophilic pro-drug for morphine with no effects of its own. Research into the treatment of heroin abuse and clinically relevant new opioid analgesics should regard heroin as an opioid drug distinct from morphine and possibly capable of activating unique receptor variants and downstream signal transduction mechanisms.

3. Conclusion

Overall, the current studies better characterize the phenomenon of heroin dependence and demonstrate that NPW jumping is a valid measurement of heroin dependence in mice. Further, they reveal that the processes underlying morphine and heroin dependence are similar at the pharmacologic, receptor, and genetic levels. However the current data also adds to accumulating evidence that the functional consequences of acute and chronic administration of heroin on classical opioid effects can be differentiated from those seen with morphine. Though more studies need to be completed to expand on the current data and to explore the mechanistic basis for the observed effects, these studies provide a solid base on which future research can be built.

References

Abbadie C, Pasternak GW. Differential in vivo internalization of MOR-1 and MOR-1C by morphine. Neuroreport 2001;12(14):3069-3072.

Abdelhamid EE, Sultana M, Portoghese PS, Takemori AE. Selective blockage of delta opioid receptors prevents the development of morphine tolerance and dependence in mice. J Pharmacol Exp Ther 1991;258(1):299-303.

Aceto MD, Dewey WL, Portoghese PS, Takemori AE. Effects of beta-funaltrexamine (beta-FNA) on morphine dependence in rats and monkeys. Eur J Pharmacol 1986;123(3):387-393.

Aghajanian GK, Kogan JH, Moghaddam B. Opiate withdrawal increases glutamate and aspartate efflux in the locus coeruleus: an in vivo microdialysis study. Brain Res 1994;636(1):126-130.

Alger BE, Nicoll RA. Spontaneous inhibitory post-synaptic potentials in hippocampus: mechanism for tonic inhibition. Brain Res 1980;200(1):195-200.

Ammon-Treiber S, Hollt V. Morphine-induced changes of gene expression in the brain. Addict Biol 2005;10(1):81-89.

Arvidsson U, Dado RJ, Riedl M, Lee JH, Law PY, Loh HH, Elde R, Wessendorf MW. delta-Opioid receptor immunoreactivity: distribution in brainstem and spinal cord, and relationship to biogenic amines and enkephalin. J Neurosci 1995;15(2):1215-1235.

Arvidsson U, Riedl M, Chakrabarti S, Lee JH, Nakano AH, Dado RJ, Loh HH, Law PY, Wessendorf MW, Elde R. Distribution and targeting of a mu-opioid receptor (MOR1) in brain and spinal cord. J Neurosci 1995;15(5 Pt 1):3328-3341.

Bailey CP, Connor M. Opioids: cellular mechanisms of tolerance and physical dependence. Curr Opin Pharmacol 2005;5(1):60-68.

Bare LA, Mansson E, Yang D. Expression of two variants of the human mu opioid receptor mRNA in SK-N-SH cells and human brain. FEBS Lett 1994;354(2):213-216.

Belknap JK. Components of the opioid withdrawal syndrome in mice are thermoregulatory responses. Pharmacol Biochem Behav 1989;34(2):241-245.

Bell J, Adler MW. Comparison of peripheral and central administration of naloxone in precipitating abstinence in morphine-dependent rats. Drug Alcohol Depend 1988;21(3):189-194.

Berrendero F, Kieffer BL, Maldonado R. Attenuation of nicotine-induced antinociception, rewarding effects, and dependence in mu-opioid receptor knock-out mice. J Neurosci. 2002 Dec 15;22(24):10935-40.

Bisaga A, Comer SD, Ward AS, Popik P, Kleber HD, Fischman MW. The NMDA antagonist memantine attenuates the expression of opioid physical dependence in humans. Psychopharmacology (Berl) 2001;157(1):1-10.

Blasig J, Herz A, Reinhold K, Zieglgansberger S. Development of physical dependence on morphine in respect to time and dosage and quantification of the precipitated withdrawal syndrome in rats. Psychopharmacologia 1973;33(1):19-38.

Bonci A, Williams JT. Increased probability of GABA release during withdrawal from morphine. J Neurosci 1997;17(2):796-803.

Bozarth MA, Wise RA. Anatomically distinct opiate receptor fields mediate reward and physical dependence. Science 1984;224(4648):516-517.

Brase DA, Loh HH, Way EL. Comparison of the effects of morphine on locomotor activity, analgesia and primary and protracted physical dependence in six mouse strains.J Pharmacol Exp Ther. 1977 May;201(2):368-74.

Brown GP, Yang K, King MA, Rossi GC, Leventhal L, Chang A, Pasternak GW. 3-Methoxynaltrexone, a selective heroin/morphine-6beta-glucuronide antagonist. FEBS Lett. 1997 Jul 21;412(1):35-8.

Brown, G.P., Yang, K., King, M.A., Rossi, G.C., Leventhal, L., Chang, A., Pasternak, G.P., 1997. 3-Methoxynaltrexone, a selective heroin/morphine-6β-glucuronide antagonist. FEBS Lett. 412, 35-38.

Cadet P, Mantione KJ, Stefano GB. Molecular identification and functional expression of mu 3, a novel alternatively spliced variant of the human mu opiate receptor gene. J Immunol. 2003 May 15;170(10):5118-23.

Cadet P. Mu opiate receptor subtypes. Med Sci Monit. 2004 Jun;10(6):MS28-32.

Cappendijk SL, de VR, Dzoljic MR. Excitatory amino acid receptor antagonists and naloxone-precipitated withdrawal syndrome in morphine-dependent mice. Eur Neuropsychopharmacol 1993;3(2):111-116.

