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**CONTRIBUTIONS OF SEX AND GENOTYPE TO MORPHINE ANALGESIC
TOLERANCE**

by

Eileen Hopkins

**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**

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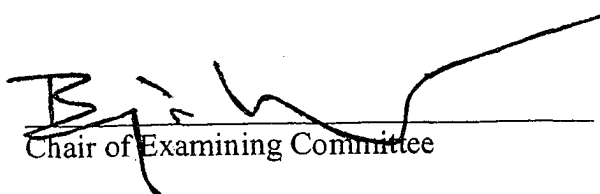
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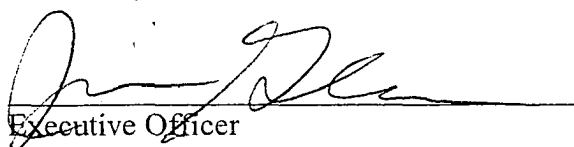
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Abstract

CONTRIBUTIONS OF SEX AND GENOTYPE TO MORPHINE ANALGESIC
TOLERANCE

by

Eileen Hopkins

Adviser: Professor Benjamin Kest

Individual differences in morphine's analgesic efficacy are important clinical concerns. While the contributions of sex and genotype to individual differences in acute morphine analgesia has received much recent attention in the research, little work has been done to explore the degree to which these factors contributed to morphine's ability to sustain its analgesic efficacy. The focus of the present dissertation was to examine the extent to which tolerance to morphine's effects varies as a function of sex and/or genotype.

It was determined, through experiments using CD-1 mice, that females show greater tolerance to the analgesic effects of morphine on the tail withdrawal test following chronically administered subcutaneous and intrathecal, but not intracerebroventricular, morphine. In contrast, male CD-1 mice showed greater tolerance to the hypothermic effects of morphine, demonstrating dissociation of tolerance development to the different behavioral/ physiological effects of the drug. Sex did not have a significant main effect on tolerance development to morphine analgesia on the acetic acid writhing

test, suggesting the possibility of dissociation in tolerance to analgesia for different types of pain. A comprehensive strain survey revealed significant genetic differences in tolerance to morphine's analgesic effects. Further, sex differences were observed within some strains under one method of calculating analgesia (ED_{50}), but not another (AD_{50}). Overall, it is clear from the experiments that sex and genotype play an important role in the development of morphine tolerance. Like findings of sex differences in morphine analgesia, however, the exact nature and direction of differences in tolerance found appears dependent on the specific experimental methods. Collectively, the findings of the present dissertation contribute to our understanding of individual differences in tolerance development, and suggest that such differences should be taken into account in the planning and interpretation of future research in this area.

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of gratitude by contributing in a meaningful way to the field of developmental disabilities.

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INTRODUCTION

Morphine has long been recognized as one of the most effective analgesics, and is one of the most frequently prescribed drugs for the treatment of moderate to severe pain across a wide range of conditions (Yaksh, 1993; Portenoy, 1997). It is well established that individual differences in the analgesic effects of morphine exist (Levine, Gordon, Smith, and Fields, 1981; Twycross, 1994; Cherny & Portenoy, 1994), and such differences pose a challenge for physicians hoping to alleviate patient pain in the most effective manner possible. Researchers have demonstrated a number of factors which contribute to the individual differences in morphine's efficacy at treating pain, including age, genetic background, hormonal status, and gender (for reviews, see Bodnar et al., 1988, Kest et al., 2000; Mogil et al., 2000).

In addition to morphine's initial efficacy in alleviating pain in an individual, its ability to sustain this efficacy over time is an important measure of its clinical utility.

However, for reasons not yet understood, the development of tolerance varies greatly among individuals, with some attaining adequate pain management with relatively stable doses for many months, and others requiring large dosage increases within days of initiating opioid therapy (Kanner & Foley, 1981). Drug tolerance poses a particularly troubling challenge in pain management for a number of reasons.

Respiratory depression can occur and can prove to be a lethal adverse effect of higher

doses of morphine administration in certain patients (Writer et al., 1985; Hanks et al., 1981). Further, concerns about inducing tolerance/dependence may cause a physician to err on the side of giving too small a dose (i.e., not achieving adequate analgesia) rather than risk inducing drug tolerance and/or dependence in the patient (Marks and Sachar, 1973). While many variables influencing morphine analgesia have been well studied, little work has been done to date to explore those factors that may influence individual differences in the development of tolerance to morphine's analgesic effects. Elucidating the contribution of two of such factors, sex and genotype, will be the objective of the present dissertation.

The specific aims of the current series of studies address this objective through the exploration of some of the organismic variables that affect the development of morphine tolerance, and attempt to clarify under what specific conditions these variables may play a role. The first specific aim of the study was to determine the degree to which tolerance to the analgesic effects of systemically administered morphine varies as a function of sex. The study involved performing dose-response analysis of morphine analgesia in male and female mice outbred (CD-1) mice before and after induction of tolerance via the administration of increasing doses of morphine injected subcutaneously three times a day. The tail withdrawal test, a commonly used assay of thermal nociception, was used to determine baseline nociception and analgesic efficacy of the various morphine doses.

Because systemically delivered morphine exerts effects both supraspinally and spinally, and each is capable of yielding analgesia (see Yaksh and Malmberg, 1994 for review), specific aims two and three explore the relative contribution of each of these sites of action to the development of morphine tolerance in male and female mice. In study two, morphine was delivered intracerebroventricularly for the tolerance induction period, and dose-response analysis of morphine analgesia was compared in each sex both prior to, and subsequent to, the tolerance induction period. Study number three involved the administration of morphine intrathecally during the tolerance induction period, again with comparisons of morphine analgesic doses before and after this period in each sex.

Research shows that the degree of analgesic efficacy of a given agent can be dependent upon the different type of pain the individual is experiencing. For example, while there is clear consensus that morphine is a very effective analgesic for severe cancer pain (Twycross, 1994), it may be less effective for other types of pain, including neuropathic pain (Arner and Meyerson, 1998). The degree to which patterns of sex differences in morphine tolerance are consistent across different types of pain has yet to be explored in the literature. Specific Aim Four examines male and female outbred mice using the acetic acid writhing assay, a test of chemical/visceral pain, to determine whether sex differences in morphine tolerance are apparent when the pain is of this typography.

Morphine's effects on the organism extend beyond analgesia, and include respiratory depression, reduced gastrointestinal transit, and thermoregulatory changes, among other effects. Tolerance development to these other effects of morphine has yet to be fully explored, and possible sex and genetic differences in tolerance development to such properties are unknown. Specific Aim Five explores differences in core body temperature that occur upon morphine administration in naïve animals, and after a three day tolerance induction period, to determine the degree to which sex differences may exist in tolerance to the thermoregulatory effects of morphine.

While there have been a handful of previous studies exploring the contribution of genotype to morphine analgesic tolerance in rodents (Mas et al., 2000; Hoffman et al., 1998), these studies compare only the same few strains. To date, a comprehensive strain survey exploring differences in tolerance as a function of sex and genotype (and any interaction effects) has not yet been conducted. Indeed, sex differences in morphine analgesia have been shown to interact with strain, such that some strains show greater male relative to female analgesia, some no sex difference, and in one strain, female greater than male analgesia (Kest et al., 1999). Such a study would not only facilitate a more complete comparison among strains, but also allow for the analysis of the degree to which the magnitude of tolerance is correlated with baseline nociception and initial morphine analgesic efficacy. Correlations may also be assessed with other morphine-related measures such as dependence (Kest et al., 2002). The identification of extreme responding strains are also requisite for any subsequent quantitative trait loci

mapping studies designed to identify chromosomal regions associated with phenotypic variability. Specific Aim Six attempts to accomplish this goal through the assessment of analgesic morphine doses prior to, and subsequent to, chronic subcutaneous morphine administration in males and females of eleven strains of inbred mice.

In order to provide the reader with an understanding of relative issues and prior findings influencing the work to be conducted (and interpretation thereof), the following sections will provide a comprehensive overview of applicable literature, including: a) basic central nervous system mechanisms of pain modulation, b) a review of the actions and mechanisms of action of morphine, c) research findings relating to the differences in morphine's effects as a function of sex or genetic makeup, d) basic concepts of tolerance and general theories surrounding its development, e) literature specifically focusing on possible mechanisms of tolerance to the analgesic effects of morphine, and e) a review of the limited available literature pertaining to the contributions of sex and genotype to the development of morphine tolerance. The final section of the introduction will provide a rationale for the specific studies and methods chosen as part of the present dissertation.

A. Central Nervous System Mechanisms of Pain Modulation

i. Basic Findings

Pain can be defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (International Association for the Study of Pain, 1979). In reviewing literature regarding individual differences in pain related traits/ behaviors, the multitude of mechanisms through which such differences might originate quickly becomes apparent. In its simplest description, the sensation of pain occurs when a stimulus of sufficient intensity leads to activation of specific populations of nociceptive sensory afferents, which in turn serve to induce activity in a complex of dorsal horn neurons within the spinal cord and other sensory cranial nerve nuclei. This activity includes induction of the release of a variety of chemical neurotransmitters, including the neuropeptide substance P, nerve growth factor, neurotrophic factors, and the amino acid transmitter glutamate (Urban and Gebhart, 1999). From there, action potentials project an excitatory outflow via long tracts, typically in the ventrolateral quadrant into the brainstem and diencephalon. These projections reach other areas of the brain (such as the limbic system and somatosensory cortex) where higher level processing occurs. In reality, the manner and extent of central nervous system modulation of pain processing is considerably more complex than this brief description implies. The movement of excitatory input through the dorsal horn and into the brainstem is highly regulated by local circuits which may

modulate both the excitability of the synapse and of postsynaptic neurons. Data from research during the past thirty years suggest clearly that sensitivity to afferent input may be either augmented or reduced through a number of mechanisms, of both endogenous and exogenous origin. The perceived pain from a given stimulus results from a balance between the activity of inhibitory (or antinociceptive) and facilitatory (or pronociceptive) circuits in action. While antinociceptive circuits inhibit the ascending transmission of nociceptive information from the spinal cord dorsal horn producing analgesia, pronociceptive circuits facilitate this transmission thereby either inhibiting analgesia (antianalgesia) or increasing basal pain sensitivity (hyperalgesia) (McNally, 1999). Differences in these mechanisms as a function of sex, genotype, or other organismic variables may account for behavioral differences observed in many studies of pain related behaviors in these groups (for example, see Staud et al., 2003; Flores et al., 2001; Bereiter et al., 2002; Stephan et al., 2002; Bradshaw et al., 2000). As such, observed patterns of behavior that differ as a function of sex or genotype are likely subserved by neuroanatomical, neurochemical, and/or neurophysiological differences (at the systems, cellular, and/or molecular levels). While the complexity of information gained through pain research over the last three decades precludes a complete review of each of these systems, a historical overview of some of the major findings will be presented. Following this, the research to date regarding individual differences in pain related behaviors, and those specifically related to sex and genotype, will be reviewed.

It has been known for some time that discrete analgesic circuits exist within the CNS. This idea was first suggested by Reynolds (1969) who demonstrated that focal electrical stimulation of the midbrain periaqueductal gray (PAG) in the rat produced profound analgesia (sufficient to allow abdominal surgery in the absence of exogenous analgesics or anesthetics). This report was closely followed by numerous other demonstrations of “stimulation produced analgesia” from several structures, including the nucleus raphe magnus and medullary reticular formation, locus coeruleus, habenula, and lateral hypothalamus (McNally, 1999; see Oliveras & Bensen, 1988 for review). Subsequent studies identified binding sites for morphine and naloxone in the CNS (e.g., Kuhar, Pert, & Snyder, 1973; Pert & Snyder, 1973), and it became apparent that regions where stimulation would produce analgesia were included in these sites. This, in conjunction with the isolation of the peptides met-enkephalin, leu-enkephalin, and B-endorphin which exhibited opiate-like (opioid) activity (e.g., Hughes, 1975; Hughes et al., 1975), suggested that, in addition to their activation by focal electrical stimulation, these circuits may subserve the analgesic effects of morphine. Over the next few years, a multitude of divergent lines of evidence supported this conclusion. This evidence included the finding that stimulation-produced analgesia displayed cross-tolerance to morphine (Mayer & Hayes, 1975) and that direct microinjection of morphine into regions where analgesia could be produced by electrical stimulation would itself produce analgesia (Akaike et al., 1978, Yaksh et al., 1976). In addition, it was found that the competitive opioid receptor antagonist naloxone, when administered systemically, attenuated not only morphine-induced analgesia but stimulation-produced

analgesia as well (Akil, Mayer, & Liebeskind, 1976). From these early findings, an entire field of research in central modulation of nociception emerged, leading to an increasingly complicated view of the locations and systems that modulate pain, and the neurotransmitters through which these systems exert their effects.

As discussed above, the PAG, originally found to be the most effective site for the production of stimulation-produced analgesia, was subsequently determined to be a prominent component of an endogenous pain inhibitory system (Fields and Basbaum, 1999). The PAG contains high levels of endogenous opioid peptides and receptors, and ventral lateral microinjections of opioids, nonspecific excitants, or neuropeptides in the PAG produces analgesia in animals (Urban & Gebhart, 1999). The majority of descending PAG efferents project to the rostral ventromedial medulla (RVM), which, in turn, sends bilateral descending projections through dorsolateral spinal funiculi that terminate in the spinal dorsal horn (Basbaum and Fields, 1978). The significance of the RVM as a relay in mediating descending pain inhibition was demonstrated by the fact that lesions of the RVM, or dorsolateral funiculi transection, blocked the analgesia produced by PAG stimulation. Additionally, electrical stimulation or local injections of opioids, excitatory amino acids, or neuropeptides into the RVM produces analgesia similar to that found after PAG stimulation (Fields et al., 1991). Systems of descending inhibition appear to be activated by a variety of stimuli, such as aversive environmental events or signals that predict such events, leading to stress-induced analgesia (Faneslow, 1991). In fact, supraspinal sites such as the locus coeruleus and the

amygdala, involved in regulating the organism's response to environmental events, have been shown to modulate descending inhibitory control of pain transmission.

Experimental data show that this regulation occurs through regional interaction with the RVM (as in the case of the locus coeruleus) and the ventral lateral PAG (as for the central and basolateral nuclei of the amygdala) (for review, see Bodnar, 2000; Basbaum and Fields, 1999). These inhibitory pathways, through activation of descending (brain stem to spinal cord) norepinephrine, enkephalin, and serotonin releasing neurons, activate enkephalinergic and dynorphinergic neurons in the dorsal horn of the spinal cord, which, in turn, exert an analgesic action by inhibiting release of transmitters from the primary afferent nociceptive neurons. Descending pain modulation systems are important not only for endogenous regulation of pain control in response to environmental events, but also contribute to the mechanisms of action of analgesic agents (as evidenced by the findings that selective supraspinal lesions significantly reduce the analgesic actions of systemic opioids, reviewed by Urban and Gebhart, 1999).

While much of the literature regarding central mechanisms of pain control is focused on methods of inhibitory / analgesic circuits, it is becoming increasingly evident that there similarly exist multiple systems and mechanisms of descending pain facilitation (or pronociceptive circuitry). For example, Fields and others (Fields et al., 1983; Fields and Heinricher, 1985; Morgan and Fields, 1994) characterized descending pain modulatory systems which are comprised of bulbospinal projections traveling from the

rostral ventromedial medulla along the dorsolateral funiculus to the spinal dorsal horn, which can mediate both descending pain inhibition and facilitation. RVM neuronal populations include “OFF” cells that comprise a descending inhibitory system and “ON” cells that facilitate nociceptive processing through spinopetal fibers (Fields et al., 1983, 1991; Fields and Heinricher, 1985). Low intensity electrical or chemical stimulation of the RVM has been shown to increase the spontaneous activity and evoked responses of spinal dorsal horn nociceptive neurons, and enhances the spinal behavioral responses to acute noxious stimuli (Gebhart, 1993). While the stimulation of the same site (the RVM) can both inhibit and facilitate spinal nociceptive transmission, these opposing descending influences appear to be mediated by independent and distinct mechanisms. Descending inhibitory influences from the RVM are conveyed bilaterally in the DLF, and involve descending cholinergic and monoaminergic systems, while descending facilitory appear to involve different spinal mediators, including serotonin₁, kappa opioid, and cholecystokinin_b receptors (as reviewed by Umans and Gebhart, 1999).

Administration of morphine and other opioids activates both endogenous pain inhibitory and facilitory systems (e.g., Gardell et al., 2002). It is assumed that activation of descending facilitory systems from the RVM serves in an antianalgesic manner to oppose opioid action and to maintain a basal level of pain responsivity. There is evidence, however, to suggest that this role is not solely antianalgesic, but in fact also pro-nociceptive. For example, a number of studies report “opioid induced pain” in

individuals who have been chronically exposed to morphine, in which hyperalgesia and allodynia are apparent (see Arner et al., 1988, for review). Certain descending projections from the RVM have been shown to contain glutamate and substance P, the principal transmitters in primary afferent nociceptors believed to mediate central (spinal) sensitization.

The presence of such descending pain facilitation systems led Vanderagh et al. (2001) to hypothesize that opioid induced pain might be the consequence of inappropriate tonic descending facilitation, possibly because of increased ON-cell discharge. Thus, lidocaine administered into the RVM blocked (in a short acting and reversible manner) opioid induced allodynia and thermal hyperalgesia in rats that received morphine though either subcutaneous pellets or chronic infusion via mini osmotic pumps. To further confirm the role of this pain modulatory system, they administered chronic morphine to groups of rats with dorsal lateral funiculus (DLF) lesions and sham lesions. DLF lesioned rats did not show morphine pellet-induced allodynia and hyperalgesia, whereas sham-lesioned rats did. They interpreted their findings as support for the view that abnormal pain caused by tonic descending facilitation from the RVM decreases the antinociceptive potency of spinal (and systemic) morphine, and hypothesized that approaches that block the RVM plasticity associated with sustained opioid exposure (or nerve injury) may allow sustained analgesic efficacy of opioids and their use in the treatment of chronic pain. Other studies by their group (Gardell et al., 2002; Vanderah et al., 2001) have suggested that sustained morphine exposure may also induce a spinal

dynorphin-dependent enhancement of excitatory transmitter release from primary afferent fibers.

While supraspinal contributions, both pronociceptive and antinociceptive, are clearly complex and still being elucidated, limiting examination to the spinal cord does not yield further simplicity or clarity. At the spinal level, there are a number of important mediators in pain transmission including Substance P, a neurokinin acting principally at the NK1 receptor (Dougherty et al., 1994). Glutamate, another ligand present in the spinal cord, also plays a very important role in pain facilitation. The role of NMDA receptors, ionotropic glutamate receptors which are widely distributed throughout the central nervous system, has been extensively studied, and established to be central to the development and maintenance of hyperalgesia and morphine analgesic tolerance (Coderre et al., 1993; Wilcox, 1991). NMDA receptors have been localized to presynaptic terminals of fine-diameter nociceptors in the spinal dorsal horn, where it is believed they function as autoreceptors to facilitate and prolong nociceptor input by increasing the release of substance P (reviewed by Willis and Coggeshall, 1991). Alternatively (or additionally) the synthesis and diffusion/release of the second messenger NO by NMDA receptors may enhance the release of substance P from the presynaptic terminals, thus contributing to the development of hyperalgesia (and tolerance, as discussed in the tolerance section of the present dissertation) (see Mao et al., 1995 for review). Inhibitory neurotransmitters, such as GABA and glycine also play a role in modulating dorsal horn transmission of nociceptive impulses (e.g., Curtis

et al., 1968; Willcockson et al., 1984; Lin et al., 1994). As such, the spinal cord dorsal horn provides the opportunity for sensitive and complex pain modulating circuitry, the exact nature of which is only beginning to be understood.

ii. Individual Differences

Individual variability in a number of pain-related traits has been well-established in the literature. As far back as 1934, Libman reported that pressure applied to the mastoid bone produces “marked” pain in 60-70% of human patients, but little or no pain in 30-40%. Over the subsequent 70 years, numerous studies expanded our appreciation of the extent of variability present in traits such as threshold sensitivity to pain, efficacy of opioid analgesics, side effects of analgesics, and tolerance liability. Specifically, early studies confirmed the presence of large individual differences in threshold sensitivity and tolerance to several noxious stimuli applied to cutaneous tissue, including pressure (Sherman, 1943; Chapman and Jones, 1944; Woodrow, Friedman, Siegelau, and Collen, 1972), heat (Clark and Bindra, 1956, Wolf and Jarvik, 1963, Wolf and Jarvik, 1964), and electrical current (Clark and Bindra, 1956; Lanier, 1943), as well as tolerance to visceral (Chapman and Jones, 1944) and deep muscle (Wolf and Jarvik, 1963, 1964) pain (as reviewed by Mogil, 1999). More recent studies have confirmed that interindividual variability in pain thresholds greatly exceeds intraindividual variability for pressure pain thresholds (Brennum, Kjeldsen, Jensen and Jensen 1989; Isselee, DeLaat, Lesaffre, and Lysens, 1977), cold pressor tolerance (Chen, Dworkin, Haug, and Gehrig, 1989) and pain associated with manual palpation of tender points in

fibromyalgia sufferers (Jacobs, Geeman, van der Heide, Rasker, and Bijlsma, 1995).

Further, in the cold pressure test, Chen et al. (Chen, Dworkin, Haug, and Gehrig, 1989; Chen, Dworking, and Haug, 1989) observed a minority “pain sensitive” group tolerating the test for a duration of 50 seconds in contrast to a majority “pain tolerant” group able to tolerate the test for a full 300 seconds. These findings (reviewed by Mogil, 1999) highlight the extent of individual variability observed in basal thresholds for nociceptive stimuli.

Individual variability in efficacy of opiate analgesics has been also been well demonstrated in the literature, both in clinical and experimental studies (which will be discussed in more detail in the morphine section of the present dissertation). The analgesic effects of morphine in individuals who have undergone dental extraction break into dichotomous “responder” and “non responder” groups being easily identified at a range of doses (Levine, Gordon, Smith, and Fields, 1981). Further, Chapman et al. (1990), testing analgesic efficacy of bolus doses of alfentanil, fentanyl, and morphine on healthy volunteers, while finding noteworthy individual differences in analgesic doses for all three drugs, found that intersubject variability was greatest for morphine. Finally, the degree of individual differences in drug effects on pain modulation are vividly observed in a study conducted by Buschbaum et al. (1977), using the opioid antagonist naloxone, typically thought to block analgesia. They found that intravenous injection of 2mg naloxone produced (expected) hyperalgesia, but only in a group shown to be relatively insensitive to electric shock to the forearm. Paradoxically, in a group

shown to be sensitive to the pain of electrical shock to the forearm, the same 2mg dose of naloxone produced an analgesic effect (Buschbaum, Davis, Bunney, 1977).

iii. Sex Differences

There exists a large body of literature studying gender differences in pain-related traits / behaviors. Recent evidence suggests that one of the factors that may influence both the assessment and management of pain is a person's gender. Researchers have noted gender differences in pain thresholds and tolerance to painful stimuli (see Fillingim & Maixner, 1995 for review), specific gender distribution for certain painful conditions (see Unruh, 1996 for review), and gender differences in response to opioid analgesic medications (see Kest et al., 2000 for review).

Regarding gender differences in pain thresholds, as reviewed by Bodnar et al. (1988) and Berkley (1997), there are sex differences reported in basal nociception, with females generally showing lower thresholds to identify a stimulus as painful, higher ratings of the intensity of a given painful stimulus, and less tolerance to painful stimuli. The differences, however, are often small and inconsistently observed, and the presence or direction of differences can be influenced by age, situational, health related, hormonal, motivational and/or nutritional factors (Berkley, 1997). Further, Mogil et al. (2000), in an attempt to perform a meta-analysis of sex differences reported in the rodent literature, found that the direction of sex differences observed (male > female

versus female>male) was strikingly dependent on the nociceptive assay employed. Specifically, they report that while sensitivity to electric shock and chemical/inflammatory nociception (abdominal constriction, cystitis, and formalin tests) appear to be higher in females than in males, findings of sex differences in assays involving noxious heat (e.g., hot plate, tail-flick/withdrawal) are less consistently observed. For example, out of 23 studies using tail-flick/withdrawal from either radiant heat or hot-water immersion as a dependent variable, eight reported increased sensitivity in males (all in rats), two reported increased sensitivity in females (both in mice), and 12 studies reported no significant differences between the sexes. Given the complexities of findings to date, predictions regarding the manner and mechanism of sex differences are difficult, as are interpretations of findings of sex differences when they do occur.

Sexual dimorphism in brainstem pain modulating circuitry likely underlies sex differences when they are, in fact, observed. Recent studies have begun to look at neuroanatomical areas and systems involved in descending inhibitory controls, and how these systems respond in a different manner to certain stimuli in males and females. For example, it has been demonstrated in the rat that central administration of morphine, into the lateral ventricles (Kepler et al., 1989; tailflick and shock-jump test), the ventral lateral PAG (Krzanowska and Bodnar, 1999; tailflick and shock-jump test), and the RVM (Boyer et al., 1998; tail withdrawal test), produces greater analgesia in males than females. Sex differences have also been shown in analgesic effects of intracerebroventricularly administered DAMGO, a selective mu agonist (males>females

on tailflick, no difference on shock-jump test), but not analgesia produced by the selective delta agonist DSLET (Kepler et al., 1991). Further, activation of kappa opioid receptors within the rostral ventral medial medulla has been shown to have an anti-mu opioid analgesic action in male rats, while the same dose of kappa opioid agonist that reduces the mu opioid-mediated increase in tail flick latency in males produces a moderate increase in tail flick latency in female rats (Tershner et al., 2000).