Chen Y, Mestek A, Liu J, Hurley JA, Yu L. Molecular cloning and functional expression of a mu-opioid receptor from rat brain. Mol Pharmacol. 1993;44(1):8-12.

Choi HS, Kim CS, Hwang CK, Song KY, Wang W, Qiu Y, Law PY, Wei LN, Loh HH. The opioid ligand binding of human l-opioid receptor is modulated by novel splice variants of the receptor. Biochem Biophys Res Commun. 2006;343:1132–1140.

Connor M, Kitchen I. Has the sun set on kappa3-opioid receptors? Br J Pharmacol 2006;147(4):349-350.

Contarino A, Picetti R, Matthes HW, Koob GF, Kieffer BL, Gold LH. Lack of reward and locomotor stimulation induced by heroin in mu-opioid receptor-deficient mice. Eur J Pharmacol. 2002 Jun 20;446(1-3):103-9.

Couceyro P, Douglass J. Precipitated morphine withdrawal stimulates multiple activator protein-1 signaling pathways in rat brain. Mol Pharmacol 1995;47(1):29-39.

Cowan A, Zhu XZ, Mosberg HI, Omnaas JR, Porreca F. Direct dependence studies in rats with agents selective for different types of opioid receptor. J Pharmacol Exp Ther. 1988 Sep;246(3):950-5.

Craft RM, Stratmann JA, Bartok RE, Walpole TI, King SJ. Sex differences in development of morphine tolerance and dependence in the rat. Psychopharmacology (Berl) 1999;143(1):1-7.

Crain, S.M., Shen, K., 2000. Enhanced analgesic potency and reduced tolerance of morphine in 129/SvEv mice: evidence for a deficiency in GM1 ganglioside-regulated excitatory opioid receptor functions. Brain Res. 856, 227-35.

Cui CL, Wu LZ, Han JS. Spinal kappa-opioid system plays an important role in suppressing morphine withdrawal syndrome in the rat. Neurosci Lett 2000;295(1-2):45-48.

Dahan A, van Dorp E, Smith T, Yassen A. Morphine-6-glucuronide (M6G) for postoperative pain relief. Eur J Pain. 2007 Sep 12;

Daniels DJ, Lenard NR, Etienne CL, Law PY, Roerig SC, Portoghese PS. Opioid-induced tolerance and dependence in mice is modulated by the distance between pharmacophores in a bivalent ligand series. Proc Natl Acad Sci U S A 2005;102(52):19208-19213.

Deffner-Rappold, C., Azorlosa, J. L., Baker, J. D., 1996. Acquisition and extinction of context-specific withdrawal. Psychobiology 24, 219-226.

DeLander GE, Portoghese PS, Takemori AE. Role of spinal mu opioid receptors in the development of morphine tolerance and dependence. J Pharmacol Exp Ther 1984;231(1):91-96.

Dingledine R, Iversen LL, Breuker E. Naloxone as a GABA antagonist: evidence from iontophoretic, receptor binding and convulsant studies. Eur J Pharmacol. 1978 Jan 1;47(1):19-27.

Drug Enforcement Administration, Drugs of Abuse, 2005

Duman RS, Tallman JF, Nestler EJ. Acute and chronic opiate-regulation of adenylate cyclase in brain: specific effects in locus coeruleus. J Pharmacol Exp Ther 1988;246(3):1033-1039.

Easterling KW, Plovnick RM, Holtzman SG. Acute opioid but not benzodiazepine dependence in rats responding for intracranial self-stimulation. Psychopharmacology (Berl) 2000;148(3):263-271.

Eisenberg, R.M., 1982. Further studies on the acute dependence produced by morphine in opiate naive rats. Life Sci. 31, 1531-40.

el-Kadi AO, Sharif SI. The influence of various experimental conditions on the expression of naloxone-induced withdrawal symptoms in mice. Gen Pharmacol 1994;25(7):1505-1510.

Elliott K, Kest B, Man A, Kao B, Inturrisi CE. N-methyl-d-aspartate (NMDA) receptors, mu and kappa opioid tolerance, and perspectives on new analgesic drug development, Neuropsychopharmacology 1995;13:347–356.

Evans CJ, Keith DE Jr, Morrison H, Magendzo K, Edwards RH. Cloning of a delta opioid receptor by functional expression. Science. 1992;18:258(5090):1952-5.

Farrell M. Opiate withdrawal. Addiction 1994;89(11):1471-1475.

Finn AK, Whistler JL. Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. Neuron 2001;32(5):829-839.

Fukagawa Y, Katz JL, Suzuki T. Effects of a selective kappa-opioid agonist, U-50,488H, on morphine dependence in rats. Eur J Pharmacol 1989;170(1-2):47-51.

Fundytus ME, Coderre TJ. Attenuation of precipitated morphine withdrawal symptoms by acute i.c.v. administration of a group II mGluR agonist. Br J Pharmacol 1997;121(3):511-514.

Fundytus ME, Coderre TJ. Effect of activity at metabotropic, as well as ionotropic (NMDA), glutamate receptors on morphine dependence. Br J Pharmacol 1994;113(4):1215-1220.

Fundytus ME, Ritchie J, Coderre TJ. Attenuation of morphine withdrawal symptoms by subtype-selective metabotropic glutamate receptor antagonists. Br J Pharmacol 1997;120(6):1015-1020.

Gackenheimer SL, Suter TM, Pintar JE, Quimby SJ, Wheeler WJ, Mitch CH, Gehlert DR, Statnick MA. Localization of opioid receptor antagonist [3H]-LY255582 binding sites in mouse brain: comparison with the distribution of mu, delta and kappa binding sites. Neuropeptides. 2005 Dec;39(6):559-67.

Gellert VF, Holtzman SG. Development and maintenance of morphine tolerance and dependence in the rat by scheduled access to morphine drinking solutions. J Pharmacol Exp Ther 1978;205(3):536-546.

George SR, Fan T, Xie Z, Tse R, Tam V, Varghese G, O'Dowd BF. Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. J Biol Chem 2000;275(34):26128-26135.

Georges F, Le MC, ston-Jones G. No effect of morphine on ventral tegmental dopamine neurons during withdrawal. J Neurosci 2006;26(21):5720-5726.

Gilbert, A.K., Hosztafi, S., Mahurter, L., Pasternak, G.W., 2004. Pharmacological characterization of dihydromorphine, 6-acetyldihydromorphine and dihydroheroin analgesia and their differentiation from morphine. Eur. J. Pharmacol. 492, 123-30.

Goldstein A, Cox BM. Opioid Peptides (Endorphins) In Pituitary And Brain. Psychoneuroendocrinology 1977;2;PP.I 1-16.

Goldstein A, Tachibana S, Lowney LI, Hunkapiller M, Hood L. Dynorphin-(1-13), an extraordinarily potent opioid peptide. Proc Natl Acad Sci U S A. 1979;76(12):6666-70.

Gomes I, Jordan BA, Gupta A, Trapaidze N, Nagy V, Devi LA. Heterodimerization of mu and delta opioid receptors: A role in opiate synergy. J Neurosci 2000;20(22):RC110-

Granados-Soto V, Kalcheva I, Hua X, Newton A, Yaksh TL. Spinal PKC activity and expression: role in tolerance produced by continuous spinal morphine infusion. Pain 2000;85(3):395-404

Gumulka SW, Dinnendahl V, Schönhöfer PS. The effect of naloxone on cerebellar cGMP content. A possible GABA-antagonistic action? Naunyn Schmiedebergs Arch Pharmacol. 1979 Mar 20;306(2):169-72.

Hadjimarkou MM, Khaimova E, Pan YX, Rossi GC, Pasternak GW, Bodnar RJ. Feeding induced by food deprivation is differentially reduced by opioid receptor antisense oligodeoxynucleotide probes in rats. Brain Res. 2003 Oct 17;987(2):223-32.

Hadjimakou MM, Silva RM, Rossi GC, Pasternak GW, Bodnar RJ. Feeding induced by food deprivation is differentially reduced by G-protein α-subunit antisense probes in rats. Brain Research 2002;955:45-54

Hadjimarkou MM, Singh A, Kandov Y, Israel Y, Pan YX, Rossi GC, Pasternak GW, Bodnar RJ. Opioid receptor involvement in food deprivation-induced feeding: evaluation of selective antagonist and antisense oligodeoxynucleotide probe effects in mice and rats. J Pharmacol Exp Ther. 2004 Dec;311(3):1188-202.

Hamdy MM, Noda Y, Miyazaki M, Mamiya T, Nozaki A, Nitta A, Sayed M, Assi AA, Gomaa A, Nabeshima T. Molecular mechanisms in dizocilpine-induced attenuation of development of morphine dependence: an association with cortical Ca2+/calmodulin-dependent signal cascade. Behav Brain Res 2004;152(2):263-270.

Han W, Soichiro I, Sora I, Yamamato H, Ikeda K. A possible genetic mechanism underlying individual and interstrain differences in opioid actions: Focus on the mu opioid receptor gene. Ann. NY Acad. Sci. 2004;1026:370-375.

Harris AC, Hanes SL, Gewirtz JC. Potentiated startle and hyperalgesia during withdrawal from acute morphine: effects of multiple opiate exposures. Psychopharmacology (Berl) 2004;176(3-4):266-273.

Hayward MD, Duman RS, Nestler EJ. Induction of the c-fos proto-oncogene during opiate withdrawal in the locus coeruleus and other regions of rat brain. Brain Res 1990;525(2):256-266.

He L, Lee NM. Delta opioid receptor enhancement of mu opioid receptor-induced antinociception in spinal cord. J Pharmacol Exp Ther. 1998 Jun;285(3):1181-6.

Hegmann, J.P., Possidente, B., 1981. Estimating genetic correlations from inbred strains. Behav. Genet. 11, 103-14.

Heishman, S.J., Stitzer, M.L., Bigelow, G.E., Liebson, I.A., 1989. Acute opioid physical dependence in humans: effect of varying the morphine-naloxone interval I. J. Pharmacol. Exp. Ther. 250, 485-91.

Herman, B.H., Vocci, F., Bridge, P., 1995. The effects of NMDA receptor antagonists and nitric oxide synthase inhibitors on opioid tolerance and withdrawal. Medication development issues for opiate addiction. Neuropsychopharmacology 13, 269-93.

Hoffmann O, Plesan A, Wiesenfeld-Hallin Z. Genetic differences in morphine sensitivity, tolerance and withdrawal in rats. Brain Res. 1998 Sep 28;806(2):232-7.

Holmlund JT. Applying antisense technology: Affinitak and other antisense oligonucleotides in clinical development. Ann NY Acad Sci. 2003 Dec;1002:244-51

Huffman RD, Simmons KE, Lum JT. An intraventricular infusion model for inducing morphine dependence in rats: quantitative assessment of precipitated withdrawal. Behav Neurosci. 1985 Oct;99(5):861-80.

Hughes J, Smith TW, Kosterlitz HW, Fothergill LA, Morgan BA, Morris HR. Identification of two related pentapeptides from the brain with potent opiate agonist activity. Nature. 1975; 258(5536):577-80.

Hughes J. Isolation of an endogenous compound from the brain with pharmacological properties similar to morphine. Brain Res. 1975;88(2):295-308.

Ikeda, K., Ide, S., Han, W., Hayashida, M., Uhl, G.R., Sora, I., 2005. How individual sensitivity to opiates can be predicted by gene analyses. Trends Pharmacol. Sci. 26, 311-7.