Recently, Barrett et al. (2002) used a test of mechanical nociception to show that male rats not only had a higher threshold for nociception (ie., they could tolerate longer periods of a constant pressure applied to their paw before withdrawing), they also were more sensitive on this assay to the analgesic effects of multiple opioids acting at the mu receptor (levorphanol, morphine, dezocine, buprenorphine, butorphanol, and nalbuphine). In contrast, they found that on this test of nociception sex differences were not consistently observed with the kappa receptor agonists they studied (including spiradoline, U69593, U50488, enadoline, ethylketozyclazocine, and nalorphine). In another study by their group (Terner et al., 2003) the warm water tail withdrawal procedure was used to assess antinociceptive efficacy of morphine, spiradoline, and (-)-pentazocine in four strains of rats. They found morphine to be generally more potent in males, but sex differences in spiradoline antinociception were not consistently observed. In contrast, there were clear sex differences in (-)-pentazocine, which varied markedly across the four strains (from being 2.5 times more potent in male than female F344 rats, to being 11-fold more potent in males than females of the Wistar strain).

The degree to which gonadectomy alters the effects of various analgesic agents has also been studied. Kepler et al. (1989) demonstrated that castration produced small but significant reductions in the magnitude of central (ICV) morphine analgesia in male rats. Krzanowska and Bodnar (1999) showed that sham-operated female rats tested during the estrus phase were significantly less sensitive to morphine administered to the vIPAG than ovariectomized rats. Turner et al. (2002), using the tail withdrawal test with F344 and SD strains of rats, found that for the opioids they examined (including high efficacy opioids etorphine and morphine, low efficacy opioids buprenorphine and dezocine, and low efficacy, mixed action opioids butorphanol and nalbuphine) potency in males was generally higher than females, with larger differences observed with less effective opioids. They found that the effect of gonadectomy on drug potency varied as a function of the drug and strain used, but in general, resulted in a decrease in drug potency in males. In females, gonadectomy increased the antinociceptive effect of most of the drugs studied, again varying in the degree of increase by the drug and strain. In general, it appears that the influence of gonadal hormones on analgesic systems is a complex one, which likely involves both organizational and activational mechanisms. For example, Cicero et al. (2002) masculinized female rats by large doses of testosterone early in prenatal life, and found the morphine dose response curve resembled that typically seen in normal males. Similarly, Krzanowska et al. (2002), using male and female rats, were able to demonstrate reversal of sex differences in morphine analgesia elicited from the vIPAG through neonatal hormone manipulations. Activational influences of hormones on both basal nociception and sensitivity to opioid

analgesia have also been reported. For example, the potency of morphine has been shown to change across the estrus cycle in female rats and mice (Mogil et al., 2000; Kepler et al., 1989; Banerjee et al., 1983; Berglund and Simpkins, 1988). Further, Craft reports (1993) that morphine's analgesic potency is lowest (that is, female rats are most different from males), when females are in estrus, while when in diestrous, morphine's efficacy is not significantly different than males.

Findings from a recent human study (Bragdon et al., 2002) suggest that men and women might call upon different pain modulatory circuitry under stressful conditions, and that individuals with chronic pain (in their study, women with temporomandibular disorder) may be unable to effectively engage normal pain inhibitory systems. Further, Staud et al. (2003) found that diffuse noxious inhibitory control significantly inhibited thermal wind-up pain caused by repeated heat taps to the hand in normal males, but not in normal females or fibromyalgia patients. Continued exploration of sexual dimorphism in pain modulation (both facilitory and inhibitory circuits) is likely to help clarify the nature of sex differences in many pain related traits / behaviors.

iv. Genetic Differences:

To date, there have been only a few single gene pain pathologies identified, including congenital insensitivity to pain (resulting from exonic mutations of the NTRK1/TRKA gene, Indo et al., 1996) and gene mutations leading to familial hemiplegic migraine

(Ophoff et al., 1996). However, familial aggregation of pain pathologies and extremes of pain sensitivity have been repeatedly noted in humans (Bachiocco et al. 1993; Edwards et al., 1995; Violon & Giurgea, 1984; Pollard, 1985; Lester et al., 1994; Leone et al., 2001) but such findings are often attributed to shared environments and/or family modeling. Twin studies (comparing monozygotic and dizygotic twin pairs), employed across many areas of behavioral research to identify relative contribution of environmental and genetic factors, have also been used in pain research. Heritability (the proportion of overall phenotypic variance accounted for by genetic factors) has been estimated at 39-58% for migraine (Ziegler et al., 1998; Honkasalo et al., 1995; Larsson et al., 1995), 55% for menstrual pain (Treloar et al., 1998), 50% for back pain (Bengtsson & Thorson, 1991) and 21% for sciatica (Heikkila et al., 1989) (as reviewed by Mogil, 1999), 46% for carpal tunnel syndrome pain (Hakim et al., 2002), and 30-46% for osteoarthritis pain (Kirk et al., 2002). In contrast to these relatively high heritability estimates for pain associated with the above conditions, MacGregor et al. (1997) estimated heritability for a nonpathological, basal pain sensitivity (forehead pressure pain thresholds) to be only 10%, suggesting that shared environmental factors were largely responsible for correlations in pain thresholds among twins. Little other work has been done to date regarding heritability of non-pathological pain related traits in humans.

Several studies of experimental and acute pain have found that cultural or ethnic background is associated with significant variation in pain intensity reports as well as

attitudes, emotions, and behaviors associated with pain (e.g., Zborowski, 1952; Lipton and Marbach, 1984). Bates et al. (1993) reported that ethnic group affiliation was one of the best predictors of pain intensity variation, although they attributed this finding to reported pain intensity being affected by differences in attitudes, beliefs, and emotional and psychological states associated with the different ethnic groups, steering clear of any suggestion of a genetic contribution to their findings. Indeed, the large number of factors which can not be controlled for in human studies (for example, attitudes, emotions, cultural norms, etc.) make any interpretations of findings of sex differences or genetic differences in pain behaviors (baseline nociception, reported analgesic efficacy, etc) inherently suspect.

In animal studies, the contribution of genotype to individual differences in pain related behaviors is somewhat more easily (and lately, more frequently) studied. Work done with standard outbred strains, selectively bred strains, inbred strains, recombinant inbred strains, and genetic knock-outs have all advanced our understanding of individual differences in pain related measures. For example, inbred strains are derived from repeated (>20 generation) full-sibling pairing (i.e. brother x sister) mating (Festing, 1996). Mating of individuals with common ancestors increases the probability of offspring inheriting two copies of the same allele. During inbreeding, therefore, genetic heterozygosity is progressively lost as alleles of initially segregating genes are fixed into a homozygous state (Mogil, 1999). Comparison of strain differences, when environmental factors are controlled, allows for the observation of the impact of genetic

contributions to behavior. Mogil et al. (1999) reviewed many of the pain-related findings to date using inbred strains. As one example, the DBA/2J strain is relatively sensitive to opioid analgesia compared with the C57BL/6 strain (Belknap et al., 1989; Frischknecht et al., 1983). However, the C57BL/6 strain is markedly more sensitive than the DBA/2J strain to other opioid-mediated phenomena, including locomotor activity, learning/memory, and muscular rigidity (Straub tail). The dissociation of these behaviors suggests different underlying genetic control. In addition to the studies above, other experiments using inbred strains have found genetic contributions to measures such as morphine analgesic efficacy (Elmer et al., 1998; Mogil et al., 1996; Semenova et al., 1995; Belknap et al., 1989), non-mu analgesic efficacy (Wilson et al., 2003; Pick, 1996), electroacupuncture analgesia (Wan et al., 2001), nociception modulating effects of supraspinal orphaninFQ / nociceptin (Mogil et al., 1999), and cold water swim –induced analgesia (Marek et al., 1988).

As mentioned earlier, a growing literature documents the important influence of organismic factors such as sex and genotype on pain sensitivity and pain modulation. Some recent studies have examined the role of both of these factors, with interesting results. For example, Kest et al. (1999) studied supraspinal morphine analgesia in male and female mice of different strains, and found that the presence, direction, and magnitude of sex differences observed were determined by genotype. Further, in 1997, Mogil's lab determined that 3-min forced swims in 15 °C water produced non-opioid (i.e., naloxone-insensitive) analgesia in outbred Swiss-Webster mice of both sexes; this

form of stress-induced analgesia (SIA) was significantly attenuated by the *N*-methyl-*D*-aspartate (NMDA) antagonist, dizocilpine (MK-801) in males, but not females. Then, in 2000, their lab conducted and published results of experiments designed to confirm the non-opioid and (in male mice) NMDAergic nature of 15 °C swim SIA in the C57BL/6J and DBA/2J inbred strains (used widely in gene mapping). In female mice of both strains, 15 °C swim SIA was insensitive to antagonism by either naloxone or dizocilpine. In male C57BL/ 6J mice, the observed SIA was naloxone-insensitive, but was attenuated by dizocilpine. This pattern of results was virtually identical to that obtained using Swiss-Webster mice in their previous studies. However, male DBA/2J mice displayed SIA that was significantly attenuated by naloxone, but insensitive to dizocilpine antagonism. Their findings support the hypothesis that genetic factors and sex, in addition to stressor parameters, can determine the selective recruitment of alternative central mechanisms of pain inhibition.

The sex differences in analgesic effects of kappa opioid agonists demonstrated by Gear et al. (1995) have recently been studied from a different angle by Mogil et al. (2003). Via quantitative trait locus mapping followed by a candidate gene strategy using both mutant mice and pharmacological tools, they recently demonstrated that the melanocortin-1 receptor (*Mclr*) gene mediates kappa-opioid analgesia in female mice only. This finding suggested that individuals with variants of the human MC1R gene, associated in the human species with red hair and fair skin, might also display altered kappa-opioid analgesia. Mogil's group found that women with two variant MC1R

alleles displayed significantly greater analgesia from the kappa-opioid, pentazocine, than all other groups. This study demonstrates not only that pain modulation in the two sexes can involve neurochemically distinct substrates, but also provides an example of a direct translation of a pharmacogenetic finding from mouse to human.

Taken together, the last thirty years of analgesia research has led to tremendous improvements in our understanding of pain and the pathways that regulate it. Research into organismic variables influencing pain-related behaviors has not only clarified how individuals may differ on pain related measures and outcomes as a function of sex and genotype, it has also begun to help clarify some of the basic mechanisms of central modulation of pain transmission. This research helps lay the foundation for the discovery and use of increasingly effective analgesic agents and therapies.

Interestingly, however, at the end of this thirty year period of considerable progress in pain research, the drug which is perceived as the most effective analgesic agent and “gold standard” of analgesics is the same as it was at the beginning of this period: morphine. The following chapter will review this important chemical compound, its effects, and its mechanisms of action as we now understand them.

B. Morphine Mechanism of Action and Effects

i. Basic Findings

Morphine and morphine-like opiates, providing analgesia without a loss of touch, proprioception, or consciousness, are described as being the most important class of compounds used in the treatment of severe acute pain and severe cancer pain (Nestler et al., 2001). Morphine, named after Morpheus, the Greek god of dreams, was isolated by Sertuner in 1806, and remains the prototype agonist with which all opioids are compared (Astrup & Korean, 1999). To date there is no evidence that any other opioid has greater analgesic efficacy than morphine (Inturrisi, 2002), and it is thought to be the best painkiller in contemporary medicine, being commonly used for acute and chronic pain, and premedication preoperatively (Omoigui, 1995).

While preparations of opiates may have been used medicinally as far back as 300AD (Van Ree et al., 1999), it was only in the later part of the twentieth century that a number of findings began to advance our understanding of the mechanisms of actions of these substances. The structural similarity between all substances with an opiate-like action, and the discovery of opiate agonists, mixed agonist-antagonists, and antagonists, generated the concept that there existed a new class of receptors through which these agents exerted their actions (Van Ree, 1999). Discovery of endogenous opioid peptides and confirmation of multiple opioid receptors occurred during the 1970s. Three major receptor subtypes in the mammalian CNS, mu, delta, and kappa, were in close

proximity identified and differentiated on the basis of binding and distribution studies (Kuhar, Pert, & Snyder, 1973; Lord, et al., 1977; Pert & Snyder, 1973; Simon et al., 1973; Terenius, 1973). At around the same time, a number of studies suggested the existence of endogenous opioids, including those that identified substances with opioid-like activity in brain extracts (Terenius and Wahlstorm, 1974; Kosterlitz and Waterfield, 1975), and those that showed opioid antagonists exerted some intrinsic activity in opiate naïve subjects, diminishing non-drug induced analgesia (Lasagna, 1965; Jacob et al., 1974; Akil et al., 1976; Buchsbaum et al., 1977).

Over time, the work of several groups clarified the presence of discrete branches of the opioid peptide family: the proopiomelanocortin (POMC)-derived peptides, the proenkephalin-derived peptides, and the prodynorphin-derived peptides (see review by Akil et al., 1984). In the past few years, novel peptides have been discovered which may be determined to have a place within the opioid peptide family. These include endomorphin (1 and 2) (Zadina et al., 1997), which exhibits highly specific binding with the mu receptor, and orphanin FQ, also called nociceptin, identified as an activator of an opioid-like orphan receptor (Meunier et al., 1995; Reinscheid et al., 1995).

Drugs classified as opioid analgesics exert their actions at the mu, delta, and/or kappa receptors, which are G protein coupled receptors that signal via a second messenger (cyclic AMP) or an ion channel (K^+) (Gutstein and Akil, 2001). Cloning and sequencing of opioid receptors in the 1990's has greatly furthered our understanding of their mechanisms of action in terms of receptor-effector coupling. The isolation and

cloning of the rat delta (Evans et al., 1992; Kieffer et al., 1992), mu (Chen et al., 1994; Fukuda et al., 1993; Thompson et al., 1993) and kappa (Meng et al., 1993; Minami et al., 1993) receptors confirmed that opioid receptors belong to the family of seven-trans-membrane spanning receptors coupled to G-proteins inhibiting adenylyl cyclase, and have revealed significant sequence homology among the three receptor classes (approximately 60%), as well as between rodent and human mu receptors (approximately 90%). Later research has demonstrated that as part of the superfamily of G-protein coupled receptors, opioid receptors (mu, delta, and kappa) couple via heterotrimeric G proteins to a variety of downstream effectors that include not only adenylyl cyclase, but also phospholipase C, and mitogen activated protein kinase (for review see Koch et al., 2003). Opiate ligands bind to a membrane receptor, which transduces this signal into the cell via activation of a guanosine triphosphate (GTP)-binding protein (G_i/G_o) and involves inhibition of intracellular cAMP production, activation of K^+ channels, and inhibition of voltage dependent Ca^{2+} channels (reviewed by Harrison et al., 1998).

Morphine belongs to the chemical class of phenanthrene opioid alkaloids (Astrup and Korean, 1999). The phenanthrene nucleus contains three rings of carbon atoms and the fourth ring, a tertiary amine nitrogen, which is ionized at physiological pH. This ionized state enhances the water solubility and receptor binding. The stereochemical structure of the opioid is important as the levorotatory isomers are more active. The opioid effect is proportional to opioid binding, which through inhibition of adenylyl

cyclase activity via G proteins, results in hyperpolarization and suppression of discharges and evoked responses. Opioids may also interfere with calcium-ion transport across membranes and the presynaptic release of excitatory neurotransmitters (Prada, 2001).

Morphine is a potent mu receptor agonist with additional kappa receptor and delta activity (Lippman, 1990; Reisine and Pasternak, 1996). Mu opioid receptors are thought to be the most significant opioid receptors involved in analgesia, and are found in the periphery (following inflammation), spinally, and supraspinally. Specifically, they are located at pre and post synaptic sites in the spinal cord dorsal horn, within the ascending pain transmission system including the brain stem, thalamus, and cortex, and within the descending inhibitory system that modulates spinal cord pain transmission, including the rostral ventral medulla, nucleus raphe magnus, and periaqueductal grey (Inturrisi, 2002). The mu opioid receptors at which morphine exerts its main effects are subdivided pharmacologically into μ_1 and μ_2 receptors. μ_1 receptors are located outside the spinal cord, and as such are responsible for supraspinal contributions to analgesia and the perception of pain. μ_2 receptors are located throughout the CNS and are responsible for spinal analgesia, respiratory depression, bradycardia, and physical dependence (Astrup and Korean, 1999).

Spinally, opioid receptor agonists such as morphine selectively inhibit the activity of cells in the substantia gelatinosa, in the dorsal horn of the spinal cord. Opioid receptors

are highly concentrated in this region of the spinal cord. Consequently, intrathecal morphine administration results in the inhibition of painful cutaneous stimuli at the dorsal horn, and inhibition of nociceptive impulse transmission via ascending pathways. Spinal action of opioids at the dorsal horn involves inhibition of release of nociceptive neurotransmitters, such as N-methyl-D-aspartate, substance P, and glutamate, by type C pain fibers (Austup & Korean, 1999). Considerable data emphasize that opioids serve to reduce the slope of the intensity-response curve (Yaksh, 1978) and diminish the magnitude of the response evoked by small, high-threshold afferent input, with minimal effects upon the excitation caused by low-threshold afferents (Yaksh & Malmberg, 1994). Hence, they are able to produce analgesia without interfering with the individual's ability to feel non-painful stimuli.

Morphine's supraspinal modes of action, as discussed in the previous section, include activation of descending inhibitory neurons releasing neurotransmitters such as norepinephrine, serotonin, gamma aminobutyric acid, and enkephalin, providing antinociceptive action at the spinal cord. Recording from dorsal horn neurons, it has been shown that microinjection of morphine into the PAG, the locus coeruleus/subcoeruleus region, and into the raphe magnus will significantly reduce the activity evoked by noxious stimuli (Bennet and Mayer, 1979; Gebhart et al. 1984; Gebhart and Jones, 1988). Morphine administered to these sites also produces analgesia, as noted above. Further, subanalgesic doses of morphine, when administered simultaneously to certain supraspinal sites (such as the RVM, vlPAG, and locus coeruleus), interact

synergistically, producing notable analgesia (for review see Bodnar, 2000). Likewise, morphine's analgesic synergy has also been demonstrated when administered supraspinally and spinally (to the dorsal horn) (Yeung and Rudy, 1981); Roerig and Fujimoto, 1989).

The pharmacokinetics of morphine depend on the route of administration. With intravenous administration, morphine provides peak analgesic effects within 20 minutes, as compared with 30-60 minutes with intramuscular administration (Puntillo, 1994). For treating patients in pain, oral administration of morphine and other opioid drugs is usually effective as long as oral intake is possible. Unfortunately, at times adverse gastrointestinal effects or failing patient compliance may make the oral route impractical or unreliable. More invasive routes like subcutaneous, intravenous, and intrathecal infusion of opioids can improve bioavailability at the receptor sites and increase analgesic effect (Kalso, 1999). Clinically, intrathecal administration results in more effective analgesia compared with intramuscular or intravenous administration, allowing direct and more access the receptor site (although is clearly a more invasive procedure which may not always be warranted) (Astrup and Korean, 1999).

Upon administration, morphine, as a water soluble molecule, crosses the blood-brain barrier relatively slowly in comparison with other opioids such as heroin and fentanyl. Morphine is distributed through the blood and plasma throughout the body, and is known to have two main metabolites, morphine-3-glucoronide (M6G) and morphine-6-

glucoronide, both generated by hepatic metabolism. Morphine -6-glucoronide has been found to itself possess significant analgesic potency. The half-lives of both morphine and morphine-6-glucoronide are both 3-5 hours (Julien, 2002). Because the kidney plays a major role in the elimination of most opioid metabolites, their accumulation occurs in patients with renal insufficiency, and can result in an opioid-induced neurotoxicity. This is a syndrome consisting of a number of neuropsychiatric symptoms (cognitive impairment, severe sedation, hallucinations, myoclonus, seizures, and generalized hyperalgesia) that has been recognized as a problem in high-dose and prolonged opioid treatment (Hanks, De Conno, Hanna, et al., 2001, Sjogren, Jonsson, and Jensen, 1993; Daeninck and Bruera, 1999). The symptoms of opioid-induced neurotoxicity are more often seen in dehydrated, severely sick patients, especially those with failing renal function (Sjogren et al., 1993; Daeninck and Bruera, 1999).

Studies of morphine have shown a poor correlation between concentrations and effect, possibly because of relatively poor lipid solubility (Aitkenhead et al., 1984). Hug and Murphy (1981) showed that CSF concentrations of morphine peak 15-30 minutes after intravenous injection and decay more slowly than do plasma concentrations.

Therefore, peak analgesic and respiratory effects probably occur after the initial peak plasma concentration and persist after the plasma concentration has decreased (Stoelting, 1989).

Systemic administration of opioids such as morphine remains the most effective means of alleviating severe pain across a wide range of conditions (Yaksh, 1997, Portenoy and Payne, 1997). However, their clinical use has been limited by undesirable side effects that can occur in a substantial proportion of patients. These side effects include tolerance, dependence, respiratory depression, nausea, pruritus, constipation, cognitive impairment, orthostatic hypotension, sedation, altered mentation, and other aversive reactions (Jaffee and Martin, 1990; Cherny, 1996; Reisine and Pasternak, 1996; Portenoy and Payne, 1997). Morphine has also been shown to effect thermoregulation in a dose dependent manner (Jorenby et al., 1989; Rosow et al., 1980) and in rodents, frequently produces hyperlocomotion and muscular rigidity (as evidenced by the Straub tail effect) (Aceto et al., 1969). Individual differences in morphine's effects, particularly those that vary as a function of sex and genotype, will be discussed in detail below.

ii. Individual Differences: Morphine

Individual differences in morphine's analgesic effects are well documented in the literature, and it is clear that large interindividual differences exist in doses of morphine required to produce analgesic effects. Twycross (1994) reports that effective oral doses of morphine range from as little as 5mg to more than 1 gram every four hours. Similarly, in a study of postoperative morphine requirements in patients using Patient Controlled Analgesia devices (PCA), Beeton et al. (1992) found extremely wide

variation in morphine required to produce acceptable levels of analgesia. Further, Chapman et al. (1990), examining three opioid analgesics (alfentanil, fentanyl, and morphine), found noteworthy individual differences in all drugs, but the greatest level of inter individual variability for morphine (which also showed the weakest relationship between plasma concentration and analgesic effect in their study, suggesting individual differences in morphine's analgesic effects are likely strongly mediated by pharmacodynamic differences).

iii. Sex Differences

Sex differences in the analgesic effects of opioids and other drugs have been frequently reported. In the majority of studies examining possible sex differences in morphine analgesia, male rats and mice typically show greater analgesia from systemically administered morphine than do females (Kest et al., 2000). This enhanced sensitivity, shown by increased potency (i.e. lower ED₅₀ values) and/or efficacy (i.e. drug induced increase in pain response latency), has been documented across several pain assays, including thermal (hot plate tests: Cicero et al., 1996, 1997; Kavaliers & Innes, 1987, 1990; Kepler et al., 1989), somatic (tail flick-withdrawal tests: Cicero et al., 1996; Islam, Cooper, & Bodnar, 1993; Candido et al., 1992), chemical (abdominal writhing after acetic acid: Cicero et al., 1996), and visceral (hypertonic saline: Baamonde et al., 1989). It should be noted that virtually every animal study performed in which sex differences to morphine antinociception have been reported used Sprague Dawley rats, Swiss-derived mice, or deer mice (Mogil et al., 2000), with most studies finding males

to be more responsive to morphine's antinociceptive effects than females (e.g., Cicero et al., 1996; Baker and Ratka, 2002; Kepler et al., 1989; Krzanowska and Bodnar, 1999; Boyer et al., 1998; Kavaliers and Innes, 1987; Mogil et al., 2000). Therefore, the extent to which differences observed generalize beyond these particular strains is unknown.

The reported enhanced analgesic effect of morphine in males versus female is also seen in studies using a central route of administration, whether this administration is intracerebroventricular (Kepler et al., 1989; Kepler et al., 1991) or into supraspinal regions critically involved in descending opioid pain inhibition, such as the PAG and rostral ventral medulla (Krzanowska & Bodnar, 1999; 2000). The magnitude and direction of sex differences in morphine analgesia appears to vary with other intrinsic factors, however, such as genotype (Kest, Sarton, and Dahan, 2000). For example, male Sprague-Dawley and Wistar-Furth rats displayed greater morphine analgesia than their female counterparts after systemic administration, but sex-related potency differences varied in magnitude between those strains (Kasson & George, 1884). In mice, the presence, magnitude, and direction of sex differences in morphine analgesia after intracerebroventricular administration differed among eleven genetically distinct inbred mouse strains (Kest, Wilson, & Mogil, 1999). Overall, it is suggested that sex differences in morphine analgesia are mediated, at least in part, by CNS mechanisms.

Research into sex differences in analgesic effects of different opioids has not been limited to morphine. Sex differences have been reported for selective agonists and peptides acting at the mu, kappa, and delta receptors. For example, the selective mu agonist DAMGO has been reported to be more effective in the tail flick test in male than female rats when injected intracerebroventricularly (Kepler et al., 1991). Conversely, it is more effective in females than males when injected into the vIPAG (Tershner et al., 2000). The selective delta agonist DSLET, however, produced equivalent analgesia in males and females (Kepler et al., 1991). Further, a study using other delta agonists (deltorphan and DPDPE) found that the drugs did not have analgesic effects in male or female rats on the tail withdrawal test when administered intracerebroventricularly, but that with high doses, analgesia could be produced on the hotplate assay, where males were in general more responsive to morphine's effects than females (Bartok and Craft, 1997). Considerable work has also been done recently with agonists known to act preferentially at the kappa opioid receptor. Findings have varied in the nature and direction of sex differences, with some studies reporting greater analgesic efficacy in females (e.g., U69,593 injected into the rat RVM, tail withdrawal assay, Tershner et al., 2000; subcutaneous bremazocine in the rat, tail withdrawal assay, Bartok and Craft, 1997), and others reporting no sex differences (e.g., enadoline (Barrett et al., 2002) or U50488 (Patrick et al., 1999) injected intraperitoneally or subcutaneously on the tail withdrawal and acetic acid writhing assay in the mouse), or males presenting with greater analgesic effects (e.g., intraperitoneally injected bremazocine on the tail withdrawal in rats, Barrett et al., 2002).

Recently, sex differences were also reported in antinociception elicited through vIPAG microinjections of beta-endorphin in rats, with males showing increased latencies relative to females on the tail flick test (Krzanowska and Bodnar, 2000). Their group also explored the effect of D-Pro(2)-endomorphin 2 administered into the vIPAG, and found that the substance produced greater response latencies in females rats relative to male rats on the tail flick, but not the jump test (an unexpected result given morphine's greater antinociceptive effects in males than females when administered similarly). However, they found extreme overall behavioral activation (excessive grooming, seizures, barrel rolls, etc.) in males (but not females) during testing, and attributed the results to sex-dependent alterations in behavioral activation (and not nociceptive processing).

Although it is clear that sex differences in pain modulatory mechanisms exist in animals, the mechanisms at work to produce these differences are poorly understood. The importance of achieving a better understanding of these mechanisms will undoubtedly aid our understanding, and treatment, of pain in humans. However, as more and more work is done in this area, the picture appears to get even more confusing. Some researchers suggest that in general, variation in the response to opioids seems to be related to individual differences in metabolite turnover. For example, Ravenscroft and Schneider (2000) looked at steady state concentrations of morphine and its metabolites (M-6-G and M-3-G) in two patients as they took

increasing doses ordered by their physician. They found large individual differences in AUC's of morphine and its major metabolites, which represent the exposure of the patient to the drug or metabolite and the different rates of accumulation of the drug. They interpreted these findings as suggestive of an underlying pharmacokinetic basis for the individual differences in the responses to the drug by different individuals. However, Dahlstrom et al. (1992) evaluated the pharmacokinetics of morphine in patients (males and females) who received the drug through a PCA device. No gender differences were found in the amount of morphine consumed, mean plasma concentration, or minimum effective concentration. In fact, the vast majority of the literature suggests that mechanisms underlying sex differences appear to be much more complex than simply pharmacokinetic differences. For example, while two studies demonstrating larger concentration of morphine in the brain of male mice relative to female mice after systemic administration might suggest simple sex differences in drug disposition might underlie sex differences seen in morphine's analgesic effects (Candido et al., 1992; Craft et al., 1996), an extensive investigation did not support this conclusion, finding that there are in fact no differences in morphine serum levels between the sexes at times corresponding to the drug's peak analgesic effects (Cicero et al., 1997). Also, the fact that sex differences are apparent following central administration (Boyer et al., 1998; Kepler et al., 1989; Krzanowska et al., 1999) would suggest factors beyond basic pharmacokinetics clearly play a role.

Investigation of some of the “primary suspects” with regard to pharmacodynamic differences has to date only further complicated the picture. For example, there are no differences in male and female rats in brain mu or delta receptor populations (Kepler et al., 1991). In mice, different studies have reported conflicting results, with Mogil et al. (1994) reporting increased opioid receptor population levels in males, and Candido et al. (1992) reporting no differences in whole brain opioid binding. The disconnect between analgesia and receptor density is supported by findings that male mice exhibit a significantly greater morphine analgesic supersensitivity than females after chronic treatment with the opioid antagonist naltrexone, despite the absence of sex differences in the magnitude of opioid receptor up-regulation (Candido et al., 1992).

Many investigators have attempted to relate sex differences in opioid analgesic efficacy to gonadal hormone levels. In studies involving rats, it has been demonstrated that estrus phase modulates the expression of morphine analgesia, whether systemically (Banarjee et al., 1983; Berglund & Simpkins, 1988) or centrally administered (Kepler et al., 1991). In mice, however, it has been shown that opioid sensitivity does not vary throughout the estrus cycle (Moskowitz et al., 1985). Pinsky et al. (1975) suggested, from their observations, that male gonadal hormones play a permissive or agonistic role in morphine-induced antinociception, and La Bella (1975) further proposed a receptor interaction between gonadal steroids and opiates. In contrast, Slivko and Stets (1978) suggested an antagonistic role for gonadal hormones in opiate actions. In a study by Kasson and George (1982) however, both castrated and testosterone treated rats showed

no significant changes in opiate response. They proposed that, at least for the three morphine actions studied (analgesia, hyperthermia/hypothermia, and catalepsy), there is no apparent interaction between acute morphine actions and male gonadal hormones. Testosterone treatment did, however, appear to suppress tolerance to the antinociceptive effects of morphine (and not to the temperature regulation or catalepsy effects). Interestingly, while gonadectomized male rats displayed tolerance similar to control males, testosterone treated males, while having similar Day 1 ED₅₀ values to controls, showed a trend toward suppression of tolerance (to antinociception only). Taken together, the literature suggests that a role exists for modulation of pain and pain-related behaviors through gonadal hormones, but that this role may vary according to species, nociceptive assay, and/or other factors. The organizational effects of gonadal hormones play a critical role in morphine analgesia following either systemic or vIPAG administration. Thus, male rat pups neonatally castrated one day after birth display morphine analgesia as adults that is comparable to female rats and significantly lower than male rats. Female rat pups neonatally androgenized with testosterone one day after birth display morphine analgesia as adults that is comparable to male rats and significantly higher than female rats (Cicero et al., 2002; Krzanowska et al., 2002).

Some of the inconsistency of findings in sex difference literature, or the inability at times for one lab to replicate a finding reported by another, has been attributed to estrus cycle variability, test specificity, and experimental parameters (e.g., Bartok and Craft, 1997; Kavaliers and Innes, 1987). Genotype of test subjects is likely to be another

variable which contributes to these disparate findings. For example, Mogil (2000) surveyed supraspinal morphine analgesia in 11 inbred strains and found no difference in morphine analgesia in 7 of the strains, three strains in which males displayed higher sensitivity to morphine's analgesic effects (AKR, B6, SWR), and one strain in which females were more sensitive (CBA). While far from simplifying the already complex question of sex differences in pain related behaviors, studies involving multiple inbred strains provide a unique opportunity to better understand individual differences and guide future work in the area.

Miaskowski and Levine (unpublished article) reviewed the human literature for studies evaluating possible gender differences in analgesic consumption through PCA use in the immediate postoperative period. In four studies they reviewed (together reflecting over 900 men and 900 women) it was found that when morphine was the drug used, males consumed significantly more morphine than did females (on average 2.4 times more opioids when morphine data was collapsed with studies of other opioid drugs). While the assumption in interpreting these studies might be that both males and females titrate their medication to the minimum effective plasma concentration (i.e., a concentration that would produce an optimal balance between adequate analgesia and an acceptable level of side effects), the findings suggest only that differences existed in the amount of morphine consumed. The lack of data on pain intensity ratings subsequent to analgesic dosing in these studies precludes any interpretations regarding gender differences in the drug's actual analgesic effect in each sex.

As noted earlier, systemic morphine antinociception partially involves mu opioid receptor activation of a descending pain inhibitory circuit that includes neuronal connections between the ventrolateral periaqueductal gray and rostral ventromedial medulla (Fields and Basbaum, 1994). Mu opioid receptor agonist injection into either the vIPAG or RVM produces antinociception and mu opioid receptor antagonists at either site block the antinociceptive effect of systemically administered mu agonists (Dickenson et al., 1979; Azami et al., 1982; Jensen and Yaksh, 1986; Bodnar et al., 1988; Smith et al., 1988; Manning and Franklin, 1998). In contrast to mu agonists, Pan et al., 1997 showed that a kappa opioid receptor selective agonist in the RVM attenuates antinociception produced by mu opioid receptor agonists in the vIPAG in male rats. However, Tershner et al. (2000) found that in the RVM, the same dose of kappa opioid receptor agonist that reduces mu receptor-mediated increase in tail flick latency in male rats produces a moderate increase in tail-flick latency in female rats. In addition, they found that female rats were significantly more sensitive to the mu opioid agonist DAMGO, injected into the ventrolateral PAG. The results suggest that these two brain structures, which mediate the analgesic effects of opioids, are sexually dimorphic with regard to opioid receptor function (although to date this finding has not been replicated.)

iv. Genetic Differences

A number of genetic correlations among pain phenotypes have been reported to date, including those related to morphine's analgesic efficacy. It has been well established by a number of groups using rodents that there exists a negative genetic correlation

between initial nociceptive sensitivity and subsequent morphine analgesia (Elmer et al., 1997, 1998; Panocka et al., 1986; Mogil et al., 1996). Animals that were initially sensitive to noxious stimuli tend to exhibit modest analgesic responses to morphine. Most recently, Mogil's lab (Mogil et al., 1999), using 11 inbred mouse strains and 12 separate commonly used nociceptive assays, identified three clusters of genetically correlated nociceptive assays. By using multivariate analyses, they found that for three identified clusters, within cluster genetic correlations greatly exceeded between cluster correlations. These clusters were as follows: "thermal" (Hargreaves' tests, hotplate test, tail-immersion/withdrawal test, and, surprisingly, autotomy), "chemical" (acetic acid abdominal constriction, magnesium sulfate abdominal constriction, acute and tonic- phase formalin test), and "mechanical-hypersensitivity" (von Frey test, carrageenan thermal hypersensitivity, peripheral nerve injury thermal, and mechanical, hypersensitivity). They later refined their analysis through the inclusion of additional assays, resulting in their identification of at least five fundamental types of nociception and hypersensitivity: baseline thermal nociception, spontaneous responses to noxious chemical stimuli, thermal hypersensitivity, mechanical hypersensitivity, and afferent-input dependent hypersensitivity (Lariviere et al., 1992). Interestingly, in mice at least, morphine's efficacy at alleviating pain caused by these assays may vary as a function of the pain subtype, with low doses of morphine being effective for tests in the "spontaneous responses to noxious chemical stimuli" subgroup (such as the acetic acid test), and larger doses for tests in the baseline thermal nociception group (hot plate and tail withdrawal) (see Hammond, 1989).

Mice selectively bred for high/low swim stress analgesia (HA/LA mice), and high/low levorphanol analgesia (HAR/LAR mice) also demonstrate the genetic mediation of sensitivity to morphine's analgesic effects, as demonstrated by a study by Mogil et al. (1996), in which they showed an inverse relationship with respect to morphine antinociceptive potency in hot plate and acetic acid abdominal constriction tests. Likewise, there have been a number of studies showing strain differences in morphine's analgesic effects (Semenova et al., 1995; Sudakov et al., 1996; Elmer et al., 1998; Pick et al., 1991, Belknap et al., 1989, etc.), as well as studies showing altered morphine efficacy as a result of targeted mutations (Bohn et al., 2000; King et al., 2001; Bohn et al., 2002; Jasmin et al., 2002; Thompson et al., 2000).

In addition to quantitative differences listed above, some interesting qualitative strain differences of relevance to pain have been noted. For example, a number of investigations have suggested that certain strains activate opioid analgesic systems after exposure to stress, whereas others produce equivalent amounts of SIA, but through a non-opioid (naloxone-insensitive) mechanism (Helmstetter & Fanselow, 1987; Mogil & Belknap, 1997; Urca et al., 1985). Vaccarino (1998) reported that naloxone injection produced paradoxical analgesia on the formalin test in BALB/c mice, but not in standard outbred CD1 mice or inbred C57/BL6 mice. Taking studies of genetic contributions in animals to the next (and arguably, most important) step, there have been three studies demonstrating linkage of a pain related trait to chromosomal

locations in the mouse. Each of these studies used recombinant inbred strains derived from C57/BL6 and DBA/2 strain intercrosses. First, Belknap et al. (1995) found that allelic variation at the QTL identified accounted for about 30% of the observed genetic variability in morphine analgesia, and is also associated with other opioid traits as well. Subsequently, Mogil et al. (1997), mapped basal thermal nociceptive sensitivity by using the hot plate test in the same recombinant inbred strain, and found a sex-specific quantitative trait locus (QTL) for the trait, which encoded the mouse delta receptor type. Specifically, they found a sex and strain dependent pattern of responses, with the delta specific antagonist naltrindole lowering nociceptive latencies in the following order of efficacy: D2male > B6male > D2 female > B6 female. Similarly, as mentioned earlier, Mogil's lab has also identified a female specific QTL for non-opioid (naloxone insensitive) stress induced analgesia, cold-water swim induced analgesia which is attenuated by NMDA antagonists in males, but not in females (Mogil et al., 1997). Overall, it is clear that both genotype and sex, under certain circumstances, may contribute to pain-related measures / outcomes. The degree to which they exert control over the development of morphine tolerance will be the main focus of the present dissertation.

C. Putative Mechanisms of Morphine Tolerance

i. Basic Findings

One of the major limitations of the clinical use of opiates is their tendency to induce tolerance (Harrison, 1998). Koob and Bloom (1988) define tolerance as “the need, during chronic drug exposure, for increasing amounts of the drug to achieve the original effect”. During the course of drug administration, in fact, the effective dose of a drug can increase by as much as 100 or even 1000-fold (Smith & Law, 1988). In studies within the analgesic tolerance literature, tolerance is typically operationally defined either as a rightward shift in the function-relating morphine dose to pain sensitivity or as the reduction in the efficacy of a single dose of the drug across multiple administrations.

Patients vary greatly in the opioid dose required to manage pain. A survey of patients with advanced cancer observed that the average daily opioid requirement was equivalent to 400-600mg of intramuscular morphine. However, approximately 10% of patients required greater than 2000mg and one patient required over 35,000 mg per 24 hours (Coyle et al., 1990). The need for escalating doses is a complex phenomenon. While most patients reach a dose that remains stable for prolonged periods (Brescia et al., 1992) some do not, and require rapidly escalating doses. While disease progression, psychological distress, or even changes in the pharmacokinetics of the drug may be involved, true “pharmacological tolerance”, presumably involving changes at the

receptor level (Foley et al., 1991; Lufty & Yoburn, 1991), is likely to play an important role.

Clinically, tolerance to the nonanalgesic effects of opioids also occurs, albeit at varying rates for different effects. For example, while tolerance to respiratory depression, somnolence, and nausea generally develops rapidly, tolerance to opioid induced constipation and pupillary effects develop slowly, if at all (Foley, 1991).

It should be noted that tolerance to a particular effect of a drug can occur from exposure to that specific drug, a related drug, or at times even an unrelated drug. A distinction is often made between acute tolerance, decreases in sensitivity that develop during a single drug exposure, and chronic tolerance, decreases in sensitivity that develop from repeated or sustained exposure (see Rosenfeld and Burkes, 1977; Yano and Takemori, 1977; and Huidobro-Toro et al., 1978). The degree to which similar processes underlie both acute and chronic processes has yet to be fully understood. Additionally, literature relating to drug tolerance often distinguishes between “associative” (e.g., Siegel 1975; Kersner and Cook, 1983; Shapiro et al., 1983; Maude-Griffin and Tiffany, 1989; Tiffany and Maude-Griffin, 1988) and “non-associative” (e.g., Sklar and Amit, 1989; Tiffany and Maude Griffin, 1988) forms of tolerance, the former typically referring to experimental paradigms in which environmental stimuli paired with drug use become conditioned stimuli that elicit classically conditioned compensatory responses. “Non-associative” tolerance studies (often not explicitly labeled as such, but rather lacking

reference to issues of learning / conditioning) typically refer to forms of tolerance presumably caused by adaptive cellular changes in response to the chronic presence of the drug.

It is likely that most forms of behavioral tolerance reflect the convergence of contributions of both associative and non-associative factors, with drug-conditioned responses emerging in anticipation of the actual pharmacological changes elicited by the drug, and interacting with the drug induced unconditioned responses. Earlier studies dissociated the two components, noting that under conditions in which low doses of a drug, and relatively long inter-dose intervals are used, rightward shifts in drug dose response curves typically reflect associative factors, while when doses used in tolerance induction are higher, and inter-dose intervals shorter, nonassociative factors become increasingly evident (reviewed by Tiffany and Maude, 1988).

More recently, some interesting work has explored each of these contributions in isolation, and suggests that the involved processes may be quite different (in terms of their temporal and neurochemical parameters). For example, Azorlosa (1994) demonstrated that naloxone pretreatment prior to morphine injections over a 14 day period attenuated tolerance development in a group of rats receiving their injections in their home cage (unpaired), but had no effect on context-specific (associative) tolerance demonstrated in a group which received their injections in a special room. Grisel et al. (1996), also using context paired and context unpaired testing groups, found that spinal

administration of a selective neurotensin antagonist selectively abolished associative tolerance, while pretreatment with MK801 (the NMDA receptor antagonist) had generally equal effects in both groups (suggesting a role in non-associative tolerance). Further, Mitchell et al. (2000) showed that morphine given in an associated context increased Fos-like immunoreactivity in the lateral amygdala and CA1 region of the hippocampus, and that microinjection of the cholecystokin B antagonist L-365,260 into the amygdala blocked associative tolerance. (While clearly the biological bases of the relative contributions of associative and non-associative factors have just begun to be explored, issues relating to possible contributions of learning to findings of the present studies will be discussed as they arise.)

Overall with regard to tolerance, potential mechanisms underlying its occurrence are commonly divided into two categories: pharmacokinetic and pharmacodynamic processes (Julien, 2001). Pharmacokinetic, or dispositional, processes, are those which reduce the concentration of a drug or its duration of action in a target system. These include changes in absorption, distribution, metabolism, or excretion that affect the concentration of a drug at effector sites. Pharmacodynamic, or functional processes, are those adaptive processes which reduce the sensitivity of the site of action to a given drug concentration.

Pharmacodynamic processes which underlie tolerance development may involve a number of mechanisms which can be viewed as either decremental or oppositional (Littleton and Harper, 1989). Any reduction in the activity of a drug at its receptors,

such as receptor down-regulation or desensitization which might occur in the sustained presence of the drug, and result in a reduction of the intensity of the drug-induced biological signal, would be considered decremental processes. Oppositional processes, conversely, would involve counter adaptations in response to the sustained presence of the drug. In effect, continued presence of a drug would recruit processes that oppose the initial effects of the drug. Thus, there could be a constant signal intensity (the interaction between the drug and its receptors remains unchanged in the presence of the drug chronically being “on board”), but the initial effects of the drug are opposed or counteracted by other systems. (For example, homeostatic changes in biochemical, cellular, and/or effector systems that mediate the primary drug effect, or even in other systems that are changed by these effects). Decades of research in opioid tolerance has established that the changes caused by sustained opioid receptor activation are complex and multifaceted (for reviews, see de Leon-Casasola, 2002; Kieffer and Evans, 2002; Nestler, 2001; Williams et al., 2001; Mayer et al., 1999; Harrison et al., 1998). In fact, a complicating factor in the interpretation of mechanisms by which modulation of antinociceptive tolerance might occur is the great diversity of substances that have been reported to produce such effects (Vanderah, 2001). These include, among others, CGRP antagonists (Menard et al., 1996; Powell et al., 2000), NO synthase inhibitors (Powell et al., 1999), calcium channel blockers (Aley and Levine, 1997), CCK antagonists (Xu et al., 1992), cyclooxygenase inhibitors (Powell et al., 1999) and protein kinase C inhibitors (Mao et al., 1995). Relevant research findings for putative

mechanisms of morphine analgesic tolerance, both decremental and oppositional, will be discussed in turn.

Receptor Downregulation / Upregulation

One of the earliest explored potential contributions to morphine tolerance was the process of receptor down regulation. Much of the work exploring down-regulation (reduction in available receptors or their sensitivity to the drug) as a mechanism of morphine analgesic tolerance has been done in vitro (see Harrison et al., 1998, for review). For example, studies using mu receptor containing mouse pituitary tumor cells showed down regulation in response to prolonged exposure to high concentrations of morphine (Puttfarken et al., 1988). Further, Zadina et al. (1993, 1994) showed that morphine decreased both mu and delta receptor Bmax in human neuroblastoma cells. More recently, it has been shown that while DAMGO (a selective mu agonist) treatment causes labeling of endocytic vesicles in transfected HEK 293 cells, morphine did not have an affect the cellular distribution of mu receptors (Arden et al., 1995). Similarly, Keith et al. (1996) found that while enkephalins and etorphine could cause internalization of opiate receptors into transferin-containing endosomes, morphine did not internalize either mu or delta receptors, even at high doses capable of inhibiting cAMP. These studies contrast the ligand binding studies which indicate that morphine and specific agonists can both decrease the Bmax of mu and delta receptors (as reviewed by Harrison et al., 1998), but support the findings of in vivo studies, which show that down regulation of opiate receptors in response to chronic agonist treatment

is not a consistent finding in adult animals. In fact, either down-regulation, up-regulation, or no change in receptor number has been found after agonist treatment (Tao et al., 1987a, Tao et al. 1987b, Yoburn et al., 1993). In general, while down regulation has been observed for mu and delta agonists (PL017, DPDPE, etc.), studies of chronic morphine treatment in adult animals have often shown up-regulation instead. Several studies have suggested specifically that morphine up-regulates a subset of opiate receptors, those in the mu-delta complex (Rothman et al., 1989; Rothman et al., 1986). It has also been shown that up-regulation of such sites occurs preferentially in the striatum, and in distinct nuclei of the hypothalamus and the amygdala (Bhargava & Gulati, 1990). The few studies that show down-regulation of receptors after morphine treatment indicate that this occurs only in select brain areas (pons, medulla, and cerebral cortex) (Brady et al., 1989) and that only some high affinity sites are down regulated (Werling et al., 1989). Since morphine can clearly induce behavioral tolerance in these animals, such studies have seriously challenged the role of receptor down-regulation in the mechanism of tolerance (Harrison et al., 1998).

The study of mRNA levels after chronic drug treatment presents another avenue to explore possible mechanisms of tolerance. However, two different morphine treatment regimens in rats have failed to decrease mu receptor mRNA. Specifically, despite the presence of behavioral tolerance (Brodsky et al., 1995) or withdrawal (Castelli et al., 1997), both of these studies found that there were no significant decreases in mRNA levels in several CNS areas involved in pain modulation: dorsal horn of the spinal cord,

nucleus raphe magnus, PAG, hypothalamus, medial thalamus, or sensory motor cortex, striatum, nucleus accumbens, or septum. Overall, with regard to the study of mRNA levels following chronic morphine treatment, it has been demonstrated that changes in mRNA levels do not appear to be correlated with either receptor binding or behavioral measures of tolerance (Harrison et al., 1998). What is clear from the studies presented above, however, is that in *in vitro* studies, mu and delta receptors do indeed have the ability to down-regulate when exposed chronically to agonists, including morphine. However, down-regulation does not consistently occur *in vivo*. Further, the time course of down-regulation does not match the time course for tolerance, and down regulation can not alone account for the high degree of tolerance that can be attained (Smith and Law, 1988).

Desensitization:

Agonist induced receptor-effector uncoupling, or desensitization, is another possible mechanism of morphine tolerance (Arden et al., 1995). Desensitization can involve agonist-induced opioid receptor phosphorylation resulting in the uncoupling of the receptor from the G-proteins, or a loss of the ability of a drug to inhibit cAMP to below control levels (Koch et al., 2003). Early studies demonstrated that the ability of morphine to inhibit AC acutely (Sharma et al., 1975a) appears to be lost during chronic treatment, as cAMP levels return to normal (Sharma et al., 1975b). It has been postulated by Sharma's group that chronic exposure of cells to morphine resulted in a compensatory increase in AC activity (and thus cAMP levels), rather than a loss in the

ability of morphine to inhibit AC (thus suggesting the “decremental” process of desensitization itself involves “oppositional” processes).

Studies of desensitization in receptors as a result of morphine administration show inconsistent results. For example, in Neuro2A cells transfected with the mu receptor, both DAMGO and morphine desensitized receptors in a time and concentration dependent manner (Chakrabarti et al., 1995). In contrast, in mu receptor transfected HEK 293 cells, morphine, DAMGO, and levorphanol all failed to desensitize the receptor and in fact caused a compensatory increase in forskolin-stimulated cAMP accumulation after chronic treatment, a phenomenon referred to as super sensitization (Blake et al., 1997). Intrinsic differences in the two cell lines into which receptors were transfected could account for the differences in these two studies. However, desensitization could be detected in the HEK 293 cells in response to other drugs (buprenorphine and methadone). Other possible explanations for the contrasting results are species differences in the mu receptor used (unknown for Chakrabarti et al., 1995; and mouse for Blake et al., 1997) or differences in the expression level of the transfected receptor (as reviewed by Harrison, 1998)

Phosphorylation has been suggested to play a role in the desensitization of mu, delta, and kappa receptors (Koch et al., 2003). In mu receptor transfected CHO cells, a phosphoprotein can be immunoprecipitated by use of an antibody to the mu receptor. The levels of this protein are increased five-fold by morphine treatment or by activation

of PKC. The time course of this event was correlated temporally with desensitization in a separate electrophysiological preparation (Zhang et al., 1996). Similarly, in HEK 293 cells transfected with the mu receptor, basal levels of an immunoprecipitated phosphoprotein are enhanced 1.8 fold by morphine treatment (Arden et al., 1995). In vivo studies have also explored the role of cAMP in tolerance and dependence. It has been shown that acute opiates can decrease levels of cAMP in the striatum (Bhoola et al., 1986; Blume et al., 1979), and that after chronic treatment with opiates the ability to inhibit CAMP is lost. For example Noble and Cox (1996) show that chronic morphine treatment results in desensitization of both mu and delta receptors in the thalamus and PAG. This desensitization is heterologous, with metabotropic glutamate and 5-HT_{1a} receptors also desensitizing. In the same animals there is homologous desensitization of delta receptors in the nucleus accumbens and caudate-putamen. Thus, even within the same animals, there can be different adaptations to chronic treatment, depending on the brain area studied.