Inoue M, Mishina M, Ueda H. Locus-specific rescue of GluRepsilon1 NMDA receptors in mutant mice identifies the brain regions important for morphine tolerance and dependence. J Neurosci 2003;23(16):6529-6536.

Inturrisi CE. Clinical pharmacology of opioids for pain. Clin J Pain. 2002;18(4 Suppl):S3-13.

Inturrisi, C.E., Schultz, M., Shin, S., Umans, J.G., Angel, L., Simon, E.J., Evidence from opiate binding studies that heroin acts through its metabolites. Life Sci. 1983;33:773-6.
Iorio LC, Deacon MA, Ryan EA. Blockade by narcotic drugs of naloxone-precipitated jumping in morphine-dependent mice. J Pharmacol Exp Ther 1975;192(1):58-63.

Jang CG, Rockhold RW, Ho IK. An autoradiographic study of [3H]AMPA receptor binding and in situ hybridization of AMPA sensitive glutamate receptor A (GluR-A) subunits following morphine withdrawal in the rat brain. Brain Res Bull 2000;52(3):217-221.

Jasinski, D.R., 1977. Assessment of the abuse potentiality of morphine like drugs (methods used in man). In: Martin, W.R., (Ed.), Handbook of Experimental Pharmacology, Springer-Verlag, Berlin, pp. 197-258.

Johnson SW, North RA. Opioids excite dopamine neurons by hyperpolarization of local interneurons. J Neurosci 1992;12(2):483-488.

Jones KL, Zhu H, Jenab S, Du T, Inturrisi CE, Barr GA. Attenuation of acute morphine withdrawal in the neonatal rat by the competitive NMDA receptor antagonist LY235959. Neuropsychopharmacology 2002;26(3):301-310.

Juni A, Klein G, Kest B. Morphine hyperalgesia in mice is unrelated to opioid activity, analgesia, or tolerance: evidence for multiple diverse hyperalgesic systems. Brain Res. 2006 Jan 27;1070(1):35-44.

Kendall JM, Latter VS. Intranasal diamorphine as an alternative to intramuscular morphine: pharmacokinetic and pharmacodynamic aspects. Clin Pharmacokinet. 2003;42(6):501-13.

Kenny PJ, Chen SA, Kitamura O, Markou A, Koob G. Conditioned withdrawal drives heroin consumption and decreases reward sensitivity. J Neurosci 2006;26(22):5894-5900

Kenny PJ, Markou A. The ups and downs of addiction: role of metabotropic glutamate receptors. Trends Pharmacol Sci 2004;25(5):265-272.

Kenny, P.J., Chen, S.A., Kitamura, O., Markou, A., Koob, G.F., 2006. Conditioned withdrawal drives heroin consumption and decreases reward sensitivity. J. Neurosci. 26, 5894-900.

Kest B, Lee CE, McLemore GL, Inturrisi CE. An antisense oligodeoxynucleotide to the delta opioid receptor (DOR-1) inhibits morphine tolerance and acute dependence in mice. Brain Res Bull 1996;39(3):185-188.

Kest B, Palmese CA, Hopkins E, Adler M, Juni A, Mogil JS. Naloxone-precipitated withdrawal jumping in 11 inbred mouse strains: evidence for common genetic mechanisms in acute and chronic morphine physical dependence. Neuroscience 2002;115(2):463-469.

Kest B, Palmese CA, Hopkins E, Adler M, Juni A. Assessment of acute and chronic morphine dependence in male and female mice. Pharmacol Biochem Behav 2001;70(1):149-156.

Kest B, Palmese CA, Juni A, Chesler EJ, Mogil JS. Mapping of a quantitative trait locus for morphine withdrawal severity. Mamm Genome. 2004 Aug;15(8):610-7.

Kieffer BL, Befort K, Gaveriaux-Ruff C, Hirth CG. The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. Proc Natl Acad Sci U S A. 1992;89(24):12048-52.

Kishioka S, Inoue N, Nishida S, Fukunaga Y, Yamamoto H. Diltiazem inhibits naloxone-precipitated and spontaneous morphine withdrawal in rats. Eur J Pharmacol 1996;316(1):7-14.

Kitanaka N, Sora I, Kinsey S, Zeng Z, Uhl GR. No heroin or morphine 6beta-glucuronide analgesia in mu-opioid receptor knockout mice. Eur J Pharmacol 1998 Aug 14;355(1):R1-3.

Klein, G., Juni, A., Inturrisi, C.E., Kest, B. Heroin Dependence in Mice: Contribution of Opioid and Excitatory Amino Acid Receptors. 2007 (In Press)

Koch T, Schulz S, Pfeiffer M, Klutzny M, Schroder H, Kahl E, Hollt V. C-terminal splice variants of the mouse mu-opioid receptor differ in morphine-induced internalization and receptor resensitization. J Biol Chem 2001;276(33):31408-31414.

Koch T, Schulz S, Schroder H, Wolf R, Raulf E, Hollt V. Carboxyl-terminal splicing of the rat mu opioid receptor modulates agonist-mediated internalization and receptor resensitization. J Biol Chem 1998;273(22):13652-13657.

Kolesnikov, Y., Jain, S., Wilson, R., Pasternak, G.W. Lack of morphine and enkephalin tolerance in 129/SvEv mice: evidence for a NMDA receptor defect. J. Pharmacol. Exp. Ther. 1998;284:455-9.

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

Koob GF, Maldonado R, Stinus L. Neural substrates of opiate withdrawal. Trends Neurosci 1992;15(5):186-191.

Kosersky DS, Harris RA, Harris LS. Naloxone-precipitated jumping activity in mice following the acute administration of morphine. Eur J Pharmacol. 1974 Apr;26(1):122-4.