A growing body of evidence indicates a specific role for protein kinases, particularly protein kinase C (PKC), in the development of antinociceptive tolerance (Narita et al., 1994; Narita et al., 1994; Mayer et al., 1995). It has been shown that intrathecal administration of GM1 ganglioside, a reported inhibitor for PKC translocation from cytoplasm to cell membrane, attenuates the development of tolerance to the antinociceptive effects of morphine in rats (Mayer et al., 1995). In addition, Mayer's lab also showed that spinal cord levels of membrane-bound PKC increase reliably as

morphine tolerance develops (1995). This increase occurs mainly within laminae I-II of the spinal cord dorsal horn, a region similar to the one showing increased levels of PKC translocation in nerve injured animals with demonstrable thermal hyperalgesia (Mao et al., 1992; Mao et al., 1993). This, and other (Mao et al., 1995), work leads to a strong suggestion of a common mechanism, or at least location, for the development of hyperalgesia and morphine tolerance in the dorsal horn regions of the spinal cord.

The exact mechanism of desensitization (an up-regulation of cAMP versus physical uncoupling of receptors and subsequent loss of ability to inhibit cAMP) remains controversial in in vivo studies. The discovery that opiate receptor activation is linked to GTPase activity (Koski and Klee, 1981) implicated G-proteins in mediating the coupling of receptors to AC and focused attention to the uncoupling of receptors and G-proteins as a mechanism of desensitization and thus tolerance. As reviewed by Harrison et al. (1998), some studies in rats implicated uncoupling from G-proteins during morphine tolerance by showing that stimulation of GTPase is decreased in tolerant animals (e.g., Barchfield and Medzihradsky, 1984). More recent studies have measured [³⁵S]GTPγS binding, as an indication of activation of G-proteins and thus receptor-G protein coupling. In rats treated chronically with morphine, there is a decrease in activation of G-proteins in some brain areas (dorsal raphe, locus coeruleus, lateral and medial parabrachial nuclei, commissural nucleus tractus solitarius) but no change in others (nucleus accumbens, amygdala, thalamus, substantia nigra). Thus, again there are differences among brain areas of the same animal with regard to desensitization

during chronic agonist exposure, with changes apparent only in brainstem nuclei involved in physiological homeostasis and autonomic function (Sim et al., 1996).

However, another study by the same lab examined changes in G-protein activation in the locus coeruleus in more detail, and indicated that there was a decrease in both basal and opiate-stimulated G-protein activation, with no change in the percent stimulation by agonist. Thus, in this case, there was a decrease in G-protein activity in the locus coeruleus with chronic morphine, but no desensitization (Selley et al., 1997).

Numerous *in vitro* studies indicate that arrestins are involved in desensitization of G-protein coupled receptors (see Claing et al., 2002 for review), and it has been proposed that this family of proteins could be involved in the development of tolerance *in vivo*. Interestingly, morphine tolerance was delayed in mice lacking the B-Arr2 (Bohn et al., 2002). Future work in this area will likely clarify the role that arrestins may play in morphine tolerance in animals which lack this genetic alteration.

Receptor Internalization

With the cloning of opioid receptors has come a better understanding of the mechanisms that regulate the life cycle of receptors. It is clear that opioid receptors, as is the case with many G protein linked receptors, are not static and cycle to and from the plasma membrane (Williams et al., 1999). Receptor trafficking initiated by agonist binding and internalization through the endosomal pathway may be involved in desensitization and /or the initiation of nuclear signaling. While receptor internalization

may subserve aspects of tolerance to some agents, it has been determined that morphine is among the agonists that do not, under most circumstances, cause internalization.

Given the idea that receptor internalization may be a mechanism that mediates receptor turnover and desensitization, the inability of morphine to mediate this basic response could result in chronic receptor activation. The continuous stimulation of transduction pathways may recruit signaling pathways to cause downstream adaptations (counter adaptations) with repercussions unrelated to direct actions of opioids (Williams et al., 1999).

Whistler and colleagues (Whistler et al., 1999) suggested that receptor uncoupling, endocytosis, and adaptive responses could be linked, and showed that some agonists, like methadone, are efficient at causing receptor internalization whereas other agonists, such as morphine, are not. From those initial observations, a ratio was calculated in which the relative ability of a given agonist to activate potassium currents (relative activity: RA) was divided by its ability to cause receptor internalization (versus endocytosis: VE). Agonists with large RA/VE numbers activated potassium currents but were poor at triggering internalization. This ratio, termed RA/VE, was hypothesized to determine which form of tolerance developed in response to chronic agonist treatment. Agonists with high RA/VE values would activate various effectors for a prolonged period, because of the inability to signal the endocytic pathway, and compensatory cellular adaptations would mediate tolerance. Agonists with low RA/VE

values, on the other hand, would be uncoupled from effectors, removed from the membrane, and no compensatory mechanism would develop.

To further explore this hypothesis, Finn and Whistler (2002) used different mutated forms of the mu opioid receptor to examine the role of receptor internalization in the development of tolerance and withdrawal to opioids. Their results showed that when activated receptors remain on the surface of the plasma membrane, there is an upregulation of adenylyl cyclase and increased gene expression through CREB. This adaptive response results in cellular tolerance. Under conditions in which receptors are internalized, however, adenylyl cyclase activity and CREB-mediated gene expression were reduced. These findings support the RA/VE hypothesis, and suggest that the degree to which receptors internalize can be predictive of the manner of tolerance development.

Stafford et al. (2001) examined the differences in opioid tolerance and changes in receptor number following chronic treatment with morphine and etorphine. Etorphine is a potent agonist and an efficient activator of receptor internalization so that it has a RA/VE value that is much lower than that of morphine (Whistler et al., 1999).

Stafford's group showed that morphine treatment showed a decrease in the analgesic potency, an effect that was linearly dependent on the dose. This decrease occurred despite no change in opioid receptor number, consistent with previous studies. A very different pattern was observed in animals treated chronically with etorphine. Little or

no tolerance developed with lower doses of etorphine, but as the dose of etorphine was increased, tolerance increased sharply. Interestingly, at high doses, there was also a 50% downregulation of receptors. The interpretation based on the RA/VE hypothesis would be that morphine treatment induced an adaptive mechanism that resulted in tolerance. Etorphine, on the other hand, being both a potent agonist and an efficient activator of receptor internalization, caused little tolerance at lower doses and at higher doses caused tolerance through a downregulation of the receptors.

Given that there are multiple downstream signals induced by chronic treatment with morphine, it is possible that treatment of cells with morphine for periods of hours (as in most in vitro studies) may not model the full complexity of changes seen with much more prolonged treatment periods in the whole animal. Downstream events not captured by in vitro studies may include upregulation of key components of the endocytic pathway, which could dramatically change receptor trafficking, and promote internalization of mu receptors following chronic morphine administration. For example, an increase in the level or activity of key components of the endocytic pathway, such as G protein receptor kinase (GRK), arrestin, or dynamin, have all been shown to increase morphine-mediated receptor internalization (Whistler and von Zastrow, 1998; Zhang et al., 1998; Noble et al., 2000; Hurle, 2001). In fact, simple co-administration of DAMGO, the selective mu receptor agonist, with morphine was shown to facilitate endocytosis of the mu opioid receptor (He et al., 2002). As a result, rats treated chronically with both drugs showed reduced analgesic tolerance compared to rats treated with morphine alone. These studies suggest that in the case of morphine,

promoting endocytosis of the mu opioid receptor can actually reduce the development of tolerance.

Other cellular and pharmacological properties potentially involved in the development of tolerance include differences in intrinsic efficacy between agonists, receptor reserve, constitutive activity of receptors, and rate of receptor recycling (see Chavkin et al., 2001, for review). Further, a large body of literature suggests that neither receptor down regulation, receptor trafficking, nor desensitization may entirely explain the phenomena of tolerance. Other mechanisms, such as the presence of “antiopiate” systems, or “between systems adaptations” may also play a role.

“Anti-opiates”

The sustained presence of exogenously delivered opiates may induce the action of a compensatory opioid modulating system. This system could act at the levels of receptor number and desensitization, or exert opposing behavioral effects through distinct mechanisms. A few of such agents studied to date include neuropeptide FF, the Tyr-MIF-1 family of peptides, orphan FQ/nociception, and CCK-8 (Harrison et al., 1998).

NPFF binding sites, among other areas, are found densely in the dorsal horn of the spinal cord, with binding site localization roughly paralleling those of peptide localization and suggesting a role in analgesic responses (Allard et al., 1992). Initially described as an antiopiate peptide, it was shown to decrease tail flick latency in rats,

inducing hyperalgesia, as well as attenuated morphine induced analgesia (Yang et al., 1985). It was subsequently shown, using the formalin assay, that NPFF delivered directly into the ventral tegmental area can block analgesia caused by morphine injected into the same area, as well as analgesia induced by foot shock stress (Altier et al., 1997). However, there have also been several reported cases of the peptide producing analgesia and potentiating morphine -induced analgesia (Kontinen and Kaslo, 1995), complicating our understanding of the role this peptide and its analogues may play. Some role for NPFF in tolerance is also suggested, but by no means definitively confirmed. Chronic morphine increases levels of NPFF in cerebrospinal fluid (Mallin et al., 1990) and stimulates its release from spinal cord slices in vitro (Devillers et al., 1995). Continuous infusion of NPFF decreases mu opiate receptor number in areas of the brain rostral to the PAG (Goodman et al., 1996). However, chronic morphine treatment does not have reciprocal effects on NPFF receptors (Payza et al., 1993). Finally, antibodies to the peptide can reverse morphine tolerance (Lake et al., 1991).

As with NPFF, orphanin FQ has been variously shown to display analgesic and antianalgesic properties, and receptors have been localized in areas compatible with a role in modulating nociception (Harrison, 1998). Recent findings suggest a possible role for nociceptin in modulating morphine tolerance. Tian et al. (1998) found that intracerebroventricular administration of an anti-OFQ antibody (OFQ-ab) resulted in a 50% reduction in the development of chronic morphine tolerance in rats, but did not affect acute tolerance. Their lab subsequently showed an 81% increase in OFQ

immunoreactivity in the PAG after a five-day period of morphine administration (elevations were 17% and 34% on the first and third day of this treatment, respectively) (Yuan et al., 1999). Lufty et al. (2001) found that intracerebroventricular OFQ attenuated chronic morphine tolerance on the hot plate test, and even further reductions in tolerance with OFQ administered to the hippocampus, suggesting an effect on learning may form the basis for its modulation of tolerance. However, Kest et al. (2001), using mice lacking the OFQ peptide due to targeted gene mutation, found no differences in the degree of morphine tolerance seen in knock out animals as compared with mice heterozygous for the mutation and wild type animals. While clearly compensatory changes in these animals could have been responsible for their apparently normal behavioral response to chronic morphine treatment, the exact role that the OFQ peptide or its receptor may play in morphine tolerance is still unknown.

The Tyr-MIF-1 peptides, which have been demonstrated to bind to the mu receptor, are also potential modulators of opioid tolerance. They can act as either opiate agonists or antagonists when binding to opiate receptors in the guinea pig ileum preparation, with the antagonist properties being most evident when the receptor reserve is low, as in tolerance (Zadina et al., 1992). Chronic, but not acute morphine, has been shown to reduce binding of Tyr-MIF (Zadina, 1989). Additionally, in *in vitro* studies, Tyr-MIF-1 has been shown to attenuate the effects of chronic opiates on receptor number (Harrison et al., 1998). As with the other potential “anti-opiates” mentioned, the exact mechanism, if any, by which this peptide might contribute to tolerance development is

as yet unknown. Interestingly, sex differences in the antiopioid effects of the endogenous peptides Tyr-MIF-1 and neuropeptide FF on morphine analgesia have been reported (Kavaliers and Innes, 1992 a, b), although the connection of this finding, if any, to possible sex differences in morphine tolerance, has not yet been explored.

CCK-8, another neuropeptide with reported “anti-opioid” activity, is perhaps the most well studied of the above substances with regard to its potential role in tolerance. Chronic administration of opiates (as well as spinal cord and other CNS injuries) elevates the level of cholecystokinin (CCK) in the spinal cord, and such elevated levels appear to antagonize opioid effects, resulting in significantly diminished analgesia (Watkins et al., 1984; Xu et al., 1993, 1994). Administration of CCK inhibitors such as proglumide (Watkins et al., 1984) or other selective CCK antagonists (Idanpaan-Heikkila et al., 1997; Xu et al., 1992), themselves having no analgesic properties, have nonetheless been shown to reverse morphine tolerance and increase analgesic effects of morphine. This effect, however, has been determined to be limited in duration, with its benefits waning after about a week (Kellstein & Mayer, 1990), and the mechanisms by which these effects are accomplished are poorly understood.

“Between-Systems Counter Adaptations”

It should be noted that adaptations subserving tolerance may occur either within a given drug-sensitive neuronal system or between systems. In the case of within-system adaptations, repeated drug treatment would elicit opposing activity in the system

through which a drug induces its primary pharmacological actions. For example, Nestler et al. (2001) review evidence indicating that, in the locus coeruleus, opioid tolerance involves up-regulation of cyclic adenosine monophosphate (cAMP) systems that oppose an initial sustained opioid-induced inhibition of adenyl cyclase. In the case of between-system adaptations, initial changes in primary drug-sensitive neurons would trigger adaptations in biochemical, cellular, or behavioral pathways different from those directly involved in the initial pharmacological actions of a drug. Such between-systems adaptations may be implicated, for example, in the apparently pervasive role of *N*-methyl-D-aspartate receptors in modulating tolerance to opiates and other drugs. Trujillo and Akil (1991) reported that systemic co-administrations of morphine and the non-competitive NMDA receptor antagonist MK-801 prevented the development of morphine analgesic tolerance but did not reverse the expression of this tolerance once established. These actions of MK801 were mediated, at least in part, by the drug's actions on the spinal cord (Gutstein & Trujillo, 1993). Subsequently there have been several reports that systemic, intracerebroventricular, or intrathecal administration of NMDA receptor antagonists prevent the development and/or attenuate the expression of morphine analgesic tolerance (Ben-Eliyahu et al., 1992; Mao et al., 1994; Marek et al., 1991; Marek et al., 1991). Moreover, the same intracellular processes linked to NMDA receptor activation and critical for the production of hyperalgesia (e.g. synthesis of nitric oxide, increases in the levels of protein kinases) have been implicated in the development of morphine analgesic tolerance (e.g. Elliot et al., 1994; Kolesnikov, 1993; Mao et al., 1994). If hyperalgesia and morphine analgesic tolerance are mediated by the

same spinal mechanisms, then rats tolerant to morphine analgesia should also be hyperalgesic. Indeed, Mao et al. (1994) reported that rats subjected to repeated intrathecal injections of morphine developed a considerable hyperalgesia when they were tested for pain sensitivity using the tailflick test between injections of morphine, and both the tolerance and hyperalgesia which accrued from these repeated intrathecal injections of morphine were prevented by coadministration of an NMDA receptor antagonist. These results are clearly consistent with a role for spinal mechanisms for hyperalgesia in tolerance development.

The spinal cord has been suggested as a site of this NMDA action, since spinalization does not stop systemic MK-801 from preventing the development of morphine tolerance (Gutstein et al., 1992). Also, an identified consequence of opioid and NMDA receptor interactions is the formation of dark neurons, a form of degenerative neuronal changes that are observed in the spinal cord dorsal horn following both peripheral nerve injury (Sugimoto et al., 1989; 1990) and the induction of morphine tolerance (Mao et al., 1997). The coadministration of the opioid receptor antagonist, naltrexone, and morphine reliably prevented both the development of the antinociceptive tolerance as well as the increase in dark neurons. Thus, opioid and NMDA receptor interactions could lead to potentially irreversible neuronal changes within the spinal cord dorsal horn.

Loss of Synergy

Synergistic interactions between spinal and supraspinal sites are recognized to play an important role in modulating the analgesic effects of opioids. Morphine administered simultaneously intracerebroventricularly and intrathecally exhibits profound synergistic effects, with the antinociceptive potency much greater than would be predicted from a simple addition of the potencies administered at either site alone. He and Lee (1997) found that when mice were made tolerant via implantation of a 75 mg morphine pellet for three days, this synergism was eliminated, so that morphine administered to the two sites had an additive effect only (no tolerance was evident in doses delivered to either site alone). They found that coadministration of the peptide dynorphin-A to the two sites resulted in a partial restoration of the synergistic effect. The authors interpreted their findings as evidence that morphine tolerance results not from a reduced sensitivity to morphine at discrete central nervous system sites, but rather from a reduced synergistic interaction of morphine at spinal and supraspinal sites. Similarly, Advokat et al. (1990) reported that in spinally transected rats, animals made tolerant to intrathecal morphine injections were not tolerant to a 6mg/kg dose of subcutaneously administered morphine. However, tolerance to intrathecal morphine injections developed more readily in spinalectomized than intact rats. These data suggest that, in intact animals, tolerance to the antinociceptive effect of spinal morphine is modulated by descending, supraspinal input, although the exact mechanisms of this modulation are unknown.

Opioid Receptor-Receptor Interactions and Other Phenomena

There have been several studies to date suggesting that an interaction between mu and delta receptors may mediate morphine tolerance. For example, Zhu et al. (1999) reported that mice with targeted deletions of the DOR-1 (delta opioid receptor) do not develop analgesic tolerance to morphine. Further, the highly selective delta-opioid receptor antagonists TIPP[psi] (Fundytus et al., 1995) and naltrindole (Abdelhamid et al., 1991) were shown to prevent the development of morphine tolerance in rodents. Abolition of tolerance to morphine was also observed in delta opioid receptor and preproenkephalin deficient mice (Nitsche et al., 2002). Other studies demonstrate co-localization of mu and delta receptors (Arvidsson et al., 1995; Cheng et al., 1997) and co-expression of these receptors in neuroblastomas (e.g., Yu et al., 1986; Palazzi et al., 1996), suggesting the possibility of mu and delta receptor complexes, perhaps through dimerization (Salahpour et al., 2000). In fact, Gomes et al. (2000) were able to isolate mu-delta heterodimers, and show how very low doses of certain selective delta ligands result in a significant increase in mu receptor agonist binding. While not yet fully understood, modulation of receptor function such as that demonstrated by mu-delta complexed sites present a possible mechanism for alterations in drug efficacy as seen in tolerance (possibly through loss of opiate synergy).

Bimodal inhibitory and excitatory effects, mediated by G_i and G_s proteins, have been demonstrated for most opiates (see Crain and Shen, 1996, for review). It is not known

whether such alternative coupling occurs via different isoforms of opiate receptors or whether one receptor can alternatively, or simultaneously, couple to different G-proteins. Nevertheless, coupling of opiate receptors to excitatory Gs proteins may have implications for the mechanism of tolerance. Opioid agonists such as morphine have been found to exert excitatory and inhibitory receptor mediated effects at low and high doses, respectively. Early studies of opiate receptor desensitization suggested that there is a compensatory increase in adenylate cyclase activity during chronic drug treatment and that this mechanism explains tolerance and dependence (Sharma, 1975). More recent studies have supported the theory that a compensatory increase of the AC-cAMP-PKA pathway occurs during tolerance (Nestler, 1993). Thus, documentation of the presence of a stimulatory Gs-coupled opiate receptor, which can be altered during chronic drug exposure, has important implications for tolerance. Several studies have suggested that chronic opiate treatment can alter the balance of inhibitory and excitatory opiate receptors, with excitatory effects becoming more pronounced (See Crain and Shen, 2000 for review). In addition to the adenylate cyclase signaling, other signaling pathways of the opioid receptor mediated by G_s and /or G_{By} subunits are stimulated after chronic opioid exposure. Excitatory effects mediated by G_s proteins have been observed for most opioids. After chronic opioid exposure, neurons become supersensitive to the excitatory effects of opioid agonists (Crain and Shen, 1992). Ultra low doses of opioid antagonists (such as naloxone and naltrexone which selectively inhibit the excitatory effects) have been reported to augment systemic morphine analgesia and inhibit the development of tolerance/physical dependence (Crain and

Shen, 1995a, 1998b; Shen and Crain, 1997). This restoration of morphine efficacy through coadministration of an ultra low dose opioid antagonist such as naloxone has been shown (Powell et al., 2002) to be spinally mediated.

ii. Individual Differences

Prolonged or repeated morphine administration can result in analgesic tolerance, evidenced by a diminution of morphine's analgesic potency, magnitude, or duration. The development of tolerance to morphine, and concomitantly cross-tolerance to other clinically useful opioids, renders subsequent effective pain control difficult and unpredictable. Similar to morphine analgesia, there exists interindividual variability among humans in response to chronic opioid treatment. Whereas some patients require rapidly escalating opioid doses in conjunction with escalating pain, others find adequate analgesia from stable opioid doses for periods of up to several months (Foley, 1993).

iii. Sex Differences

To date there have been a number of studies examining morphine tolerance development in male and female rats. In one study there was a significant attenuation of morphine analgesia on the hot-plate and jump tests in male rats treated with morphine for 14 days, while females displayed no tolerance on the jump test and less tolerance than males on the hot-plate test (Badillo-Martinez et al., 1984). Kasson and

George (1984) reported that the development of tolerance to morphine's antinociceptive and hypothermic effects "was slightly more rapid in males than in females". More recently, Craft et al. (1999) showed that after twice daily or once-weekly morphine injections, male rats were more tolerant than females to morphine analgesia on the hot-plate test. A similar pattern was seen in the tail-withdrawal test, in which repeated administration of morphine resulted in more tolerance for males, as determined by total morphine analgesia (area under the curve) or morphine ED50 values. In neonate rats, however, the magnitude of tolerance to morphine analgesic effect on the tail-flick test does not appear to be affected by sex (Thorton et al., 1997). Further, in a later study by Craft's group (Barrett et al., 2001), the development of comparable levels of morphine tolerance in male and female rats was described when the functional morphine dose was compared and analyzed. Specifically, in their earlier study (Craft, 1999), while a significantly greater development of tolerance was reported in male rats, this was in the context of the males initially showing much greater sensitivity to morphine's analgesic effects than females, and after repeated administration, showing similar analgesic efficacy to chronically treated females. Given that males were acutely more sensitive to morphine analgesia, the functional chronic morphine dose (i.e., chronic morphine dose / acute morphine ED50) administered to males was larger than that administered to females. When this was controlled for in the Barrett et al. experiments (2001), sex differences in tolerance development were no longer observed. Outside of the work of the laboratory in which this dissertation is to be conducted, little work has been done to date to discern any possible sex differences in morphine tolerance in the mouse.

Likewise, studies investigating the contribution of genotype to morphine tolerance are quite limited, and to date have involved only studies of a few strains of mice and rats.

iv. Genetic Differences

Hoffmann et al. (1998) examined tolerance to the analgesic effects of morphine in spontaneously hypertensive (SHR), Wistar-Kyoto (WK) and Sprague-Dawley (SD) rats. Within their paradigm calling for 10mg/kg of morphine twice a day for four consecutive days, they found Wistar-Kyoto rats demonstrating greater tolerance development than the other two strains, which displayed rates of tolerance development which were not significantly different from each other. Mas et al. (2000) conducted a study involving two substrains of Sprague-Dawley and one strain of Wistar rats in which they examined the development of tolerance, the sensitivity to morphine, and the effective morphine plasma concentrations within these two strains. Rats were treated with 10mg/kg of morphine subcutaneously every 12 hours for 9 days, and antinociception was assessed by the tail-flick test after the first administration and then on alternate days. They found decreases in morphine's antinociceptive effect from Day 1 to Day 9 in one of the Sprague Dawley strains (SD-CR; Sprague Dawley rats ordered from Charles River laboratories) and the Wistar strain, but not the other Sprague Dawley strain (SD-U; Sprague Dawley rats bred from lines in the authors' university, Universidad Miguel Hernandez in Spain). In order to assess possible pharmacokinetic differences accounting for the variable levels of tolerance development, they also analyzed plasma

concentrations of morphine and morphine-3-glucoronide after morphine dosing. No significant differences were observed among rat strains. In addition, they found that the plasma concentrations of morphine reached for a given dose did not change after chronic treatment, supporting the finding that the development of tolerance to morphine does not have a pharmacokinetic basis. Interestingly, their university bred strain (SD-U) actually failed to show the expected decrease in morphine's analgesic potency over a nine day treatment period, showing only a very slight, (non-significant) 4% decrease from Day 1 to Day 9, while 44% and 38% decreases were observed in W and SD-CR strains, respectively. Taken together, the above data suggest that genotype may play an important role in each organism's liability to morphine tolerance, although study of this area has been limited to date.

D. Rationale

As is clear from the background provided, there exists an extensive and growing body of literature regarding the contribution of sex and genotype to several pain related behaviors, including basal pain thresholds and morphine analgesic efficacy. However, morphine analgesic tolerance is an area that has yet to be fully explored with regard to the possible contribution organismic variables. The limited available data regarding the contributions of sex and genotype to morphine tolerance make proposing hypotheses regarding what role (if any) these variables may play quite difficult. For the effective clinical treatment of pain, however, this is an important area of study, given that morphine remains one of the most frequently prescribed analgesics for moderate to severe pain, and tolerance development (or physician concerns of such development) may be important limiting factors in its use. Additionally, as tolerance to drug effects reflects a very basic mechanism of neuronal plasticity, findings may be important to areas that extend beyond the clinical treatment of pain. Further, while inbred mouse strains provide opportunities for achieving a better understanding of genetic contributions to these mechanisms, to date this opportunity has not been fulfilled. The current dissertation has as its goal the expansion of our understanding of possible contributions of sex and genotype to morphine tolerance, and thereby to create a foundation for future work to be done in this area. Some general background regarding the rationale for the experimental methods chosen will be discussed below, followed by

a description of the specific studies to be conducted and hypotheses regarding expected findings.

Issues and Decisions Regarding the Use of Morphine:

Opioids are widely used as analgesics, and, to date, there is no evidence to suggest that any opioid has greater analgesic efficacy, or more clinical use, than morphine (Inturrisi, 2002). Individual variation in both the acute and chronic effects of opioids has been demonstrated in both humans and laboratory animals. While much of the currently available literature regarding individual differences was conducted with acute morphine dosing, differences in morphine-induced analgesia which become evident only after chronic dosing may be more important clinically, given the frequent need to control pain in the long term (especially pain relating to cancer and disease progression). Obtaining a better understanding of the efficacy of morphine following chronic administration, and the way that organismic variables such as sex and genotype effect sustained analgesic efficacy, will be a substantial and important scientific contribution.

Issues and Decisions Regarding the Use of Mice:

The genetics of drug tolerance, like any form of behavior, is difficult to study in humans, because of the likely contribution of multiple factors, both genetic and non-genetic. For example, the use of opioids and other drugs (for pain management or other

reasons) is well-recognized to involve a number of social, cultural, and environmental factors in humans. These may easily obscure the effect of genes. For this reason, studies of animals have advantages over humans. Mice were chosen as subjects for these studies for a number of reasons, primarily the large body of evidence already existing in the literature using this species, their shared 90+% genetic homology with human beings, and the ready availability of inbred mouse strains to study genetic contributions to the phenomenon of tolerance, and any effects of sex x strain interactions. As such, using this species for the current studies promotes opportunities for not only a better understanding of potential differences and contributory factors, but an ease of carrying this research on through various follow-up studies. The individual mouse strains selected for study were so chosen pseudo-randomly: factors considered were price/availability, use in previous studies, general genetic interest, or random choice.

Issues and Decisions Regarding Route of Administration:

In the tail withdrawal assay, a number of routes of morphine administration were chosen to perform cumulative dose response studies (aims 1, 2, 3, 5, 6). These were designed to specifically determine under what conditions, if any, sex and/or strain differences are observable. Systemic, subcutaneous injections, results in widespread morphine distribution along the neuraxis and periphery, thus eliciting analgesia acting at spinal, supraspinal, and possibly peripheral levels, and allows observance of differences

in tolerance in the “whole animal”, similar to what would be seen in clinical intravenous, subcutaneous, intramuscular, or oral dosing. While important given that each of these routes of administration are used clinically, study of the subcutaneous route of administration does not permit localization of the sites of action of any observed differences in tolerance. In contrast, intracerebroventricular injections permit the assessment of a central action, but there is little ability to define the drug effect in specific brain regions. Percutaneous intrathecal injections permit the examination of the pharmacology of spinal systems that alter nociceptive transmission, but, as with the other mechanisms, do not provide information regarding whether modification of these analgesic effects is through direct spinal action or through modulation of descending inputs. Taken together, however, study of all three routes allows meaningful comparisons to be made which may help guide future research as well as providing converging lines of evidence.

Issues and Decisions Regarding Nociceptive Assays:

In order to study tolerance experimentally, decisions have to be made regarding whether tolerance will be specifically studied within each animal (baseline analgesic efficacy versus analgesic efficacy subsequent to repeated administration) or whether between subjects data will be necessary (for example, if the pre-tolerance assessment of analgesic efficacy might confound later data, such as would occur if a saline control group had prior exposure to morphine). The current proposed studies are comprised of a mixture of these paradigms, as necessitated by the large number of issues that need to

be considered to minimize potential confounds. Many assays of pain are necessarily excluded from repeated assessment, as literature has demonstrated the occurrence of contextual/ associative learning or tissue damage. Also, some nociceptive assays involve mechanisms of pain that do not have good clinical responses to morphine (e.g., neuropathic pain). Below is a basic description of the assays chosen for use in the present studies:

The tail withdrawal response: The basic mechanisms of the tail withdrawal response are as follows: The heat of a water bath activates nociceptors in the end of the animal's tail, leading nociceptors to transduce the potential tissue damaging stimulus into a train of action potentials that are transmitted along their axons to cell bodies of the nociceptors, located in the dorsal root ganglion. Nociceptive neurons of the dorsal root ganglia synapse in the dorsal horn of the spinal cord on local interneurons and on projection neurons, which carry nociceptive information primarily to the brain stem, thalamus, and hypothalamus. Local circuit neurons in the dorsal horn process afferent information and convey nociceptive information to the autonomic nervous system and to motor neurons involved in local withdrawal reflexes. Regulation of the tail withdrawal response can occur via peripheral sensitization due to previous injury or inflammation or otherwise altered somatic processing at the peripheral level. Animals with tails that had visible signs of injuries were excluded from the study, given the likelihood that such injuries might be expected to alter peripheral processing of the water bath stimulus. Likewise, animals that failed to show a baseline behavioral response to the

water bath stimulus (withdrawal latencies exceeding 15 second in baseline conditions) were also excluded from the study.

The tail withdrawal assay (Janssen et al., 1963) is a modified version of the classic tail-flick test developed by D'Amour and Smith (1941). The distal half of the tail of a lightly restrained animal was dipped into circulating water thermostatically controlled at $49 \pm 0.2^{\circ}\text{C}$. Latency to respond to the heat stimulus, by a vigorous flexion of the tail, was measured to the nearest 0.1 second with a stopwatch. In the absence of such a withdrawal reflex, the tail was removed from the water after a cutoff latency of 15 seconds to avoid possible tissue damage. To improve accuracy, two separate withdrawal latency determinations were made and averaged. Each determination was separated by 20 seconds. The 49°C water temperature was chosen as it is used ubiquitously for this assay (Mogil, 1996). Because tail withdrawal/flick latencies are known to be affected by tail-skin temperature (Tjolsen and Hole, 1993), careful attention was paid to ensure that the ambient temperature for all TW trials remained at $22\text{--}23^{\circ}\text{C}$. Sets of tail withdrawal latency determinations were made immediately before, and 30 minutes after, drug injections. This post-injection latency, which has been used in studies of opioid analgesia dating back over twenty five years (for example, see Smith et al., 1976) was chosen as it has been demonstrated that onset of peak morphine analgesia occurs about 30 minutes post injection in rodents (Gouarderes et al., 2000). Mice were kept in their home cages in the interim. Antinociception was quantified via within-subject comparison of baseline versus post-drug latencies.

It should be noted that there has been some discussion in the literature (see Lichtman, et al., 1993) regarding the relevance of concerns that certain drugs may indirectly increase or decrease tail flick latencies by lowering or increasing tail skin temperature. Such concerns would generalize to the tail withdrawal test used in four of the six studies to be conducted. Especially given morphine's established effects on body temperature, it would follow that any observed sex or strain differences might be secondary to differences in the thermoregulatory effects of morphine rather than tolerance to the analgesic effects. However, Lichtman argues that it is doubtful that a drug can indirectly increase TF latencies by decreasing tail skin temperature because under typical laboratory conditions, the tail is at or near ambient temperature. Nonetheless in order to evaluate the impact of lowering tail skin temperature on the tail flick response, Lichtman et al. (1993) employed a non-pharmacological approach of immersing the tail into chilled water. Despite the fact that this manipulation reduced tail skin temperature more than 7 °C from baseline values, there was only a 1.6 second increase in TF latencies. They interpreted that this result, while statistically significant, could not be considered relevant as it represented less than 20% of the maximum possible effect. Conversely, increasing tail temperature, either by administration of mecamylamine or submergence of the tail into water at the quite warm temperature of 30 °C, had no significant impact on TF latencies relative to controls. These findings taken together suggest that tail temperature plays a minimal role in the TF response. Nonetheless, control of the room temperature was undertaken, and specific aim five in the current

proposal may add insight into whether such concerns are founded via comparison of correlation of temperature changes and tail withdrawal latencies.

Acetic Acid Writhing Assay: Koser et al. (1959) were the first to describe abdominal constrictions produced by injections of dilute acetic acid in mice. A number of variations of this test of chemical/visceral nociception have appeared in the literature since that time. Employed by Mogil et al. (1996) the assay was conducted as follows: Mice were placed on an aluminum table within Plexiglas cylinders (30cm high; 30cm diameter) and allowed to habituate to this novel environment for 30 minutes. They were then weighed, injected with morphine subcutaneously, and returned to the cylinders. Twenty minutes later, a 0.6% solution of glacial acetic acid was injected intraperitoneally in a volume of 10ml/kg. For the next 30 minutes, the number of abdominal constrictions - lengthwise stretches of the torso with a concomitant concave arching of the back – were counted and recorded in 5-minute blocks. Four mice were observed simultaneously by a single experimenter. In the present studies, the writhing test was carried out similarly, with the exception of the use of a slightly higher 0.9% solution of glacial acetic acid in distilled water. The slightly higher dosage solution was chosen due to its more reliable induction of the writhing response (Kest, personal communication). In addition, animals were weighed prior to acclimating to the cylinder, to minimize any possible effects of stress/handling during the weighing procedure on subsequent assessment of nociception and analgesia.

Issues and Decisions Regarding Method of Tolerance Induction:

Available literature on morphine tolerance is complicated by experimenters' use of a variety of dosing schedules and routes. For example, in studies of neonatal morphine tolerance, van Praag and Frenk, 1991 and Barr and Wang, 1992, found that tolerance could be obtained in 9 day old rats, while Fanselow and Cramer (1998) and Windh et al. (1995) both reported that tolerance could not be produced prior to 15 days postnatally. In each of these studies, morphine was repeatedly administered by bolus injection with a variety of dosing and injection schedules. Such differences could account for the divergent results among the studies. In addition, it is likely that repeated administration leads to wide fluctuations in CNS opioid concentrations that could affect the development of tolerance (Thorton and Smith, 1996). Furthermore, bolus injections require repeated handling and stress of the animals that could affect the development of tolerance, and such stress effects may vary by sex or genotype, thus posing a considerable confound to the studies to be undertaken. These concerns suggest that subcutaneously implanted pumps (Alzet osmotic minipumps) might be a better route to induce tolerance. However, although morphine sulfate has generally good solubility (~50 mg/ml), the maximum concentration of morphine that could be added to the pump was insufficient to produce tolerance in several attempts within our laboratory (pumps infusion rate: 1 microliter /hour /72 hours). Consideration of using two pumps simultaneously was ruled out as an option, given the cost and possible variable impact,

stress-wise and dosage-wise, on mice of different weights (females being typically smaller than males, mice of some strains being as small as 17grams, others being as large as 40 grams). Further, the administration of opioids in spaced boluses (through PCA devices or injection by medical professionals), or oral doses is actually the more common route of administration clinically, and for which information regarding tolerance induction would be most clinically relevant. In addition, spaced boluses allow for greater dose ranges in analgesic assessment and tolerance induction. Thus, greater magnitudes of tolerance, which facilitate teasing apart one group from another, are possible. In order to observe and control for the effects of handling and repeated injections, a saline group received equivalent numbers of saline injections across the tolerance induction period. Further, a “no injection” control, or “Day 1” CDR was conducted for each group in order to be able to assess any effects of handling (observed by Day 1 naïve CDR versus Day 4 saline control comparisons).

Subcutaneous injections were delivered via 28 (1/2 inch) gauge needles to minimize the pain and stress of repeated injections. All injections (subcutaneous, intrathecal, intracerebroventricular) were conducted by an experienced person proficient in the technique, in order to best minimize undue stress on the animals. Due to the difficulty of intrathecal injections (often taking some time or repeated attempts to ensure that the solution has reached the spinal cord), as well as the limited existing data regarding intrathecal dose response curves for morphine in mice, dose response studies for

animals with intrathecally-induced tolerance were conducted with systemic injections on the test day. Intrathecal injections during tolerance induction were delivered in 2 microliter amounts to minimize swelling/inflammation of the spinal cord which might result due to repeated (9) injections.

Issues and Decisions Regarding the Definition / Calculation of Morphine

Antinociception and Tolerance:

Another important choice in the proposed studies was the method used to calculate morphine's antinociceptive effects. Prior research in Dr. Kest's lab has used ED₅₀ (Effective Dose 50) values, which are derived from dose-response curves and estimate the morphine dose necessary to double baseline latencies in 50% of the animals examined. This method stands in contrast to AD₅₀ values (or Analgesic Dose 50), which typically refer to the doses at which animals of a particular group/sex/strain achieve 50% MPE (MPE: maximum possible effect, defined by the formula $[\%analgesia = [(post\ morphine\ latency - baseline\ latency)/(cut-off\ latency - baseline\ latency)] \times 100]$). There are benefits and limitations of each measure. ED₅₀ values are quantally tied to each animal's baseline response latency, and as such responses meeting the criterion for a finding of "analgesia" may substantially differ between strains. For example, if one strain has an average baseline withdrawal latency of 2.2 seconds, the dose at which the animal's withdrawal latencies exceed 4.4 seconds would be considered the analgesic dose for that animal (a response time increase of 2.2

seconds). In contrast, if another strain has a baseline latency of 4.2 seconds, an “analgesic” dose would mean the animal’s latency would increase to 8.4 seconds (a response time increase of 4.2 seconds). AD_{50} values, which incorporate the arbitrarily set cutoff latency into the calculation of analgesia, can also be seen as an imperfect measure. In all studies where possible, and in order to ethically minimize the number of mice needed for the proposed studies, cumulative dose response curves have been chosen as the method by which to assess morphine’s analgesic efficacy, with ED_{50} values representing analgesic doses. Following the collection of baseline data regarding nociception on the tail withdrawal assay, animals received logarithmically increasing doses of morphine, their withdrawal latencies measured 30 minutes subsequent to each dose. In the strain survey, collection of %MPE/ AD_{50} values also occurred, and differences in the findings as a result of these different measures will be discussed. In the acetic acid study to be conducted as part of the current proposed research, cumulative dose response studies are not possible due to the sustained effects of the painful stimulus. As such, a limited range of doses was used (0.5mg/kg, 1mg/kg, 2mg/kg) to minimize the number of animals needed for the study.

Rationale for Specific Aims of the Present Dissertation:

As it is clear from the literature reviewed in the background section, there has been quite a bit of scientific effort focused on bettering our understanding of the contributions of gender and genotype to individual differences in the pain related

behaviors of baseline nociception and responsivity to morphine analgesia. To date, however, little work has been undertaken to explore those factors that may influence individual differences in the development of tolerance to morphine's analgesic effects. It is hoped that the present dissertation will result in a meaningful contribution to our understanding of two of those factors, sex and genotype.

The first specific aim of the study was to determine the degree to which tolerance to the analgesic effects of systemically administered morphine varies as a function of sex. Given the substantial literature indicating findings of sex differences in morphine analgesia (see Craft, 2003 for review), it was hypothesized that differences similarly exist in the development of tolerance to morphine's analgesic effects, although clear predictions can not be made regarding the likely direction of those differences (m>f versus f>m). The study involved performing dose-response analysis of morphine analgesia in male and female mice outbred (CD-1) mice before and after induction of tolerance via the administration of increasing doses of morphine injected subcutaneously three times a day. The tail withdrawal test, a commonly used assay of thermal nociception, was used to determine baseline nociception and analgesic efficacy of the various morphine doses. Through exploring analgesic tolerance after 3 days and 7 days of morphine administration, this study aimed also to clarify whether the direction of sex differences remains the same, or changes, over different time periods.

Estrus effects were not examined in either specific aim one or any of the other studies in the present dissertation. At the outset of each of the studies, it was determined that meaningful analysis and interpretation of estrus data was unlikely, given that each study of tolerance spanned a period of several days. As such, even in the event that the animals were not rendered anoestrus due to same sex housing (see McClintock, 1983 for review of Lee-Boot effect and other issues concerning pheromonal regulation of the ovarian cycle), and had no other disruptions in their estrus cycle due to the presence of chronic morphine (such as those reported in Craft et al., 1999), it is not clear exactly which estrus data could be used soundly to further our understanding of any sex differences observed in morphine tolerance. For example, assessing estrus phase on the same day as assessing morphine's analgesic efficacy on "Day 4", after three days of chronic morphine, might allow for the comparison of morphine analgesia in *tolerant* female mice at different phases of their cycle (the mouse's estrus cycle spanning a period of 4-5 days). It does not, however, add much information to our understanding of hormonal influences on tolerance development, as all mice would have received morphine injections throughout each phase of their cycle. As such, the true impact, if any, of hormonal factors would not be apparent given the current experimental design.

The question of whether sex hormones may interact with the development of tolerance to the analgesic effects of morphine is an interesting one, which is deserving of future study. The experimental design of such studies would need to be carefully considered, however, to overcome the design challenges produced by the chronic administration

paradigm while minimizing the number of mice used for ethical purposes. For example, while Craft et al. (1999), in their study of sex differences in the development of morphine tolerance in the rat, did examine estrus phase throughout their experiment, their morphine-treated females showed significantly different cycling patterns than their saline-treated females, with fewer proestrus and estrus days, and more diestrus days in morphine treated compared to saline-treated rats. As such, other than the finding that chronic morphine does in fact disrupt estrus cycling [a finding not unexpected given morphine's ability to inhibit hypothalamic pulsatile luteinizing hormone secretion, necessary for normal estrus cycling, in females of various species (Pfeiffer & Herz, 1984; Williams et al., 1990)], Craft et al. were unable to draw any conclusions regarding possible effects of estrus on the development of tolerance. In addition, even in the analgesia literature, which is theoretically a much more easily studied area, conflicting reports of the effects of estrus are apparent. For example, female rats are reported by some authors to display greater analgesic sensitivity to systemic morphine on the mornings of diestrus (Banarjee et al., 1983) and proestrus (Banarjee et al., 1983; Berglund and Simpkins, 1988), while Kepler et al. (1989) reported a relative *decrease* in sensitivity during met/diestrus. Studies with gonadectomized subjects are even more conflicting. For example, although it appears that the effect of adult ovariectomy on morphine analgesia may be nociceptive modality specific (Baamonde et al., 1989; Ali et al., 1995), reductions (Banarjee et al., 1983; Kepler et al., 1989), increases (Kasson and George, 1984), and no alterations (Cicero et al., 1996) in morphine analgesia have all been reported just for the tail-withdrawal test.

Given all of the above issues, estrus effects were not specifically explored in the current studies. Informal control of estrus effects, however, was accomplished through each study being conducted with several different sets of animals over time, which in theory would somewhat randomize the phase in the estrus cycle that each group and/or individual animal began and finished the study at. Additionally, while in no way confirming the role of the estrus cycle in any sex differences in morphine tolerance observed, data collected in the present studies will include confidence intervals and other methods of assessing variability in the responses of each sex. Greater variability in female animals, if found, might be suggestive of possible differences as a function of estrus phase (although, again, it is difficult to speculate on how these findings might be interpreted, given that the tolerance induction period would span throughout the estrus cycle). Thus, the degree to which hormonal differences may underline any sex differences in morphine analgesic tolerance observed in the current series of studies is an interesting research question, which, while deserving of further study, will not be specifically addressed in the current dissertation.

Because systemically delivered morphine exerts effects both supraspinally and spinally, and centrally administered morphine is capable of yielding sex differences in analgesia (see Craft, 2003 for review), Specific Aims Two and Three respectively explore the relative contribution of intracerebroventricular and intrathecal sites of action to the development of morphine tolerance in male and

female mice. To date, no other studies of sex differences in morphine tolerance have involved central administration of morphine (either spinally or supraspinally), and as such these experiments are likely to make an important contribution to our understanding of the possible mechanisms involved. In study two, morphine was delivered intracerebroventricularly for the tolerance induction period, and dose-response analysis of morphine analgesia was compared in each sex both prior to, and subsequent to, the tolerance induction period. Study number three involved the administration of morphine intrathecally during the tolerance induction period, again with comparisons of morphine analgesic doses before and after this period in each sex.

Research shows that pain produced by different types of stimuli has varying responses to analgesic agents. For example, while there is clear consensus that morphine is a very effective analgesic for severe cancer pain (Twycross, 1994), it may be less effective for other types of pain, including neuropathic pain (Arner and Meyerson, 1998). In fact, findings relating to sex differences in morphine analgesia in humans have varied according to the type of nociceptive stimulus. For example, Zachny (2002) found that females had a better analgesic response than males on the cold pressor test, but no sex differences were observed when the stimulus was mechanical pressure. The degree to which patterns of sex differences in morphine tolerance are consistent across different types of pain has yet to be explored in the literature. **Specific Aim Four examined male and female outbred mice using the acetic acid writhing assay, a test of chemical/visceral pain, to determine whether sex differences in morphine tolerance**

are apparent when the pain is of this typography. While this study was undertaken without a clear hypothesis regarding the presence, or direction, of sex differences (should they be observed), the information gained, in conjunction with that of the other studies to be conducted, would help determine the degree to which findings in one test might generalize to others.

Morphine's effects on the organism extend beyond analgesia, and include respiratory depression, reduced gastrointestinal transit, and thermoregulatory changes, among other effects. While a handful of studies have explored contributions of genetic factors to some of these effects (e.g., Belknap et al., 1998; Belknap et al., 1989), little work has been done looking at whether tolerance to these effects varies as a function of sex.

Specific Aim Five thus explores differences in core body temperature that occur upon morphine administration in naïve animals, and after a three day tolerance induction period, to determine whether or not sex differences exist on this measure.

There have been a number of studies reporting differences in morphine analgesia in mice and rats of different strains (see Mogil, 2001 for review). In addition, recent work has begun to explore the contribution of genotype to morphine analgesic tolerance in rodents (e.g., Mas et al., 2000; Hoffman et al., 1998). Unfortunately, these studies compare only two or three strains at a time. To date, a comprehensive strain survey exploring differences in tolerance as a function of sex and genotype (and any interaction

effects) has not yet been conducted. Indeed, sex differences in morphine analgesia have been shown to interact with strain, such that some strains show greater male relative to female analgesia, some no sex difference, and in one strain, female greater than male analgesia (Kest et al., 1999). Applying this strain-survey methodology to morphine tolerance would not only facilitate a more complete comparison between strains, but also allow for the analysis of the degree to which the magnitude of tolerance is correlated with baseline nociception and initial morphine analgesic efficacy.

Correlations may also be assessed with other morphine-related measures such as dependence (Kest et al., 2002). The identification of extreme responding strains are also requisite for any subsequent quantitative trait loci mapping studies designed to identify chromosomal regions associated with phenotypic variability. **Specific Aim Six attempts to accomplish this goal through the assessment of analgesic morphine doses prior to, and subsequent to, chronic subcutaneous morphine administration in males and females of eleven strains of inbred mice (129/J, A/J, CBA/J, BALB/C, C57/BL6/J, SJL/J, AKR/J, C3H/HE/J, DBA/2J, LP/J, SWR/J).** Upon embarking on this experiment, it was expected that, as demonstrated for morphine analgesia, differences in the magnitude of tolerance, and the direction of sex differences should they be found, would be dependent upon the animal's genetic makeup (strain). Taken together, it is hoped that the present series of studies will contribute to our understanding of factors contributing to morphine tolerance, an area that, despite a wealth of research, still remains poorly understood.

Given the various findings of studies of sex and strain differences in morphine analgesia, it was the expectation of the investigators in the current series of studies that sex differences, if and when observed, would be dependent on the assay used, as well as the particular strain of animals tested. More specific hypotheses regarding the qualitative and quantitative aspects of the results were not made in advance, largely due to the paucity of prior work in this area (which would ordinarily guide such hypotheses). However, it was interesting to speculate in advance regarding possible findings of the present studies, given our current knowledge of sex and strain differences in other measures, such as baseline nociception and/or morphine antinociception. For example, strain differences in baseline tail withdrawal latencies have been reported, with Kest et al. (1999) reporting strains such as 129/J, A/J, BALB/c/J, DBA/2/J, and LP/J showing relatively longer baseline tail withdrawal latencies (i.e., showing less behavioral responsivity to that particular nociceptive stimulus) than strains such as SWR/J, SJL/J, C3H/He/J, and C57BL/6/J. It might be hypothesized that those strains with higher initial sensitivity to the particular nociceptive stimulus would experience higher levels of behaviorally defined tolerance (i.e., being that they are more sensitive to the painful stimulus to begin with, morphine's ability to attenuate this pain may be more easily lost in these animals).