Kosterlitz LW. The Wellcome Foundation lecture. Opioid peptides and their receptors. Proc R Soc Lond B Biol Sci. 1985;225(1238):27-40.

Kowalczyk BJ, Klein G, Juni A, Kest B. A model for quantitative assessment of spontaneous morphine withdrawal in the mouse. Soc Neurosci Abstr 2004

Kvam TM, Baar C, Rakvag TT, Kaasa S, Krokan HE, Skorpen F. Genetic analysis of the murine mu opioid receptor: increased complexity of Oprm gene splicing. J Mol Med 2004;82(4):250-255.

Lane-Ladd SB, Pineda J, Boundy VA, Pfeuffer T, Krupinski J, Aghajanian GK, Nestler EJ. CREB (cAMP response element-binding protein) in the locus coeruleus: biochemical, physiological, and behavioral evidence for a role in opiate dependence. J Neurosci 1997;17(20):7890-7901.

Lange, D.G., Roerig, S.C., Fujimoto, J.M., Wang, R.I. Absence of cross-tolerance to heroin in morphine-tolerant mice. Science. 1980;208:72-4.

Langerman L, Piscoun B, Bansinath M, Shemesh Y, Turndorf H, Grant GJ. Quantifiable dose-dependent withdrawal after morphine discontinuation in a rat model. Pharmacol Biochem Behav 2001;68(1):1-6.

Liang DY, Guo T, Liao G, Kingery WS, Peltz G, Clark JD. Chronic pain and genetic background interact and influence opioid analgesia, tolerance, and physical dependence. Pain. 2006 Apr;121(3):232-40.

Liang, D.Y., Guo, T., Liao, G., Kingery, W.S., Peltz, G., Clark, J.D., 2006. Chronic pain and genetic background interact and influence opioid analgesia, tolerance, and physical dependence. Pain 121, 232-40.

Liu J, Schulteis G. Brain reward deficits accompany naloxone-precipitated withdrawal from acute opioid dependence. Pharmacol Biochem Behav 2004;79(1):101-108.

Maldonado R, Feger J, Fournie-Zaluski MC, Roques BP. Differences in physical dependence induced by selective mu or delta opioid agonists and by endogenous enkephalins protected by peptidase inhibitors. Brain Res. 1990 Jun 18;520(1-2):247-54.

Manning BH, Mao J, Frenk H, Price DD, Mayer DJ. Continuous co-administration of dextromethorphan or MK-801 with morphine: attenuation of morphine dependence and naloxone-reversible attenuation of morphine tolerance. Pain 1996;67(1):79-88.

Marshall I, Weinstock M. Quantitative method for assessing one symptom of the withdrawal syndrome in mice after chronic morphine administration. Nature 1971;234(5326):223-224.

Martin, W.R., Eades, C.G. A comparison between acute and chronic physical dependence in the chronic spinal dog. J. Pharmacol. Exp. Ther. 1964;146:385-94.

Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A, Le MM, Dolle P. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. Nature 1996;383(6603):819-823.

Mayer, D.J., Mao, J., Price, D.D. The development of morphine tolerance and dependence is associated with translocation of protein kinase C. Pain 1995;61:365-74.

McLemore GL, Kest B, Inturrisi CE. The effects of LY293558, an AMPA receptor antagonist, on acute and chronic morphine dependence. Brain Res 1997;778(1):120-126.

Minami M, Toya T, Katao Y, Maekawa K, Nakamura S, Onogi T, Kaneko S, Satoh M. Cloning and expression of a cDNA for the rat kappa-opioid receptor. FEBS Lett. 1993;329(3):291-5.

Miyamoto Y, Bowen WD, Portoghese PS, Takemori AE. Lack of involvement of delta-1 opioid receptors in the development of physical dependence on morphine in mice. J Pharmacol Exp Ther 1994;270(1):37-39.

Miyamoto Y, Portoghese PS, Takemori AE. Involvement of delta 2 opioid receptors in the development of morphine dependence in mice. J Pharmacol Exp Ther 1993;264(3):1141-1145.

Miyamoto Y, Portoghese PS, Takemori AE. Involvement of delta 2 opioid receptors in acute dependence on morphine in mice. J Pharmacol Exp Ther 1993;265(3):1325-1327.

Miyamoto Y, Takemori AE. Relative involvement of supraspinal and spinal mu opioid receptors in morphine dependence in mice. Life Sci. 1993;52(12):1039-44.

Miyamoto, Y., Takemori, A.E., 1993. Relative involvement of supraspinal and spinal mu opioid receptors in morphine dependence in mice. Life Sci. 52, 1039-44.

Mogil JS, Wilson SG, Wan Y. Assessing Nociception in Murine Subjects. In: Kruger L (ed) Methods in Pain Research (Methods And New Frontiers In Neuroscience), pp. 11-40. CRC Press LLC, Florida (2001).

Nakagawa T, Ozawa T, Shige K, Yamamoto R, Minami M, Satoh M. Inhibition of morphine tolerance and dependence by MS-153, a glutamate transporter activator. Eur J Pharmacol 2001;419(1):39-45.

Nakagawa T, Satoh M. Involvement of glial glutamate transporters in morphine dependence. Ann N Y Acad Sci 2004;1025383-388.

Nakamura H, Yokoyama Y, Shimizu M. Quantitative assessment of jumping behaviour of morphine-like analgesics in mice. Arch Int Pharmacodyn Ther 1983;263(1):164-176.

Narita M, Funada M, Suzuki T. Regulations of opioid dependence by opioid receptor types. Pharmacol Ther 2001;89(1):1-15.

Narita M, Imai S, Ozaki S, Suzuki M, Narita M, Suzuki T. Reduced expression of a novel mu-opioid receptor (MOR) subtype MOR-1B in CXBK mice: implications of MOR-1B in the expression of MOR-mediated responses. Eur J Neurosci 2003;18(12):3193-3198.

National Institute on Drug Abuse, Heroin Abuse and Addiction Research Report, May 2005.

Nehmad R, Nadler H, Simantov R. Effects of acute and chronic morphine treatment of calmodulin activity of rat brain. Mol Pharmacol 1982;22(2):389-394.

Nemmani KV, Grisel JE, Stowe JR, Smith-Carliss R, Mogil JS. Modulation of morphine analgesia by site-specific *N*-methyl-d-aspartate receptor antagonists: dependence on sex, site of antagonism, morphine dose, and time, Pain 2004; 109:274–283.

Nestler EJ, Tallman JF. Chronic morphine treatment increases cyclic AMP-dependent protein kinase activity in the rat locus coeruleus. Mol Pharmacol 1988;33(2):127-132.

Nestler EJ. Historical review: Molecular and cellular mechanisms of opiate and cocaine addiction. Trends Pharmacol Sci 2004;25(4):210-218.

Nestler EJ. Molecular basis of long-term plasticity underlying addiction. Nat Rev Neurosci 2001;2(2):119-128.

Nitsche JF, Schuller AG, King MA, Zengh M, Pasternak GW, Pintar JE. Genetic dissociation of opiate tolerance and physical dependence in delta-opioid receptor-1 and preproenkephalin knock-out mice. J Neurosci 2002;22(24):10906-10913.

Noda Y, Nabeshima T. Opiate physical dependence and N-methyl-D-aspartate receptors. Eur J Pharmacol. 2004 Oct 1;500(1-3):121-8.

Ozawa T, Nakagawa T, Shige K, Minami M, Satoh M. Changes in the expression of glial glutamate transporters in the rat brain accompanied with morphine dependence and naloxone-precipitated withdrawal. Brain Res 2001;905(1-2):254-258.

Pan YX, Xu J, Bolan E, Abbadie C, Chang A, Zuckerman A, Rossi G, Pasternak GW. Identification and characterization of three new alternatively spliced mu-opioid receptor isoforms. Mol Pharmacol 1999;56(2):396-403.

Pan YX, Xu J, Bolan E, Chang A, Mahurter L, Rossi G, Pasternak GW. Isolation and expression of a novel alternatively spliced mu opioid receptor isoform, MOR-1F. FEBS Lett 2000;466(2-3):337-340.

Pan YX, Xu J, Mahurter L, Bolan E, Xu M, Pasternak GW. Generation of the mu opioid receptor (MOR-1) protein by three new splice variants of the Oprm gene. Proc Natl Acad Sci U S A 2001;98(24):14084-14089.

Pan YX. Diversity and complexity of the mu opioid receptor gene: alternative pre-mRNA splicing and promoters. DNA Cell Biol 2005;24(11):736-750.

Pan ZZ. mu-Opposing actions of the kappa-opioid receptor. Trends Pharmacol Sci 1998;19(3):94-98.

Papaleo F, Contarino A. Gender- and morphine dose-linked expression of spontaneous somatic opiate withdrawal in mice. Behav Brain Res. 2006 Jun 3;170(1):110-8.

Pasternak GW. Multiple opiate receptors: deja vu all over again. Neuropharmacology 2004; 47(Suppl 1): 312–323.

Pasternak GW. The pharmacology of mu analgesics: from patients to genes. Neuroscientist. 2001 Jun;7(3):220-31.

Pick CG, Nejat RJ, Pasternak GW. Independent expression of two pharmacologically distinct supraspinal mu analgesic systems in genetically different mouse strains. J Pharmacol Exp Ther. 1993 Apr;265(1):166-71.

Pouletty, P. Drug addictions: towards socially accepted and medically treatable diseases. Nat. Rev. Drug Discov. 2002;1:731-6.

Rady, J.J., Aksu, F., Fujimoto, J.M. The heroin metabolite, 6-monoacetylmorphine, activates delta opioid receptors to produce antinociception in Swiss-Webster mice. J. Pharmacol. Exp. Ther. 1994;268:1222-31.

Rady, J.J., Holmes, B.B., Portoghese, P.S., Fujimoto, J.M. Morphine tolerance in mice changes response of heroin from mu to delta opioid receptors. Proc. Soc. Exp. Biol. Med. 2000;224:93-101.

Rady, J.J., Roerig, S.C., Fujimoto, J.M. Heroin acts on different opioid receptors than morphine in Swiss Webster and ICR mice to produce antinociception. J. Pharmacol. Exp. Ther. 1991;256:448-57.

Rasmussen K, Kendrick WT, Kogan JH, Aghajanian GK. A selective AMPA antagonist, LY293558, suppresses morphine withdrawal-induced activation of locus coeruleus neurons and behavioral signs of morphine withdrawal. Neuropsychopharmacology 1996;15(5):497-505.

Rasmussen K, Vandergriff J. The selective iGluR1-4 (AMPA) antagonist LY300168 attenuates morphine-withdrawal-induced activation of locus coeruleus neurons and behavioural signs of morphine withdrawal. Neuropharmacology 2003;44(1):88-92.

Rasmussen K. Morphine withdrawal as a state of glutamate hyperactivity. The effects of glutamate receptor subtype ligands on morphine-withdrawal symptoms. In: B.H. Herman, J. Frankenheim, R.Z. Litten, P.H. Sheridan, F.F. Weight and S.R. Zukin, Editors, Glutamate and Addiction, Humana Press, Totowa, NJ. 2002:329–339.

Ritzmann RF. Opiate dependence following acute injections of morphine and naloxone: the assessment of various withdrawal signs. Pharmacol Biochem Behav. 1981 Apr;14(4):575-7.