Another hypothesis to consider would be that the strains which are most sensitive to morphine's effects initially (during baseline assessment of morphine antinociception) would show the greatest tolerance development after chronic administration (given that

the dosing schedule in the current series of studies was uniform across sex and strain, and individualized only by the animal's weight, NOT their initial effective doses). Thus, animals more sensitive to morphine on Day 1 could be considered to be receiving a "higher dose" throughout the tolerance induction period (relative to their initial effective dose) and, as such, demonstrate higher levels of tolerance on Day 4. For example, conducting a study in rats looking at sex differences in morphine tolerance, Craft et al. (1999) found that male rats demonstrated greater degrees of tolerance following chronic morphine administration than female rats. Subsequently, however, they conducted a similar study but controlled for the differences in baseline morphine analgesia (in their study male rats had shown significantly lower morphine ED₅₀ values at baseline). Their control consisted of using a morphine dose during the tolerance induction period that was determined by each sex's baseline ED₅₀ values. When the experimental procedure was modified in this manner (by use of this "functional" chronic morphine dose), sex differences in morphine analgesic tolerance in rats were no longer evident (Barrett et al., 2001). Using intracerebroventricular injections of morphine, Kest et al. (1999) characterized certain strains as demonstrating antinociception with doses of morphine under one microgram, such as 129/J, A/J, C3H/He/J, CBA/J, and DBA/2/J, while others required significantly higher doses to allow analgesia (e.g., C57BL/6/J, SWR/J). More data regarding the initial effective doses of morphine in different sexes and strains has been obtained in the current series of experiments, and the extent to which initial sensitivity to morphine analgesia correlates with subsequent tolerance development will be discussed.

As noted above, any finding of a correlation of morphine tolerance to initial morphine analgesia, given the experimental procedures used in the current study, would suggest that the increased tolerance is secondary only to the qualitative increase of the therapeutic agent. For example, during the period of tolerance induction in the current experiments, mice were typically administered a total of 210mg/kg of morphine subcutaneously over a three day period. If, as in Kest's study (1999), up to five-fold differences in morphine analgesic efficacy were observed in different groups, while all groups in the current study would receive the same quantitative dose (in mg/kg), for different groups this amount might be quite different qualitatively (and could in fact range from some animals receiving 35 times their effective dose throughout this period, to others receiving up to 200 times their effective dose). As such, any findings or statements regarding the correlation of baseline morphine analgesia to subsequent tolerance development would have to be qualified by the understanding that the experimental design used in the current studies did not involve matching the animal's tolerance induction dose to their initial effective dose in any way. Alternatively, however, the reverse hypothesis could be plausible, i.e., that animals who were initially the least sensitive to the analgesic effects of morphine would also be those in which morphine more quickly or more easily lost its ability to produce ANY degree of analgesia. Given that these animals would have relatively higher ED₅₀ values at baseline (i.e., require more morphine to produce analgesia), they would, in essence, also be those animals who received fewer times their analgesic dose of morphine throughout the tolerance induction period. As such, the finding of increased morphine tolerance in

these animals would be strongly suggestive of tolerance to morphine analgesia being indicative of an intrinsic vulnerability or weakness of the pain inhibition systems through which morphine exerts its effects. There is some evidence to suggest that in patients with certain types of pain (e.g. central neuropathic pain), morphine may not only have limited efficacy initially, but over the long term, even fewer patients may benefit from chronic treatment with morphine (Attal et al., 2002). A similar pattern, if demonstrated in the current studies and strain survey, would be an important finding, and could pave the way for quantitative trait loci studies and identification of the genetic basis for these individual differences.

While hypotheses such as those stated above are interesting speculations, the paucity of research in the area of differences in morphine analgesic tolerance as function of sex and strain does not allow strong support of one over the other. Further, much of the work done to date, rather than suggesting correlations between different behavioral effects of morphine, has in fact shown dissociation of these measures. For example, Belknap et al. (1989), compared three inbred strains (DBA/2J, C57BL6J, and C3H/He/J) on various measures, including morphine induced analgesia (hot plate), locomotor activity, hypothermia, Straub tail, antidiuresis (retention of water load), and constipation. The observed strain differences in each behavior/effect showed a relatively high degree of genetic control, however, there was little consistency in the rank order, suggesting dissociation of genetic control for each of these effects. Interestingly, in Belknap's study, the DBA/2J mice emerged as the most sensitive for

both analgesia and hypothermia, but also showed the lowest concentration of morphine in the brain 30 minutes after injection. Further, multiple studies have shown dissociation of morphine tolerance and dependence (e.g., Kaneto et al., 1985; Nitsche et al., 2002; Eitan et al., 2003; Rahman et al., 1994; Panerai et al., 1987), and even dissociation of multiple effects of chronic morphine administration (Bhargava, 1981). Thus, while it is interesting to speculate, the literature to date does not provide sufficient data to suggest that correlation between morphine tolerance and any of the above measures (baseline nociception, baseline morphine analgesia, etc.) is likely. Given the scope of studies undertaken in the presentation dissertation, however, it is hoped that these new data may shed meaningful light on our understanding of these issues.

GENERAL METHODS

Subjects

The following mouse strains will be used in this dissertation's experiments:

- CD1 mice: a standard, outbred strain of white mice.
- Inbred mice strains 129/J, A/J, CBA/J, BALB/C, C57/BL6/J, SJL/J, AKR/J, C3H/HE/J, DBA/2J, LP/J, SWR/J

Available descriptions of characteristics of each strain are provided below. This information is taken from the Handbook on Genetically Standardized Jax Mice (Fox and Witham, eds, 1997), Jackson Laboratory website, and available literature identified through pubmed search. (Please note that data regarding characteristics of certain strains are quite detailed, while other strains currently have limited data available on pain-relevant measures. Every effort was made to provide as thorough and relevant a general description as possible).

- 129/P3/J: A substrain of the 129 strain, these white mice are widely used in the production of targeted mutations due to the availability of several lines of embryonic stem cells. Apart from a reported high incidence of spontaneous testicular teratomas, despite a generally low tumor frequency, little work has

been done to examine the physical and behavioral characteristics of 129 mice, which is problematic given their frequent use in knockouts and other targeted mutations. High sensitivity to estrogen and high resistance to radiation is reported. Belknap et al., 1998 found that 129/J mice showed a large (205%) increase in activity levels following a high dose systemic injection of morphine (32mg/kg). Generally, 129/J mice are gentle mice (Fox and Witham, 1997).

- A/J: A cross of two albino strains, 50% of A/J mice have primary lung tumors. High frequency of renal disease in older animals. A/J's are gentle, clean mice.
- CBA/J: Hyperactive mice, older breeders prone to seizures. Fairly high tumor incidence in breeding females.
- BALB/C: These albino mice are widely distributed and among the top 2-3 most widely used inbred strains. The strain is particularly well known for the production of plasmacytomas on injection with mineral oil. Used as a general-purpose strain in many different disciplines. Generally good breeding performance and long reproductive life span. BALB/c mice show extremely high inter-and intra strain aggression. The BALB/c strain has been shown to display an increase in activity following morphine administration, although not quite as marked as the C57/BL6 strain. (Belknap et al., 1989)
- C57BL6: Black mice of the C57BL family are probably the most widely used of all inbred strains, (substrain C57BL/6 alone accounts for over 14% of occasions on which an inbred strain is used) though in many ways it seems to be

atypical of inbred strains of laboratory mice. In contrast to 36 other standard inbred strains, it carries a Y chromosome of Asian *Mus musculus* origin (c.f. AKR and SWR) and a LINE-1 element derived from *Mus spretus* the frequency of which suggests that up to 6.5% of the genome may be of *M. spretus* origin. It usually has a good breeding performance, depending on substrain, and has been used as the genetic background for a large number of congenic strains covering both polymorphic and mutant loci. The C57/BL6 strain has been shown to display a marked increase in activity following morphine administration (Belknap et al., 1989; Frishknect et al., 1988), and are more likely to show Straub tail, an index of muscular rigidity, than do DBA/2 or C3H/He strains (Belknap et al., 1989).

- SJL/J: These albino mice originated at Jackson Labs from three different sources of Swiss Webster mice. This strain carries the pink-eyed dilution gene derived from Asian mice of the *Mus musculus* type (similar to 129/J). The strain has a high incidence of reticulum cell sarcomas resembling Hodgkin's disease. Behaviorally, SJL's show high spontaneous fighting, especially severe fighting among males housed together, beginning at about 8 weeks. Most males will be killed by 4-5 months unless caged separately. Both male and female mice have short life span in conventional conditions. Known anatomical features are as follows: low brain weight, small spinal cord Cerebellum has no intraculminate fissure between vermian lobule IV and vermian lobule V (the ventral and dorsal lobules of the culmen) (contrast DBA/2). These mice show

low voluntary consumption of morphine in two-bottle choice situation (Belknap et al., 1993). Bite wounds are common in this strain. SJL/J mice have also been shown to have an increased rate of muscle regeneration after injury when compared to BALB/c mice.

- AKR/J: These albino mice originated from a dealer named Detwiler in Norristown PA and were first inbred at the Rockefeller Institute. AKR mice are widely used in cancer research for their high leukemia incidence (60-90%) and in immunology as a source of the Thy1.1 (theta AKR) antigen. Mice of this strain are viremic from birth and express in all tissues the ecotropic retrovirus AKV. AKR/J mice are relatively resistant to aortic lesion formation on a semi-synthetic high fat diet and are hyporesponsive to diets containing high levels of fat and cholesterol. AKR/J mice are generally gentle but sometimes will squeal when picked up.
- C3H/HEJ: C3H/HeJ's are agouti mice used as a general purpose strain in a wide variety of research areas including cancer, immunology and inflammation, sensorineural, and cardiovascular biology research. C3H/HeJ mice and all other Jackson substrains are homozygous for the retinal degeneration 1 mutation (Pde6b^{rd1}) causing blindness by weaning age (3 weeks). There is also a high incidence of hepatomas in C3H mice (reportedly 72-91% in males at 14 months, 59% in virgin females, 30-38% in breeding females). Despite the lack of exogenous mouse mammary tumor virus (MMTV), virgin and breeding females may still develop some mammary tumors later in life. C3H/HeJ mice, fed an

atherogenic diet (1.25% cholesterol, 0.5% cholic acid and 15% fat), fail to develop atherosclerotic aortic lesions in contrast to several highly susceptible strains of mice. The C3H/HEJ strain has been shown to display an increase in activity following morphine administration, although not quite as marked as the C57/BL6 strain (Belknap et al., 1989). These mice are generally active/jumpy. Their cages get wet quickly from urine. Females mature very early (25 days).

- DBA/2J: DBA/2J's are light grey/brown mice that are a widely used inbred strain that is valuable in a wide number of research areas including cardiovascular biology, neurobiology, and sensorineural research. Its characteristics are often contrasted with those of the C57BL/6J inbred strain. DBA/2J mice show a low susceptibility to developing atherosclerotic aortic lesions. They also exhibit high-frequency hearing loss beginning around the time of weaning/adolescence (between 3-4 weeks of age) and becoming severe by 2-3 months of age. Young DBA/2J inbred mice are also susceptible to audiogenic seizures. Aging DBA/2J mice develop progressive eye abnormalities that closely mimic human hereditary glaucoma of the peripheral iris. DBA/2J mice also show an extreme intolerance to certain effects of alcohol, morphine, and related opioids. Unlike many other strains, activity levels in DBA/2J mice remain relatively stable or decrease following morphine administration (Belknap et al., 1989). However, Belknap also showed DBA/2J's to be quite sensitive to the analgesic and hypothermic effects of morphine

(1989). These mice sometimes squeal when picked up. Females mature very early (25 days).

- LP/J: LP/J's are white-bellied agouti mice with white patches. They display a high susceptibility to audiogenic seizures. This strain is also reported to have a fairly high incidence of tumors that develop later in life, including mammary tumors, lymphoma, lung and soft-tissue sarcomas. LP/J mice are also homozygous for the piebald mutation. Mice show irregular white spotting, the amount of which is greatly influenced by minor modifying genes. They also have dark eyes. The white areas of the coat are completely lacking in neural crest-derived melanocytes, and there is a reduction in the number of melanocytes in the choroid layer of the eye. Additional characteristics reported include: high emotionality, susceptible to audiogenic seizures, long life-span in conventional conditions, low plasma cholinesterase activity in males, low brain monoamine oxidase and catechol-*O*-methyltransferase activity, large spinal cord, small kidney/body weight ratio, highly resistant to the induction of catalepsy by haloperidol associated with midbrain dopamine D2 receptor density levels. LP/J mice are generally active mice, and active nest builders.
- SWR/J: SWR/J mice are used widely in research as a general purpose strain. Aging mice exhibit a high incidence of lung and mammary gland tumors. They also develop extreme polydipsia and polyuria (nephrogenic diabetes insipidus) with increasing age. SWR/J mice have been recommended for generation and propagation of transgenic mice because they are high responders to exogenous

hormones, have large and prominent pronuclei with good resistance to lysis following microinjection, and are genetically well-defined. The SWR inbred strain was developed by Clara J Lynch at The Rockefeller Institute. SWR/J mice show: Low intrastrain aggression, high avoidance conditionability, poor T-maze learning, low spontaneous bar-pressing activity. Life-span in conventional conditions intermediate in males (14/22 = 616 days) but short in females (7/22 = 496 days). Sensitive to seizures induced by nicotine. Low voluntary consumption of morphine in two-bottle choice situation (Belknap et al., 1993). Large brain/body weight ratio. SWR/J mice are jumpy and, unlike most other inbred strains, aggressive toward people.

All mice were housed 4/5 to a cage with same sex/strain mates in the College of Staten Island Animal Facility. Mice were provided with free access to food (Purina chow) and water in a temperature controlled (22 degrees Celsius). There was a 12: 12 hour light: dark cycle (lights on at 7 am). All testing was performed following an acclimation period of at least one week after arrival and at 7-9 weeks of age. In addition, all mice were provided with a one-hour acclimation period following transportation from the vivarium to the laboratory on test days.

Drugs:

Morphine sulfate was obtained from the National Institute of Drug Abuse (Rockville, MD), and stored by Dr. Ben Kest of the College of Staten Island. Morphine was dissolved in 0.9% physiological saline for all injections.

Nociceptive Assays:

All testing was conducted near mid-photophase (10:00-15:00 hours) to reduce the circadian effects on nociceptive and analgesic sensitivity (Kavaliers and Hirst, 1993). For all experiments, strain and/or sex were completely counterbalanced so that an equal number of mice per strain/sex/condition were tested together.

For the tail withdrawal assay: Following a 1 hour habituation to the testing room, mice were assessed for baseline nociceptive sensitivity on the 49 °C tail-withdrawal test. In this assay of acute, thermal nociception, the mouse is gently restrained and the distal half of his tail is immersed in water maintained at 49 ± 2 °C by an immersion circulator pump (Fisher Isotemp Model 71). Latency to reflexive withdrawal of the tail was measured twice to the nearest 0.1 second. The two determinations were later averaged. The tail withdrawal test was chosen because of its stability even after repeated exposures to high water temperatures (D'Amour and Smith, 1941). Latencies to respond at each dose were recorded to allow for the calculation of ED50 values. A cutoff latency of

15 seconds was employed to prevent the possibility of tissue damage (and was used to calculate AD₅₀ values in the strain survey).

For the acetic acid assay: Following a 1 hour habituation to the testing room, mice were placed in 30 inch diameter by 30 inch high cylinders for another 30 minute habituation period. Following this, mice were injected subcutaneously (through the skin above the mid back area) with either morphine sulfate or a volume-matched saline control injection. Twenty minutes after this subcutaneous injection, mice were injected with a 0.9% glacial acetic acid solution intraperitoneally. Mice were then observed for the characteristic writhing response (described in detail in this study's section). Frequency of writhing responses was recorded in five-minute intervals for 30 minutes subsequent to the i.p. injection. Mice were immediately sacrificed following data recording.

Dose Response Data:

To greatly reduce the number of mice required for these studies for ethical reasons, on studies using the tail withdrawal assay, morphine analgesic potency was determined using cumulative dose-response curves. Immediately following baseline latency assessment, subjects were injected with an initial 1.0mg/kg dose of morphine, followed in succession with increasing (~0.25 log) doses (2.0, 3.6, 6.5, 11.7, 21 mg/kg). These doses have been demonstrated to provide reliable potency estimates in a variety of

mouse strains (Kest et al., 1995, 2000). Thirty minutes after injection of each dose, mice were subjected to the tail withdrawal assay as discussed above, and then injected with the next sequential dose. To ensure that all mice received the same cumulative dose of drug on Day 1, all mice were injected with identical morphine doses. In the event that any mouse did not cut off at the highest dose of 21mg/kg, repeated administrations of the 21mg/kg dose occurred up to three times or until cut off was achieved. Baseline latencies and dose-response data were collected prior to (Day 1) and after (Day 4) tolerance induction.

Immediately following dose-response studies on Day 1, subjects were injected with morphine three times daily for three days according to an escalating daily dosage schedule (for example, in the subcutaneous study, 10mg/kg TID, 20mg/kg TID, and 40mg/kg TID on days 1, 2, and 3 respectively). To assess for possible repeated handling/injecting effects, control mice of each strain received an equal number of saline injections throughout the three days, except on Day 4 when morphine dose-response curves were collected in them similarly to those mice undergoing tolerance induction.

Tolerance Induction:

Tolerance induction in all studies consisted of thrice daily administrations of morphine through the route of administration of the particular study (subcutaneous, intrathecal, or intracerebroventricular). These doses were spaced out throughout the day, with a

minimum of two hours between doses. Increasing doses were given each day throughout the tolerance induction period, which was typically 3 days (extended to 7 days in the first study presented). All studies included saline control animals that received matched numbers of saline injections throughout the tolerance induction period.

Data Analysis:

For dose response studies, ED₅₀ values (effective dose for 50% of animals, individual analgesic doses for each animal being defined as doubling of the animal's baseline tail withdrawal latency) were analyzed and reported. For strain studies, a two way ANOVA was used to examine the main effects of strain and sex, and their interaction, on tail withdrawal baseline latencies. Subjecting Days 1 and 4 latencies to repeated measures ANOVA allowed assessment of changes in baseline latencies after chronic morphine treatment. ED₅₀ values and associated 95% confidence intervals (CI) were calculated for all dose response data using the method of Tallarida and Murray (1981). ED₅₀ values with no overlapping CI's were considered significantly different. In addition, for the strain survey, two different measures of analgesia were collected and analyzed, including ED₅₀ values and %MPE (AD₅₀) values. Percent maximum possible analgesic effect, was calculated by the formula: $\%MPE = [(post\ morphine\ latency - baseline\ latency) / (cut-off\ latency - baseline\ latency)] \times 100$. The use of this transformed dependent measure takes into account the cut-off latency and the individual baseline latency for each subject, so that any variation in baseline values between test Days 1

and 4 will not bias the quantification of analgesia obtained each day. Findings for this measure will be discussed in the context of differences, if any, in findings with use of ED_{50} values.

SPECIFIC AIM ONE

Sex differences in morphine analgesic tolerance: subcutaneously administered morphine

INTRODUCTION:

It is becoming increasingly appreciated that sex may determine the efficacy of opioid analgesic sensitivity in both human and rodent populations (see Berkley, 1997; Fillinghim and Maixner, 1995; Kest et al., 1998; Unruh, 1996 for reviews). With regard to morphine, males typically display greater sensitivity to the analgesic effects of morphine than females across several nociceptive modalities following systemic administration in both rats (Baamonde et al., 1989; Cicero et al., 1996; Islam et al., 1993) and mice (Candido et al., 1992; Kavaliers and Innes, 1987; 1992a, b; Lipa and Kavaliers, 1990; but see Ali et al., 1995; Kasson and George, 1984). A strong case that sex differences in morphine analgesia may be mediated by differential central nervous system (CNS) mechanisms can be made based on the observations that male rats display greater analgesia than females following central administration into the cerebral ventricles (Kepler et al., 1989), rostral ventromedial medulla (Boyer et al., 1998), or the ventrolateral periaqueductal gray (Kraznowska and Bodnar, 1999). In mice, sex differences in morphine analgesia following intraventricular injections, as well as its magnitude and direction, have been shown to be dependent on genotype (e.g., Kest, 1999; see Craft, 1993 for review).

One of the most consistent and noticeable effects of repeated morphine administration is analgesic tolerance, or loss in the relative analgesic potency of morphine. Tolerance results from a myriad of complex molecular and biochemical changes whose relative contributions are as yet not clearly understood (e.g., Collin & Cesselin, 1991; Elliot et al., 1995, Harrison et al., 1998; Mayer & Mao, 1999; McNally, 1999, Whistler et al., 1999). The relative participation of any or several of these variables in tolerance may depend on several interacting methodological factors such as dose and dosing schedules, route of administration, and nociceptive response under study (Badillo-Martinez, 1984; Fernandes et al., 1977; Mucha et al., 1979). It is therefore not surprising that differences in initial sensitivity to morphine in drug-naive subjects are often not predictive of the magnitude and direction of differences in tolerance subsequent to chronic morphine administration (Ho et al., 1977, Hoffman et al., 1998; Kolesnikov et al., 1998; Lufty et al., 1994; Navarro et al., 1991; Oliverio & Castellano, 1974). Thus, despite apparent sex differences in morphine analgesia, it is not possible to predict whether male and female mice will undergo similar changes in morphine analgesic sensitivity following chronic administration.

Few studies have examined sex differences in morphine analgesic tolerance. Badillo-Martinez et al. (1984) reported a greater attenuation of morphine analgesia on the hot-plate test in male relative to female rats treated daily with morphine over 14 days. A similar pattern of greater tolerance in males relative to females on the hot-plate test was

observed after twice daily or once weekly morphine injections as well (Craft et al., 1999). Studies using the tail-flick test, however, present inconsistent findings. Whereas repeated administration of morphine at weekly intervals for three weeks resulted in a greater loss of morphine analgesia (area under the curve; AUC), in males relative to females (Craft et al., 1999), no significant sex differences in morphine ED₅₀ estimates were found following twice daily morphine injections for seven days in either of the two strains (Sprague-Dawley and Wistar-Furth) of rats tested (Kasson & George, 1984). These conflicting findings may be attributable to differences in methodology between the two studies. Although the identical nociceptive assay was employed, they differed in the frequency (weekly vs. twice daily injections) and duration (three weeks versus seven days) of morphine injections, as well as in the method of quantifying analgesia (AUC versus ED₅₀ estimates).

The present study has three main goals, the first of which is to assess whether sex differences in morphine tolerance are also observed in mice. Although it is always useful to other investigators when findings from one species can be generalized to others, the increasing use of inbred and transgenic mouse models in genetic approaches to the problems of chronic opioid use makes our understanding of sex differences in this species particularly vital. Second, the study will attempt to determine whether sex differences in tolerance depend on duration of morphine exposure and/or cumulative dose administered by comparing male and females for analgesia after both three and seven days of injections using a single analgesic measure (ED₅₀ values), nociceptive

assay (tail-withdrawal), and repeated injection (three times daily) paradigm. Third, the study will assess whether sex differences in tolerance to morphine depend on the method of quantifying analgesia by comparing dose-response data (i.e., ED₅₀ estimates) with time-response data (peak analgesia and AUC) in both sexes following identical morphine administration paradigms. The findings of this study have been published in the Brain Research, Volume 879 (see Kest, Palmese, and Hopkins, 2000).

METHODS:

The following study was conducted in accordance with approved protocols of the College of Staten Island /CUNY Institutional Animal Care and Use Committee.

Subjects

Adult male and female CD-1 mice (25-35 g), 6-8 weeks of age, were housed five to a cage with same-sex littermates and maintained in a temperature controlled environment (22 °C, $\pm$ 2°C) with unrestricted food and water and a 12 hour light / 12 hour dark cycle. Estrus phase was neither controlled nor monitored in the present study.

Tail-withdrawal assay

Mice were assessed for nociceptive sensitivity on the 49 °C tail-withdrawal test. 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 49.0 $\pm$ 0.2°C by an immersion circulator

pump (Fisher Isotemp Model 71). Latency to reflexive withdrawal of the tail was measured twice by an experimenter to the nearest 0.1 s, with each determination separated by 20 s. Two determinations were made prior to dose-response studies before and after tolerance induction and averaged to reflect mean baseline withdrawal latency. The tail-withdrawal test was chosen for this nociceptive assay because of its stability even after repeated exposures at this noxious water temperature (D'Amour and Smith, 1941). A cut-off latency of 15 s was employed to prevent the possibility of tissue damage, and was used to calculate %MPE (see below). All testing was done near mid-photo phase (between 10am and 2 pm) to minimize circadian fluctuations in morphine sensitivity.

Tolerance induction

Mice in the morphine treatment group were injected three times daily (tid) for three days according to an escalating dosage schedule (10, 20, and 40 mg/kg on days 1, 2, and 3, respectively). A second group of mice received additional morphine injections on days 4-7 (40, 80, 80, and 100 mg/kg, respectively). Mice in the saline control group received an equal number of saline injections, but were not tested on Day 1. All injections within a given day took place 2-3 hours apart during daytime hours.

Dose-response studies

Tolerance was operationally defined as a significant increase in morphine ED₅₀ values. To reduce the number of mice, ED₅₀ values were derived from cumulative dose-

response curves as previously described (Kest et al., 1996). Briefly, mice were injected with a 1.0mg/kg dose of morphine and received increasing doses (~ 0.25 log units) until each became analgesic. Analgesia was operationally defined as a doubling of each subject's mean baseline withdrawal latency on consecutive determinations at a given dose. Mice were tested 30 minutes following each systemic (subcutaneous) morphine injection. ED₅₀ values were determined for all groups prior to (Day 1) and following (Day 4 and 8) tolerance induction except for mice in the vehicle control group which received an equal number of saline injections on Day 1. To insure that all mice in the morphine treatment group received the same cumulative dose of drug on Day 1, responders were injected with the same subsequent morphine doses as non-responder mice during Day 1 ED₅₀ determinations until all mice were analgesic. Morphine sulfate, supplied by the National Institute for Drug Abuse (Rockville, MD), was dissolved in 0.9% physiological saline for all doses. All systemic morphine doses were delivered in an injection volume of 10 ml/kg.

Time-Response Studies

On Day 1, baseline withdrawal latencies were assessed, and all mice received a single 10mg/kg morphine injection. Tail withdrawal latencies were then re-assessed at 30-minute intervals for 2 hours. Mice were then subjected to three days of morphine injections according to the dosing schedule above. On Day 4, baseline latencies and morphine analgesia were again determined at 30-minute intervals for 2 hours. Data from the time response study was analyzed as follows: Tail withdrawal latencies at

peak analgesia (30 minutes) for each sex on Day 1 and 4 were converted to %MPE scores $[(\text{post-drug latency} - \text{baseline latency} / \text{cutoff latency} - \text{baseline latency}) \times 100]$, and compared. The area under the time x latency curve (AUC; min x s) for males and females was used to calculate the % total analgesia (AUC/maximum possible AUC) and compared.

Data Analysis

Morphine dose-response data were analyzed using the BLISS-21 computer program. This program maximizes the log-likelihood function to fit a parallel set of Gaussian sigmoid curves to the dose-response data, and provides ED_{50} values, 95% confidence intervals (CI), and estimates of the relative potency (Umans and Inturrisi, 1981). Baseline tail-withdrawal latencies, and peak and total morphine analgesia between sexes on Day 1 were compared using an independent t-test. A two-way (one within, one between) repeated measures ANOVA was used to compare peak and %total morphine analgesia on Days 1 and 4. An alpha level of 0.05 was used for all comparisons.

RESULTS:

Dose response data:

Mean baseline tail-withdrawal latencies did not differ between males and females on either Day 1 (males: $4.1s \pm 0.63s$; females: $3.9s \pm 0.37s$), Day 4 (males: $3.8s \pm 0.41s$;

females: 4.0 ± 0.29 s) or Day 8 (males: 4.1 ± 0.37 s; females: 4.2 ± 0.40 s). In addition, ED_{50} values for each sex obtained on Day 1, for both the 3 and 7 day tolerance induction experiments, were practically identical, and were thus pooled for clarity of comparisons (i.e., Day 1 ED_{50} values for each sex reflect pooled Day 1 data from animals undergoing the 3 day paradigm, and those undergoing the 7 day paradigm.) As illustrated in Figure 1, three days of morphine administration produced a rightward shift in the morphine dose-response curve in both sexes on Day 4 and Day 8 relative to Day 1. Table 1 shows that the resultant morphine ED_{50} estimate was significantly increased on Day 4 (males: 12.2 mg/kg; females: 28.6 mg/kg) relative to Day 1 (males: 4.1 mg/kg; females: 6.2 mg/kg) in both males and females, indicative of tolerance. However, the magnitude of tolerance was significantly different between sexes. Whereas morphine potency was similar in males and females on Day 1, equivalent dose-response shifts and ED_{50} estimate increases were not obtained on Day 4 (Fig. 1 and Table 1, respectively). In females, there was an approximate 4.6 fold rightward shift in the morphine dose-response curve relative to only a three-fold shift in males (Fig. 1; Table 1). ED_{50} estimates from morphine naïve mice (Day 1 in morphine-treated mice and Day 4 in saline control mice) did not differ within or between sex, confirming that there were no significant initial differences in morphine sensitivity between the sexes, and indicating no effect of the repeated injection protocol.

Although morphine tolerance is increased in both sexes when the cumulative morphine dose is increased following 7 days of morphine treatment (Table 1 and Fig. 1), sex

differences in tolerance were still observed after this extended time period. Females displayed a significantly larger seven-fold rightward shift in the dose-response curve on Day 9 (ED_{50} : 43.1) compared to only a 4.8 fold shift for males (ED_{50} : 19.5) (Fig. 1 and Table 1). Again, a comparison of morphine naïve mice from Day 1 and Day 8 indicate no effect from repeated injection in either sex.

Time-response data:

The analgesic time course for a single 10 mg/kg injection of morphine is shown in raw data form in Fig. 2. Peak morphine analgesia occurred by 30 minutes post injection for both sexes, and there was no difference in corresponding % MPE scores at that time. A two-way (one between, one within) ANOVA for sex ($F_{(1, 14)} = 0.44$; n.s.), repeated measures (time after injection) ($F_{(1,14)} = 45.5$; $P < 0.001$), and their interaction ($F_{(1,14)} = 0.12$; n.s.) on peak morphine analgesia for Day 1 and 4 revealed decreased peak morphine analgesia on Day 4 relative to Day 1 which did not differ between sex. Although there was a significant repeated measures effect ($F_{(1,14)} = 42.41$; $P < .001$), indicating a decrease in morphine analgesia between Days 1 and 4, there was no effect of sex ($F_{(1,14)} = 0.84$; n.s.) or their interaction ($F_{(1,14)} = 0.14$; n.s.). Similar to the observations for dose-response studies, mean baseline tail-withdrawal latencies did not differ between males (4.3 ± 0.66) and females (4.2 ± 0.49).

A further analysis of Day 1 data was performed as well. A two way ANOVA was completed (dose x sex) using raw Day 1 tail withdrawal latencies at baseline and each

morphine dose. Results demonstrated a significant effect of dose (accounting for 80.69% of the variance; $F_{(5,210)}=322$; $p<.0001$). Sex had a small but significant effect, accounting for 1.87% of the variance, $F_{(1,210)}=12.32$; $p=.001$). There was no significant interaction effect ($F_{(51,210)}=2.07$; n.s.). As shown in Figure 3, significant differences in tail withdrawal latencies were not apparent at baseline, however, at all but the highest dose (where the cutoff of 15 seconds served as a ceiling), males demonstrated small but significant increases in withdrawal latencies over females (indicating greater antinociception in males at each morphine dose). This contrasts with the non-significant difference in the ED_{50} values at Day 1 for each sex, but is consistent with the trend toward higher ED_{50} doses in females at this baseline assessment.

Table 1. Morphine analgesic tolerance in male and female CD-1 mice^a

^aSeparate groups of mice received subcutaneous saline or morphine injections for 3 and 7 days using an escalating dosing schedule. Morphine ED₅₀ and 95% confidence intervals (CI) estimates (mg/kg) were derived from cumulative dose-response curves obtained on Day 1, 4, and 8, respectively (see Fig. 1). Day 1 data from the two experiments were pooled for clarity. Magnitude of potency loss indicates how much MORE morphine was needed to achieve analgesia at time point (Day 4 or 8) versus baseline ED₅₀ values (Day 4 or 8 ED₅₀ / Day 1 ED₅₀).

^bDenotes significantly higher magnitude of potency loss (i.e., tolerance) than males.

Table 1.

Treatment	Day 1	Day 4 [ED ₅₀ (95% CI) / <i>Magnitude of Potency Loss</i>	Day 8 [ED ₅₀ (95% CI) / <i>Magnitude of Potency Loss</i>
<i>Male</i> Morphine	4.1 (2.9 – 5.8)	12.2 (9.3 – 15.8)/ 2.98	19.5 (13.9 – 27.0)/4.78
Saline		5.0 (3.1 – 8.1)	6.5 (4.5 – 9.1)
<i>Female</i> Morphine	6.2 (4.8 – 8.1)	28.6 (22.5 – 36.2)/ 4.61 ^b	43.1 (29.9 – 62.1)/ 6.95 ^b
Saline		6.0 (3.7 – 9.4)	6.5 (4.6 – 9.1)

Fig.1. Cumulative dose-response curves for morphine analgesia on the tail-withdrawal test in male and female mice. Curves were obtained before (Day 1) and after 3 or 7 days of repeated (tid) and escalating morphine injections in separate experiments. For clarity, Day 1 data was pooled from the two experiments. ED₅₀ estimates derived from curves indicate significant ($p < .05$) loss of analgesic potency in both sexes, which was greater in females than males, on Day 4 and 8 relative to Day 1.

“% Animals Analgesic” indicates the percentage of animals at each dose that had doubled their baseline tail withdrawal latencies.

Figure 1.

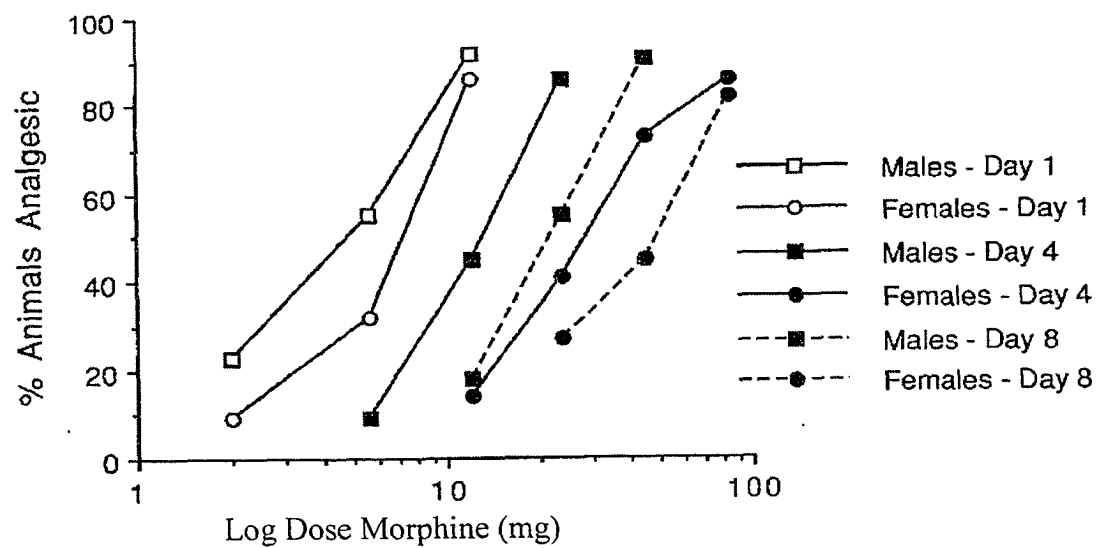


Fig. 2. Time-response curves for morphine analgesia (10mg/kg) on the tail-withdrawal test in male and female mice before (Day 1) and after (Day 4) 3 days of morphine injections. Significant ($P<.05$) reductions in analgesia on Day 4 relative to Day 1 were observed for peak %MPE (30-min post injection latency) and % total analgesia (AUC/maximum possible AUC) for males ($n=10$) and females ($n=10$).

Figure 2.

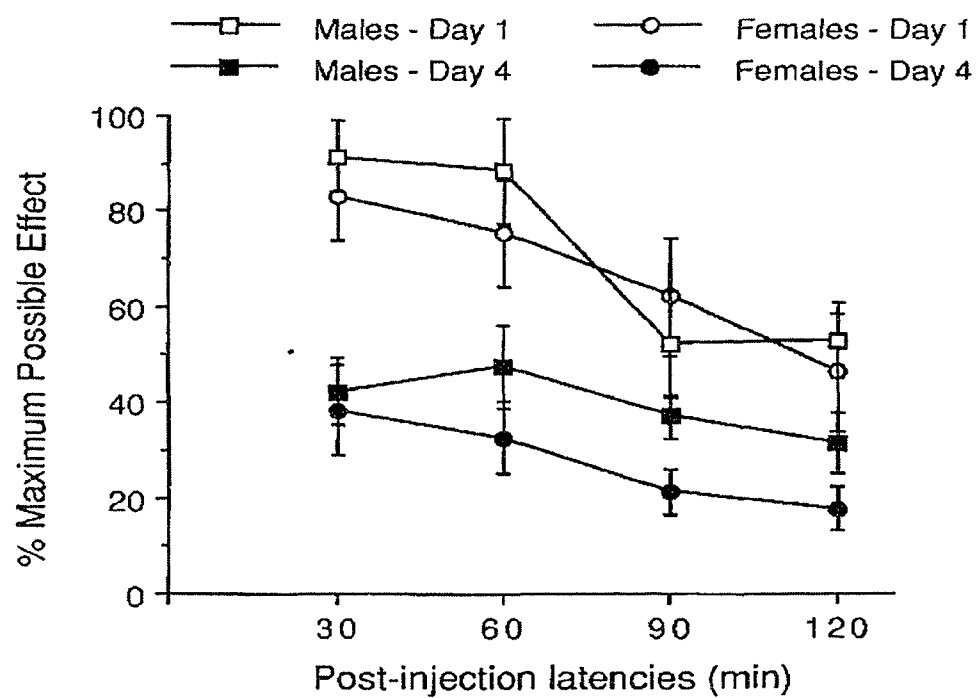
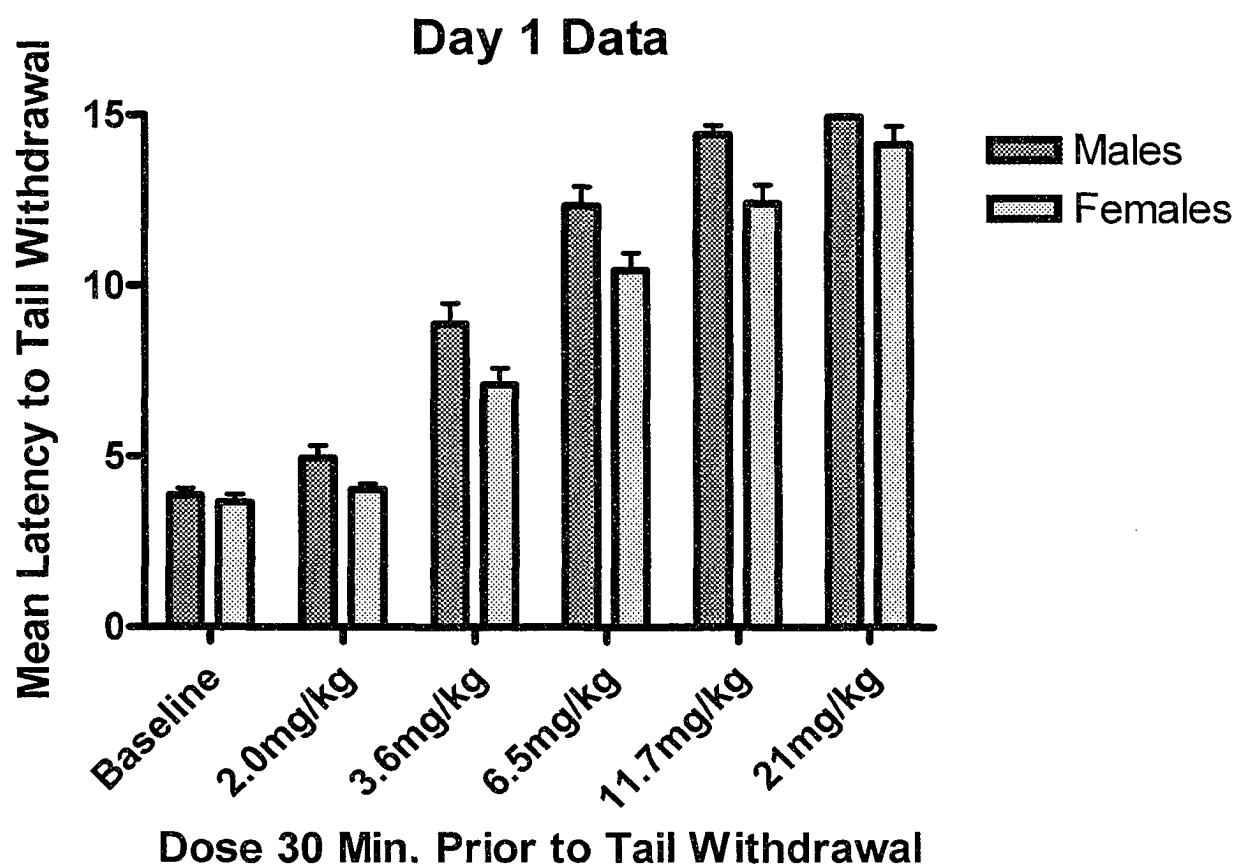


Figure 3.

Day 1 Mean Latency to Tail Withdrawal in Males and Females at Baseline and Each
Morphine Dose

Figure 3.



DISCUSSION:

A number of important findings emerge from the present study. First, under the specific conditions and tolerance induction method used, female CD-1 mice develop greater analgesic tolerance to morphine than males. Significant rightward shifts in the morphine dose-response curve and increased ED₅₀ estimates demonstrate tolerance for both male and female mice following 3 and 7 days of repeated systemic morphine injections. Whereas they were not significantly different on Day 1, the ED₅₀ estimates on Days 4 and 8 were however significantly greater in females than males, indicating the development of greater analgesic tolerance in females regardless of the morphine treatment period. This finding contrasts with previous rat studies in which greater or equal tolerance was observed in males (Badillo-Martinez et al., 1984; Craft et al., 1999; Kasson & George, 1984), and highlights an important aim of this study. That is, subject species may influence the presence and/or direction of sex differences in morphine tolerance. Although rats and mice may differ in neural substrates that may contribute to potential differences in tolerance between these species (Abdelhamid et al., 1991; Kest et al., 1996; Robson et al., 1985; Yoburn et al., 1991), a review of the literature did not find reports of any sexual dimorphism exclusively present within one species that could account for the contradictory findings between rats and mice on sex differences in tolerance. It is also worth noting that although greater tolerance was observed on Day 8 compared to Day 4, the relative sex difference in potency change remained approximately equal (approximately 1.5 fold greater loss of potency in females). This

finding suggests that the duration and/or cumulative dose of morphine administration may not impact on sex differences in tolerance in mice.

While ED₅₀ values were not significantly different in males and females on Day 1, an analysis of raw tail withdrawal latency data at each dose during the baseline (Day 1) assessment of morphine's analgesic efficacy showed a small but significant main effect of sex. Specifically, male mice showed longer latencies to tail withdrawal at most doses, suggesting a slightly greater analgesic effect of morphine in males than in females. However, while Day 1 ED₅₀ values were about 50% higher in females than in males (a non-significant difference), following the tolerance induction period, females showed ED₅₀ values over 100% greater than their male counterparts. Thus, it is suggested that differences in ED₅₀ values seen after chronic morphine administration were not simply secondary to female CD1 mice having a trend toward lower sensitivity to morphine's analgesic effects than males. It would be of interest to assess whether the greater morphine tolerance in female mice demonstrated in the current study would consistently be observed in other mouse strains in which initial morphine sensitivity was greater for males than for females. Such a finding (to be explored in Specific Aim 6 of the present dissertation) would be suggestive of shared genetic mediation and/or shared mechanisms of control for initial morphine analgesic efficacy, and morphine analgesic efficacy following chronic administration.

Since ED₅₀ values in morphine naïve mice of both sexes obtained before (Day 1 morphine treated group) and after (Day 4 saline-treated group) three days of repeated injections did not significantly differ, sex differences in response to the possible stress of repeated and chronic handling and injections also do not appear to contribute to the present sex differences in tolerance. Interestingly, in the current study there was no significant effect of sex in the magnitude of the decrease in either peak %MPE or % total morphine analgesia after 3 days of morphine. This stands in contrast to the present demonstration of greater morphine tolerance, as indicated by significantly increased ED₅₀ values, in females relative to males following the identical morphine dosing regimen. Thus, sex differences in morphine tolerance may depend on the method used to assess analgesia. Alternatively, these apparently discrepant findings may be attributable to the use of a single morphine dose on Day 1 and Day 4 to generate the AUC data as compared to multiple doses used in generating dose-response curves. That is, use of lower or higher morphine test doses on Days 1 and 4 may reveal sex differences in morphine tolerance in AUC measures of analgesia that are dose – dependent. In rats, sex differences in morphine tolerance are indeed dependent on the test dose (Craft et al., 1999). This possibility warrants further study.

It is very difficult to postulate how sex differences in morphine tolerance may be affected given the lack of studies on opioid tolerance in females in the literature, and the myriad of biochemical and structural changes that accompany tolerance in males (Collin & Cesselin, 1991; Elliott et al., 1995; Harrison et al., 1998; Mayer & Mao,

1999). Reference to the literature on sex differences in opioid analgesia per se would also not very likely be revealing as explanations of these differences are as varied as they are equivocal (see Kest et al., 2000; Craft, 1993 for reviews). Nonetheless, some systems with reported sexual dimorphism have been shown to have great import with regard to morphine tolerance, and require consideration. For example, male and female rats differ in the expression of the immediate early gene c-Fos following morphine, and the ability of antagonists of the NMDA excitatory amino acid receptor to block this response is sexually dimorphic (D'Souza et al., 1999). In the same study, a greater sensitivity to the behavioral effects of the NMDA antagonist MK-801 given without morphine was observed in females only. Since morphine tolerance has invariably been shown to be quite sensitive to NMDA receptor activation in rats and mice (Elliott et al., 1995; Kest et al., 1993; Marek et al., 1991; Mayer & Mao, 1999), functional sex differences in NMDA receptor activity following chronic morphine may reasonably contribute to sex differences in morphine tolerance. Sex differences in the anti-opioid peptides Tyr-Mif-1 and neuropeptide FF have also been documented (Kavaliers & Innes, 1990; 1992a,b), and both peptides have been implicated in the development of morphine tolerance (Harrison et al., 1998). However, since these sexual dimorphisms have been demonstrated only with regard to morphine analgesia, their mechanistic relevance to sex differences in morphine tolerance warrants further attention. Finally, we can not rule out the possible contribution of sex differences in learning processes on sex differences in morphine tolerance. Specifically, it has been repeatedly demonstrated in rats that an association between environmental cues and the systemic

effects of morphine contributes to morphine tolerance (Baptista et al., 1998; Siegel, 1976, 1999). It is also well known that sex modulates classical conditioning, as well as several other types of contextual learning paradigms, in rats (see reviews, Shors et al., 2000; Van Haaren et al., 1990). Thus, it is possible that males and females differ in the rate at which environmental and contextual cues are associated with the effects of morphine, which could in turn lead to sex differences in the magnitude of tolerance. These issues present interesting and important areas of further study.

To our knowledge, the above experiment was the first to examine possible sex differences in morphine tolerance in mice. The next five specific aims of the current dissertation were conducted in order to clarify the degree to which these findings generalize to other strains, nociceptive modalities, methods of administration, or even to non-analgesic effects of morphine.

SPECIFIC AIM TWO

Sex differences in morphine tolerance: centrally (ICV) administered morphine

INTRODUCTION:

Recent reviews of the human and animal literature indicate that males and females differ in their sensitivity to morphine analgesia (Kest et al., 2000), although the presence, magnitude, and direction of these sex differences can vary with intrinsic factors such as genetic background (Kasson & George, 1984; Kest et al., 1999; Mogil et al., 2000). Although the mechanisms underlying sex differences in morphine analgesia are unclear, the participation of centrally located neural mechanisms are indicated since analgesic differences persist in rodents following intracerebroventricular (ICV) morphine micro-injection. A reduction in morphine analgesia is commonly observed following its chronic administration, and sex differences in analgesic tolerance following chronic systemic morphine administration have been reported in rats (Badillo-Martinez et al., 1984; Craft et al., 1999).

In CD-1 mice, a comparison of morphine analgesia ED₅₀ values prior to and following 3 days of repeated systemic morphine injection revealed a greater reduction in analgesic potency in females relative to males (Kest et al., 2000; see Specific Aim One).

Findings of that study suggested that the sex difference in morphine tolerance were not simply related to initial differences in morphine sensitivity in this strain. Unlike morphine analgesia, no previous studies have addressed whether centrally located

neural mechanisms contribute to differences in morphine analgesic tolerance between the sexes. Thus, the present study again compared morphine analgesia ED₅₀ potency ratios in CD-1 mice of both sexes after three days of repeated morphine injection, but using an ICV route of drug administration. This study was published in Brain Research, Volume 892 (see Kest and Hopkins, 2001).

METHODS:

Subjects and Nociceptive Assay:

Adult male and female CD-1 mice (25-35 g) were used, and housed according to the procedures previously outlined in Specific Aim One. The tail withdrawal test was also utilized, according to the previously described procedure.

Dose-response studies:

Tolerance was operationally defined as a significant increase in morphine ED₅₀ values. To reduce the number of mice, ED₅₀ values were derived from cumulative dose-response curves as previously described (see Kest et al., 1996). Briefly, mice were injected intracerebroventricularly with a 1.0 microgram dose of morphine and received increasing doses (~0.25 log units: 2.0, 3.6, 6.5, 11.7, 21 microgram) until each became analgesic. Analgesia was operationally defined as a doubling of each subject's mean baseline withdrawal latency on consecutive determinations at a given dose. Mice were tested 30 minutes following each morphine injection. ED₅₀ values were determined for all groups prior to (Day 1) and following (Day 4) tolerance induction except for mice in

the vehicle control group which received an equal number of saline injections on Day 1. To insure that all mice in the morphine treatment group received the same cumulative dose of drug on Day 1, responders were injected with the same subsequent morphine doses as non-responders mice during Day 1 ED₅₀ determinations until all mice were analgesic. Morphine sulfate, supplied by the National Institute for Drug Abuse (Rockville, MD), was dissolved in 0.9% physiological saline for all doses. All morphine doses were delivered in an injection volume of 5 microliters.

Tolerance induction:

Mice in the morphine treatment group were injected intracerebroventricularly three times daily for three days according to an escalating dosage schedule (10, 20, and 40 micrograms TID on days 1, 2, and 3, respectively). Mice in the saline control group received an equal number of saline injections, but were not tested on Day 1. All injections within a given day took place 2-3 hours apart.

For all ICV injections (morphine and saline), mice were anesthetized via isoflourane inhalant anesthesia as previously described (Haley and McCormick, 1957; Kest et al., 1999). Prior to the first injection, a small midline incision was made in the scalp of the anesthetized animal and bregma located. Injections consisted of a bolus of 5microliters saline directly through the skull at the point 2mm caudal and lateral to bregma at a depth of 3mm using a Hamilton microsyringe with a 27 gauge needle. Following the first injection, all subsequent intracerebroventricular injections were administered

through the same hole in the skull, with a needle fitted with a stopper to ensure the exact depth of placement. The incision was closed by a stainless steel wound clip after each injection to minimize the likelihood of infection, except during dose response studies, when the incision was not stapled to prevent increased tissue damage / pain secondary to staple implantation or removal.