Romberg R, Sarton E, Teppema L, Matthes HW, Kieffer BL, Dahan A. Comparison of morphine-6-glucuronide and morphine on respiratory depressant and antinociceptive responses in wild type and mu-opioid receptor deficient mice. Br J Anaesth. 2003;91(6):862-70.

Rossi GC, Brown GP, Leventhal L, Yang K, Pasternak GW. Novel receptor mechanisms for heroin and morphine-6 beta-glucuronide analgesia. Neurosci Lett. 1996;216(1):1-4.

Rossi GC, Standifer KM, Pasternak GW. Differential blockade of morphine and morphine-6 beta-glucuronide analgesia by antisense oligodeoxynucleotides directed against MOR-1 and G-protein alpha subunits in rats. Neurosci Lett 1995;198(2):99-102.

Saelens JK, Granat FR, Sawyer WK. The mouse jumping test--a simple screening method to estimate the physical dependence capacity of analgesics. Arch Int Pharmacodyn Ther. 1971 Apr;190(2):213-8.

Sanchez-Blazquez P, Garcia-Espana A, Garzon J. Antisense oligodeoxynucleotides to opioid mu and delta receptors reduced morphine dependence in mice: role of delta-2 opioid receptors. J Pharmacol Exp Ther 1997;280(3):1423-1431.

Sanchez-Blazquez P, Rodriguez MD, Garzon J. Antibodies directed against the mu-opioid receptor alleviated multiple signs of morphine withdrawal in mice. Life Sci 1996;59(8):L87-L92.

Schiller PW, Fundytus ME, Merovitz L, Weltrowska G, Nguyen TM, Lemieux C, Chung NN, Coderre TJ. The opioid mu agonist/delta antagonist DIPP-NH(2)[Psi] produces a potent analgesic effect, no physical dependence, and less tolerance than morphine in rats. J Med Chem 1999;42(18):3520-3526.

Schuller, A.G., King, M.A., Zhang, J., Bolan, E., Pan, Y.X., Morgan, D.J., Chang, A., Czick, M.E., Unterwald, E.M., Pasternak, G.W., Pintar, J.E. Retention of heroin and morphine−6 −glucuronide analgesia in a new line of mice lacking exon 1 of MOR−1. Nat. Neurosci. 1999;2:151-156.

Sekiya Y, Nakagawa T, Ozawa T, Minami M, Satoh M. Facilitation of morphine withdrawal symptoms and morphine-induced conditioned place preference by a glutamate transporter inhibitor DL-threo-beta-benzyloxyaspartate in rats. Eur J Pharmacol. 2004;485(1-3):201-210.

Selley, D.E., Cao, C.C., Sexton, T., Schwegel, J.A., Martin, T.J., Childers, S.R. mu Opioid receptor-mediated G-protein activation by heroin metabolites: evidence for greater efficacy of 6-monoacetylmorphine compared with morphine. Biochem. Pharmacol. 2001;62:447-55.

Simonin F, Valverde O, Smadja C, Slowe S, Kitchen I, Dierich A, Le MM, Roques BP, Maldonado R, Kieffer BL. Disruption of the kappa-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal. EMBO J 1998;17(4):886-897.

Simpson, E.M., Linder, C.C., Sargent, E.E., Davisson, M.T., Mobraaten, L.E., Sharp, J.J. Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. Nat. Genet. 1997;16:19-27.

Smith AP, Lee NM. Opioid receptor interactions: Local and nonlocal, symmetric and asymmetric, physical and functional. Life Sci. 2003 73:1873–1893

Smits SE. Quantitation of physical dependence in mice by naloxone-precipitated jumping after a single dose of morphine. Res Commun Chem Pathol Pharmacol 1975;10(4):651-661.

Sora I, Elmer G, Funada M, Pieper J, Li XF, Hall FS, Uhl GR. Mu opiate receptor gene dose effects on different morphine actions: evidence for differential in vivo mu receptor reserve. Neuropsychopharmacology 2001;25(1):41-54.

Stevenson GW, Folk JE, Rice KC, Negus SS. Interactions between delta and mu opioid agonists in assays of schedule-controlled responding, thermal nociception, drug self-administration, and drug versus food choice in rhesus monkeys: studies with SNC80 [(+)-4-[(alphaR)-alpha-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide] and heroin. J Pharmacol Exp Ther. 2005 Jul;314(1):221-31.

Sporer KA, Kral AH. Prescription naloxone: A novel approach to heroin overdose prevention. Ann Emerg Med 2007;49(2):172-177.

Suzuki T, Hayashi Y, Misawa M. The role of mu1 receptor in physical dependence on morphine using the mu receptor deficient CXBK mouse. Life Sci 1992;50(12):849-856.

Suzuki T, Ikeda H, Tsuji M, Misawa M, Narita M, Tseng LF. Antisense oligodeoxynucleotide to delta opioid receptors attenuates morphine dependence in mice. Life Sci 1997a;61(11):L-70.

Suzuki T, Misawa M. Physical dependence on morphine using the mu receptor deficient CXBK mouse. Prog Clin Biol Res 1990;328519-522.

Suzuki T, Otani K, Misawa M. Differential sensitivity to physical dependence on morphine and codeine in three inbred strains of mice. Japanese Journal of Pharmacology 1991;57(4), 455-462.

Suzuki T, Tsuji M, Mori T, Misawa M, Nagase H. Involvement of delta 1 and delta 2 opioid receptor subtypes in the development of physical dependence on morphine in mice. Pharmacol Biochem Behav 1997b;57(1-2):293-299.

Takemori AE, Loh HH, Lee NM. Suppression by dynorphin A-(1-13) of the expression of opiate withdrawal and tolerance in mice. Eur J Pharmacol 1992;221(2-3):223-226.

Tang L, Shukla PK, Wang LX, Wang ZJ. Reversal of morphine antinociceptive tolerance and dependence by the acute supraspinal inhibition of Ca(2+)/calmodulin-dependent protein kinase II. J Pharmacol Exp Ther 2006;317(2):901-909.

Tao PL, Hwang CL, Chen CY. U-50,488 blocks the development of morphine tolerance and dependence at a very low dose in guinea pigs. Eur J Pharmacol 1994;256(3):281-286.

Tarabar, A.F., Nelson, L.S. The resurgence and abuse of heroin by children in the United States. Curr. Opin. Pediatr. 2003;15:210-5.

Terenius L. From opiate pharmacology to opioid peptide physiology. Ups J Med Sci. 2000;105(1):1-15.

Trujillo KA, Akil H. Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. Science 1991;251(4989):85-87.

Tsuji M, Takeda H, Matsumiya T, Nagase H, Yamazaki M, Narita M, Suzuki T. A novel kappa-opioid receptor agonist, TRK-820, blocks the development of physical dependence on morphine in mice. Life Sci 2000;66(25):L353-L358.

van der Laan JW, de GG. Changes in locomotor-activity patterns as a measure of spontaneous morphine withdrawal: no effect of clonidine. Drug Alcohol Depend 1988;22(1-2):133-140.

van Dorp EL, Romberg R, Sarton E, Bovill JG, Dahan A. Morphine-6-glucuronide: morphine's successor for postoperative pain relief? Anesth Analg. 2006 Jun;102(6):1789-97.

Vandergriff J, Rasmussen K. The selective mGlu2/3 receptor agonist LY354740 attenuates morphine-withdrawal-induced activation of locus coeruleus neurons and behavioral signs of morphine withdrawal. Neuropharmacology 1999;38(2):217-222.

Vekovischeva OY, Zamanillo D, Echenko O, Seppala T, Uusi-Oukari M, Honkanen A, Seeburg PH, Sprengel R, Korpi ER. Morphine-induced dependence and sensitization are altered in mice deficient in AMPA-type glutamate receptor-A subunits. J Neurosci 2001;21(12):4451-4459.

Vitravene Study Group. Safety of intravitreous fomivirsen for treatment of cytomegalovirus retinitis in patients with AIDS. Am J Ophthalmol. 2002 Apr;133(4):484-98.

Walker JR, King M, Izzo E, Koob GF, Pasternak GW. Antagonism of heroin and morphine self-administration in rats by the morphine-6beta-glucuronide antagonist 3-O-methylnaltrexone. Eur J Pharmacol. 1999 Oct 27;383(2):115-9.

Wang JB, Imai Y, Eppler CM, Gregor P, Spivak CE, Uhl GR. mu opiate receptor: cDNA cloning and expression. Proc Natl Acad Sci U S A 1993;90(21):10230-10234.

Wang JQ, Arora A, Yang L, Parelkar NK, Zhang G, Liu X, Choe ES, Mao L. Phosphorylation of AMPA receptors: mechanisms and synaptic plasticity. Mol Neurobiol 2005;32(3):237-249.

Watson B, Meng F, Akil, H. A chimeric analysis of the opioid receptor domains critical for the binding selectivity of mu opioid ligands. Neurobiol. Dis. 1996;3:87-96.

Way EL, Loh HH, Shen FH. Simultaneous quantitative assessment of morphine tolerance and physical dependence. J Pharmacol Exp Ther 1969;167(1):1-8.

Way, EL, Kemp JW, Young JM, Grassetti DR. The pharmacologic effects of heroin in relationship to its rate of biotransformation. J. Pharmacol. Exp. Ther. 1960;129:144-54.

Way EL, Young MJ, Kemp JW. Metabolism of Heroin and its Pharmacologic Implications. Bull. Narcotics. 1965;17:25-33.

Wiley JN, Downs DA. Naloxone-precipitated jumping in mice pretreated with acute injections of opioids. Life Sci 1979;25(9):797-801.

Williams JT, Christie MJ, Manzoni O. Cellular and synaptic adaptations mediating opioid dependence. Physiol Rev 2001;81(1):299-343.

Wolozin BL, Pasternak GW. Classification of multiple morphine and enkephalin binding sites in the central nervous system. Proc Natl Acad Sci U S A 1981;78(10):6181-6185.

Wrench IJ, Sanghera S, Pinder A, Power L, Adams MG. Dose response to intrathecal diamoprhine for elective caesarean section and compliance with a national audit standard. Int J Obstet Anesth. 2007 Jan;16(1):17-21.

Yamamoto H, Harris RA, Loh HH, Way EL. Effects of acute and chronic morphine treatments on calcium localization and binding in brain. J Pharmacol Exp Ther 1978;205(2):255-264.

Zaki PA, Bilsky EJ, Vanderah TW, Lai J, Evans CJ, Porreca F. Opioid receptor types and subtypes: the delta receptor as a model. Annu Rev Pharmacol Toxicol 1996;36379-401.

Zhu H, Barr GA. Naltrexone-precipitated morphine withdrawal in infant rat is attenuated by acute administration of NOS inhibitors but not NMDA receptor antagonists. Psychopharmacology (Berl) 2000;150(3):325-336.

Zhu H, Ho IK. NMDA-R1 antisense oligonucleotide attenuates withdrawal signs from morphine. Eur J Pharmacol 1998;352(2-3):151-156.

Zimprich A, Simon T, Hollt V. Cloning and expression of an isoform of the rat mu opioid receptor (rMOR1B) which differs in agonist induced desensitization from rMOR1. FEBS Lett 1995;359(2-3):142-146.