Data Analysis:

Data analysis of was performed as described in experiment one.

RESULTS:

As presented in Table 2, male and female mice did not differ in either baseline withdrawal latencies or morphine analgesic potency, as indicated by ED₅₀ estimates derived from cumulative dose response curves on Day 1. However, 3 days of ICV injections significantly increased ED₅₀ estimates from both sexes on Day 4 to a similar extent (approximately 6-7 fold), indicating tolerance. In both sexes, morphine ED₅₀ estimates obtained on Day 4 from saline treated (control) mice did not significantly differ from the Day 1 values of morphine treated mice, indicating no significant effect of repeated handling/injection stress in either sex. Morphine ED₅₀ values for morphine treated mice, however, were significantly increased relative to this control group on Day 4, indicative of an equivalent loss in morphine analgesic potency in both sexes. Again, mean baseline withdrawal latencies were similar for both sexes on Day 4.

Table 2.

Morphine analgesic tolerance following chronic ICV administration in male and female mice^a

^aBaseline tail withdrawal latencies [$\pm$ standard error (S.E.)] and morphine ED₅₀ estimates with 95% confidence intervals (CI) were obtained before and after 3 days of ICV morphine injection in CD-1 mice. Values (μ g) were derived from cumulative dose-response curves.

^bDenotes significant loss in morphine analgesic potency.

Table 2.

Sex	Test Day	Baseline Tail Withdrawal Latency (S.E.)	Treatment	ED ₅₀ (ug)	95% CI
Males	Day 1	3.4 (0.7)	Morphine	1.3	(0.8-2.3)
Females	Day 1	3.7 (0.6)	Morphine	1.8	(1.0-3.3)
Males	Day 4	3.6 (0.3)	Morphine	^b 10.2	(6.8-15)
Females	Day 4	3.5 (0.6)	Morphine	^b 11.1	(7.3-16.1)
Males	Day 4	3.3 (0.4)	Saline	3.3	(2.0-5.6)
Females	Day 4	3.7 (0.8)	Saline	3.6	(2.3-5.3)

DISCUSSION:

Morphine analgesic sex differences in rodents are dependent on genotype (Kasson and George, 1984; Kest et al., 1999; Mogil et al., 2000). In the present study, the absence of analgesic sex differences following ICV morphine injections on Day 1 may thus reflect low allelic frequencies of trait-relevant genes in our sample. Alternatively, differences in the subcutaneous dosing used in the prior study and intracerebroventricular doses used in the present study may have lead to these disparate results. It should be noted that subsequent to higher ICV doses, some animals (both males and females) demonstrated atypical, seizure-like motor behaviors and vocalizations. As such, it can not be ruled out that these high doses directly administered to the brain may have resulted in effects that prevented identification of sex differences.

While these caveats must be considered, the finding of similar magnitude of morphine analgesic tolerance following ICV administration does demonstrate that the mechanisms underlying tolerance to supraspinal morphine analgesia may not be sex sensitive. Although this suggests that supraspinal analgesic mechanisms do not contribute to the sex difference in analgesic tolerance following systemic morphine shown in Specific Aim One, the demonstrated significant contribution of genotype to sex differences in morphine analgesia (Kest et al., 1999; Mogil et al., 2000) cautions against drawing conclusions to mouse strains other than CD-1.

Since both supraspinal and spinal mechanisms affect systemic morphine analgesia, sex differences in tolerance to systemic morphine analgesia, if not mediated by supraspinal mechanisms of morphine tolerance, may be mediated at the spinal level. Tolerance following systemic morphine injections has also been proposed to result from loss of synergy between spinal and supraspinal opioid analgesic mechanisms (Roerig and Fujimoto, 1988), and this multiplicative interaction between CNS sites may be sexually diergic and the source of sex differences in systemic morphine tolerance. If so, sex differences in tolerance following systemic morphine injections would indeed not be evident by assessing tolerance at supraspinal sites alone, as presently assessed.

Although sex differences in spinal and supraspinal opioid analgesic synergy have not yet been studied, it is interesting that the degree of systemic tolerance in different mouse strains has been related to differential changes in spinal-supraspinal analgesic synergy (Roerig and Fujimoto, 1998). It is hoped that future studies directly comparing morphine synergy in males and females before and after chronic morphine will address its relevance to sex differences in systemic morphine tolerance. Additionally, given the finding of sex differences in morphine analgesic tolerance when a systemic route was used (in Specific Aim 1), the lack of significant sex differences with the current study's intracerebroventricular route is an important finding. As such, examination of possible spinal mediation of sex differences becomes increasingly important, and will be the focus of Specific Aim 3.

SPECIFIC AIM THREE

Sex differences in morphine tolerance – intrathecally administered morphine

INTRODUCTION:

Since both supraspinal and spinal mechanisms affect systemic morphine analgesia, sex differences in tolerance to systemic morphine analgesia, if not mediated by supraspinal mechanisms of morphine tolerance, may be mediated by tolerance in spinal morphine analgesic mechanisms. The presence of opioids in high density in the dorsal horn of the spinal cord provides the logical basis for spinal opioid use clinically (Pert & Snyder, 1973, Sabbe & Yaksh, 1990), and makes the spinal cord a strong candidate for an area subserving sex differences in tolerance development. Tolerance following systemic morphine injections has also been proposed to result from the loss of synergy between spinal and supraspinal opioid analgesic mechanisms (Roerig & Fujimoto, 1988), and this multiplicative interaction between CNS sites may be sexually dimorphic and the source of sex differences in systemic morphine tolerance. If so, sex differences in tolerance following systemic morphine administration might not be evident by assessing tolerance at either supraspinal or spinal sites alone. Regardless of the exact mechanism by which sex differences in morphine tolerance might be modulated, assessment of tolerance development through intrathecal administration will likely contribute to our understanding of the phenomenon. The aim of the present study was to assess whether sex differences in tolerance of spinal analgesic mechanisms contribute to sex differences in systemic morphine analgesic tolerance by creating dose-response curves

for systemic morphine analgesia after chronic intrathecal morphine microinjection. This study was presented as a poster at the Society for Neuroscience Annual Meeting (Hopkins, Rossi, Kest, 2003).

METHODS:

Subjects and Nociceptive Assay:

Male and female CD1 mice were studied, and housed as described in specific aim one. Likewise, the tail withdrawal assay (as previously described) was used in this study.

Tolerance induction:

Animals were injected intrathecally three times a day according to the following schedule: 10micrograms TID day 1, 20 micrograms TID day 2, and 40micrograms, followed by two 20microgram injections, on day 3. (This was an alteration of the initial planned dose on Day 3 of 40 micrograms TID, as upon the first 40 microgram dose, a number of animals showed adverse effects). Following being anesthetized with inhalant halothane, intrathecal microinjections were conducted percutaneously, with spinal injection occurring at lumbar sites. The technique produced some mild swelling/stiffness around the site of injection in some animals, and as such subsequent injections were delivered slightly more rostrally or caudally. (Given that injections were delivered percutaneously, all injections were likely delivered to slightly different sites, however, a characteristic “popping” sensation and simultaneous flicking of the animal’s tail confirmed the injection had reached the spinal cord). All injections were

delivered in a 2 microliter bolus. A saline control group received equivalent injections of 0.9% physiological saline.

Dose response studies:

On Day 4, subsequent to 3 days of thrice daily intrathecal injections, all animals were subjected to a dose response study. Baseline tail withdrawal latencies were conducted to assess baseline nociception in the 49° C tail withdrawal assay (described previously). Morphine was administered subcutaneously in increasing log doses (1mg/kg, 2mg/kg, 3.5mg/kg, 6.5mg/kg, 11.7mg/kg, 21mg/kg, 42mg/kg). Thirty minutes subsequent to each injection, each animal was tested again on the tail withdrawal assay, and response latencies recorded. Animals that did not show analgesia following the 42mg/kg dose were given one additional 42mg/kg dose and their latencies were recorded. A saline control group (having received 2ml saline intrathecally three times a day) was subjected to the same dose response study on Day 4, as was a group of naïve animals that had not undergone intrathecal injections. It should be noted that all animals were tested with subcutaneously delivered morphine due to the timing requirements of the cumulative dose response paradigm used, and the high likelihood that the stress of intrathecal injections would have made interpretation of findings questionable. Further, and most importantly, given the findings of sex differences in tolerance with subcutaneous morphine delivery, the current paradigm was chosen to determine whether or not spinal mechanisms are implicated in this difference. As such, control conditions of a saline group and a naïve group were chosen (in lieu of “Day 1” administration of a

subcutaneous cumulative dose response curve used in other studies) in order to rule out any confound caused by initial sex differences which may have resulted from the morphine administered subcutaneously (and ensure that results observed would be solely reflective of the effects of chronic intrathecal morphine).

Statistical analysis:

Statistical analyses were performed as in Specific Aim One.

RESULTS:

As shown in Table 3, while there was a trend toward higher ED₅₀ values in females, male and female naïve mice did not significantly differ in responsivity to morphine analgesia, as shown by overlapping confidence intervals. Likewise, control mice treated with i.t. injections of saline thrice daily for three days were not significantly different from naïve mice, and no sex differences were observed, indicating no general or sex-specific effect of the repeated i.t. injection process. In contrast, morphine treated mice demonstrated a significant rightward shift in their dose response curves, and a significant sex difference emerged, with females showing greater morphine analgesic tolerance than males as evidenced by significantly different ED₅₀ values on Day 4. It should be noted that some of the animals had adverse effects as a result of the higher doses of intrathecal morphine injections, a number demonstrating seizure-like activity and/or hyperflexion of hind limbs for a period of twenty minutes to an hour. This state

spontaneously resolved in most of the animals where it occurred, although there were 2 to 3 deaths in each sex. This phenomenon was not observed in saline treated animals.

Figure 4. Dose – response curves for systemic morphine analgesia in male and female mice after chronic intrathecal saline or morphine injection. Morphine ED_{50} values were significantly greater in morphine treated mice of both sexes, indicative of tolerance. The magnitude of analgesic tolerance was, however, greater in females.

Figure 4.

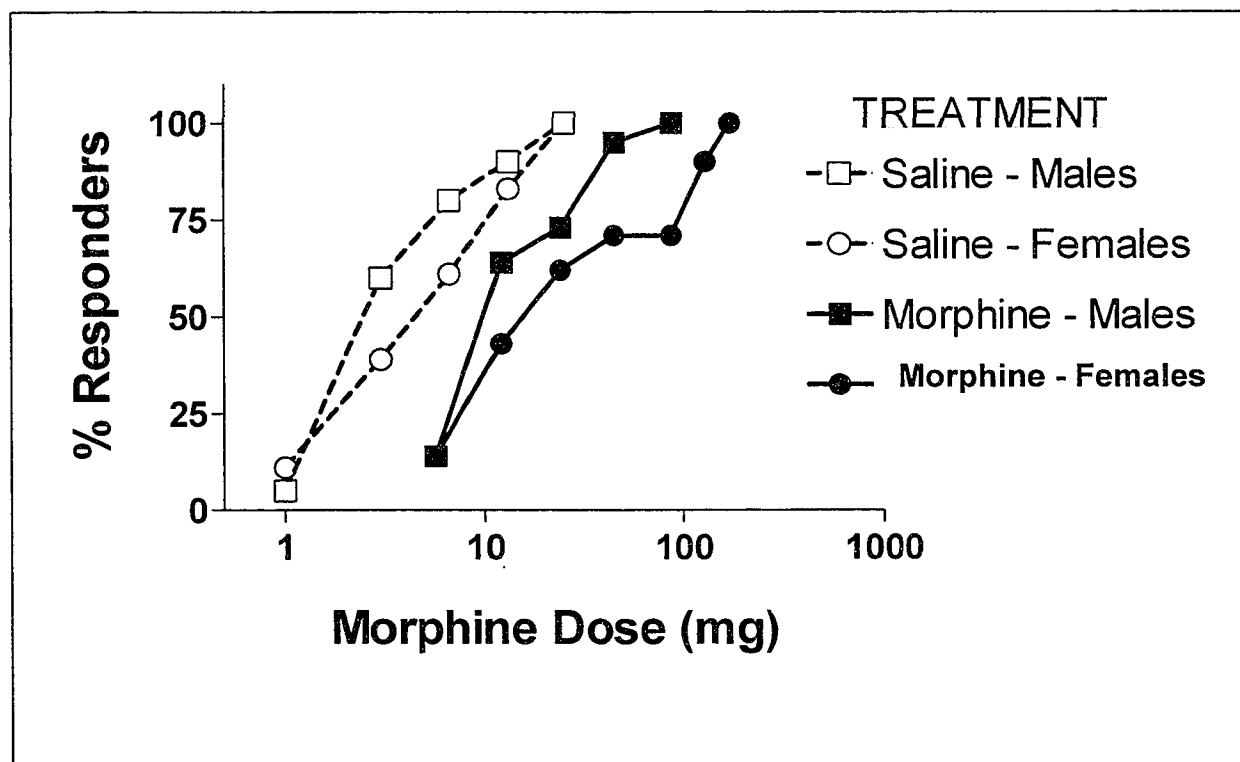


Figure 5. Dose – response curves for systemic morphine analgesia in male and female mice before (naïve group) and after chronic intrathecal saline injection. Morphine ED₅₀ values (see Table 3) indicate no significant difference between naïve and saline-treated mice in either sex.

Figure 5.

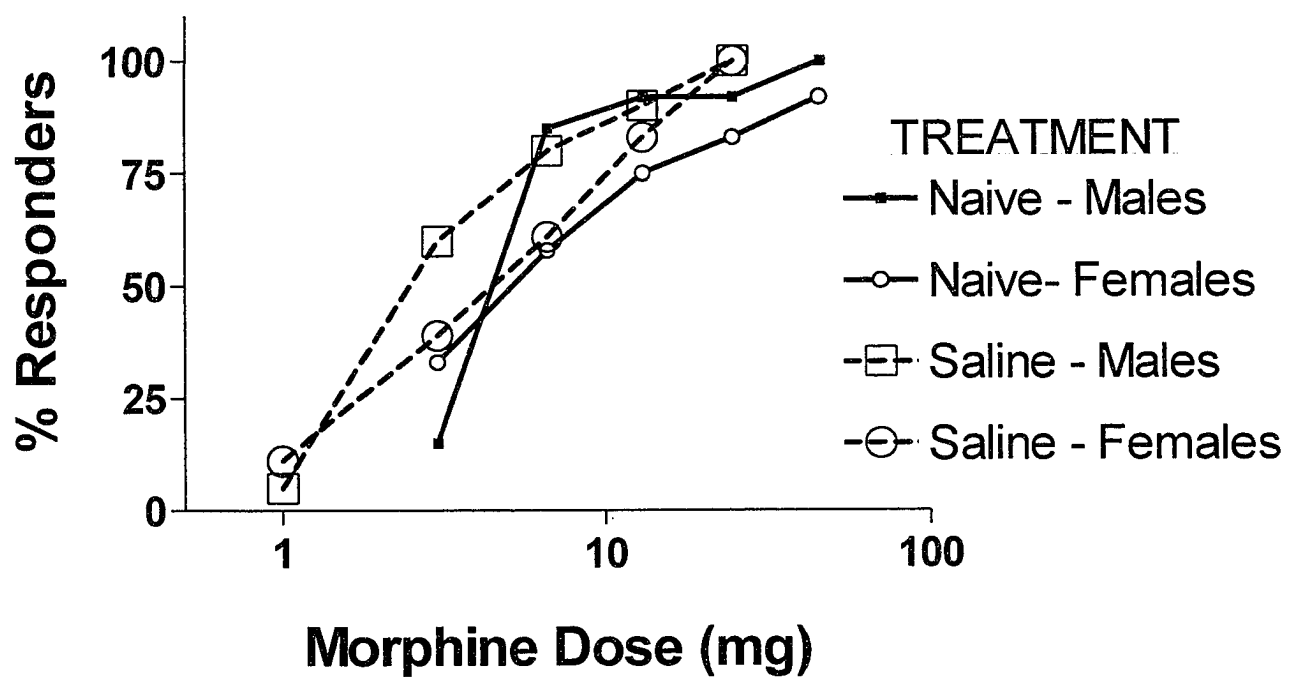


Table 3. Analgesic potency of morphine injected systemically in male and female mice following chronic intrathecal morphine treatment. Morphine ED₅₀'s and 95% confidence intervals for naïve mice, mice exposed to 3 days intrathecal saline injections, and mice exposed to three days of intrathecal morphine injections.

^aPotency Shift = Morphine Treated Mice ED₅₀ values / Saline Treated Mice ED₅₀ values.

^bRelative Potency: Male ED₅₀ / Female ED₅₀

*Indicates significant difference ($p < 0.05$) between males and females, as indicated by non-overlapping 95% confidence intervals

Table 3.

	ED50 (95% Confidence Interval)			Potency Shift^a
	Naïve	3 Days IT Saline	3 Days IT Morphine	
Males	2.9 (1.7-4.9)	2.0 (1.3-3.0)	10.3 (7.2-14.3)	5.15
Females	4.4 (2.5-6.7)	3.1 (2.0-4.6)	21.9 (14.7-30.4)	7.06
Relative Potency ^b	.66	.65	.47*	

DISCUSSION:

The results of the present study demonstrate sex-dependent decreases in the analgesic efficacy of subcutaneous morphine doses when the manner of tolerance induction is intrathecal morphine injections. The direction of the differences observed was identical to that found with subcutaneous morphine injections during the tolerance period, with females demonstrating significantly higher ED₅₀ values on Day 4 than male CD1 mice. In light of Specific Aim 2's finding of no sex difference in tolerance to centrally (ICV) administered morphine, the findings of the present study strongly suggest that spinal mechanisms may underly the sex differences observed in Specific Aim 1.

Methodologically, there are several issues to consider when interpreting these findings. First, several animals showed adverse effects of the highest morphine dose (40micrograms), resulting in a characteristic response of apparently involuntary hind leg extension, vocalizations, and at times, seizure-like activity. Several fatalities (in both males and females) were observed at this dose, and as such, findings should be interpreted cautiously. Further, while sex differences in tolerance development was apparent given the dosing regimen used, it is not clear whether such differences would also have been identified if lower doses of morphine were used during the induction period. Secondly, the current study used intrathecal injections during tolerance induction, but assessed morphine analgesic potency through subcutaneous injections. Whether or not differences in morphine analgesic tolerance would also have been observed if intrathecal injections were used on "test day" is unknown.

The spinal cord has long been recognized as the potential locus of adaptive changes underlying morphine tolerance. Several prior studies have reported that chronic exposure of the spinal cord to morphine will induce tolerance to its analgesic effects (Advocat et al., 1987; Milne et al., 1985; Yaksh et al., 1977). However, in the current study, tolerance to morphine analgesia elicited by subcutaneous injections was brought about by three days of intrathecal morphine injections. These findings of tolerance to systemic analgesia produced by intrathecal morphine injections itself stands in contrast to some published reports. For example, Siuciak and Advocat reported (1987) no effect of spinal morphine pretreatment on the analgesic efficacy of a subsequent subcutaneous morphine challenge, and concluded that chronic, intrathecal morphine is not “cross tolerant” with systemic morphine. Their design, however, called for spinally catheterized rats to be injected once daily for three days with 5micrograms of morphine (in 10microliters of saline followed by a 10 microliter saline wash). These methods stand in contrast to the present mouse study, in which distinctly higher doses were used, and the cumulative dose received after the end of the three days in mice was 170micrograms of morphine, compared to their total of only 15micrograms. (It should be noted that in their study, however, this dose was sufficient to produce notable tolerance to morphine administered to the PAG region, which was cross tolerant with systemically administered morphine.) As such, the significant findings in the current study are further evidence of the manner in which disparate experimental methodologies bring about disparate, and at times, conflicting, results. This “explanation” is also a frequent comment in research pertaining to sex differences in

morphine analgesia. A review of the literature, however, did not find other studies exploring possible sex differences in morphine analgesia elicited from intrathecal administration. The methodological difficulty of this technique may have limited study of its contribution to sex differences in analgesia, as well as analgesic tolerance. Given the important role of the spinal cord in pain modulation, and our awareness of its role in pain facilitation, more study of this area is necessary.

As demonstrated first by Yeung and Rudy in rats (1980), and later by Roerig & Fujimoto (1989), subanalgesic doses of morphine co-administered spinally and supraspinally can result in potent analgesia (displaying robust synergism). Roerig et al. (1984) also showed that when mice were made tolerant to systemic morphine, they no longer demonstrated this synergism, and instead concurrent injections to spinal and supraspinal sites resulted in only an additive response). The degree to which loss of synergism underlies sex differences in systemic morphine tolerance (or tolerance to systemic injections following chronic intrathecal morphine treatment as in the present study) is unknown, and the possibility exists that supraspinal mechanisms do play a role in sex differences in tolerance development (despite the lack of significant findings in Specific Aim 2.) Other explanations for the current findings include functional sex differences in NMDA receptor activity following chronic morphine (as suggested by D'Souza et al., 1999), or as yet unreported sex differences in any of the multitude of systems and substances that effect tolerance development, remains unknown. Importantly, however, as a step toward a better understanding of this phenomenon, the

present study is the first to clearly identify a role for spinal mechanisms in modulating sex differences in morphine tolerance in male and female CD1 mice.

SPECIFIC AIM FOUR

Sex differences in morphine tolerance: acetic acid study

INTRODUCTION:

Sex differences in morphine's analgesic effects have been reported across a number of nociceptive assays, as discussed in detail in the literature review section. In mice, the presence, magnitude, and direction of sex differences in morphine analgesia differed among eleven genetically distinct inbred mouse strains (Kest, Wilson, & Mogil, 1999). The degree to which sex differences in analgesic tolerance, should they exist, generalize to multiple typographies of pain has not to date been fully explored.

Tolerance to morphine's numerous effects does not appear to occur along the same timelines. For example, while tolerance to respiratory depression, somnolence, and nausea generally develop rapidly, tolerance to opioid-induced constipation develops very slowly, if at all (Foley, 1991). The possibility exists that these differences may extend even to multiple subtypes of analgesic tolerance. For example, tolerance to the effects of morphine at alleviating acute, thermal pain may occur in a different manner or timeline than tolerance to its effect at alleviating lasting, visceral pain. One study (Fernandes, Kluwe, & Coper, 1977), however, compared tolerance to a number of morphine's effects (including catalepsy, behavior on the tilted plane, hot plate analgesia and acetic acid writhing analgesia), and determined that they developed in a "closely similar" manner. In their study, however, only male rats were used. The extent to

which sex differences in tolerance to morphine analgesia may exist for certain assays, and not for others, may provide insights into the possible mediators of these effects.

Visceral pain is characterized by a subjectively painful perception located in the abdominal area (Gshossmann et al., 2002). In rodents, the acetic acid writhing test is used as a model of chemical visceral pain (Vycklicky, 1979). The writhing response is produced in rodents through intraperitoneal injection of pain-producing chemical substances. The acute peritonitis resulting from the injection produces a response characterized by abdominal contractions/constrictions - lengthwise stretches of the torso with a concomitant concave arching of the back. Attenuation of this writhing response by low doses of morphine has been demonstrated (Hammond, 1989).

The following study aims to observe the extent to which male and female CD-1 mice continue to benefit from the analgesic effects of morphine on a nociceptive assay of chemical/visceral pain after a three day period of morphine or saline injections.

METHODS:

The following study was completed in accordance with approved protocols of the College of Staten Island/ CUNY Institutional Animal Care and Use Committee.

Subjects:

Naïve adult male and female CD1 mice (20-35 grams) 6-8 weeks of age were housed five to a cage with same sex littermates and maintained on a 12 hour light / 12 hour dark cycle. The environment was temperature controlled ($22^{\circ}\text{C} \pm 2^{\circ}$) and the mice had unrestricted access to food and water. All testing was done near mid-photo phase (between 10am and 2 pm) to minimize circadian fluctuations in morphine sensitivity.

Drugs:

Morphine sulfate, supplied by the National Institute of Drug Abuse (Rockville, MD, USA) was dissolved in distilled water for all morphine dosing. The 0.9% saline was used alone for control injections. All morphine and saline control injections were delivered via subcutaneous injection in a volume of 10mg/kg weight per mouse. To elicit the pain response (writhing), a 0.9% solution of glacial acetic acid in saline was used. Acetic acid injections were delivered to gently restrained animals intraperitoneally in a 10ml/kg bolus.

Tolerance induction:

Mice in the morphine tolerance induction group received progressing doses of morphine delivered three times a day across a three day period (10mg/kg TID day 1, 20mg/kg TID day 2, 40mg/kg TID day 3). Saline controls received equivalent injections of physiological saline.

Dose-Response Measurement:

Morphine doses were chosen based on pre-study data to assess antinociceptive effects in both animals exposed only to saline injections and those exposed to chronic morphine treatment. On test day (Day 4), all animals received an injection of morphine (0.5 mg/kg, 1mg/kg, or 2mg/kg) or saline 20 minutes prior to an intraperitoneal injection of 0.9% glacial acetic acid. Following the injection of acetic acid, mice were observed for writhing responses and data recorded in 5 minute intervals for a 30 minute period. Data was collected on all three doses for mice receiving chronic morphine treatment and those receiving matched saline injections.

Statistical Analysis:

A two way (sex x treatment) ANOVA was used, followed by comparisons of each treatment condition with the baseline (saline TID/saline preinjection) condition using the Bonferroni procedure.

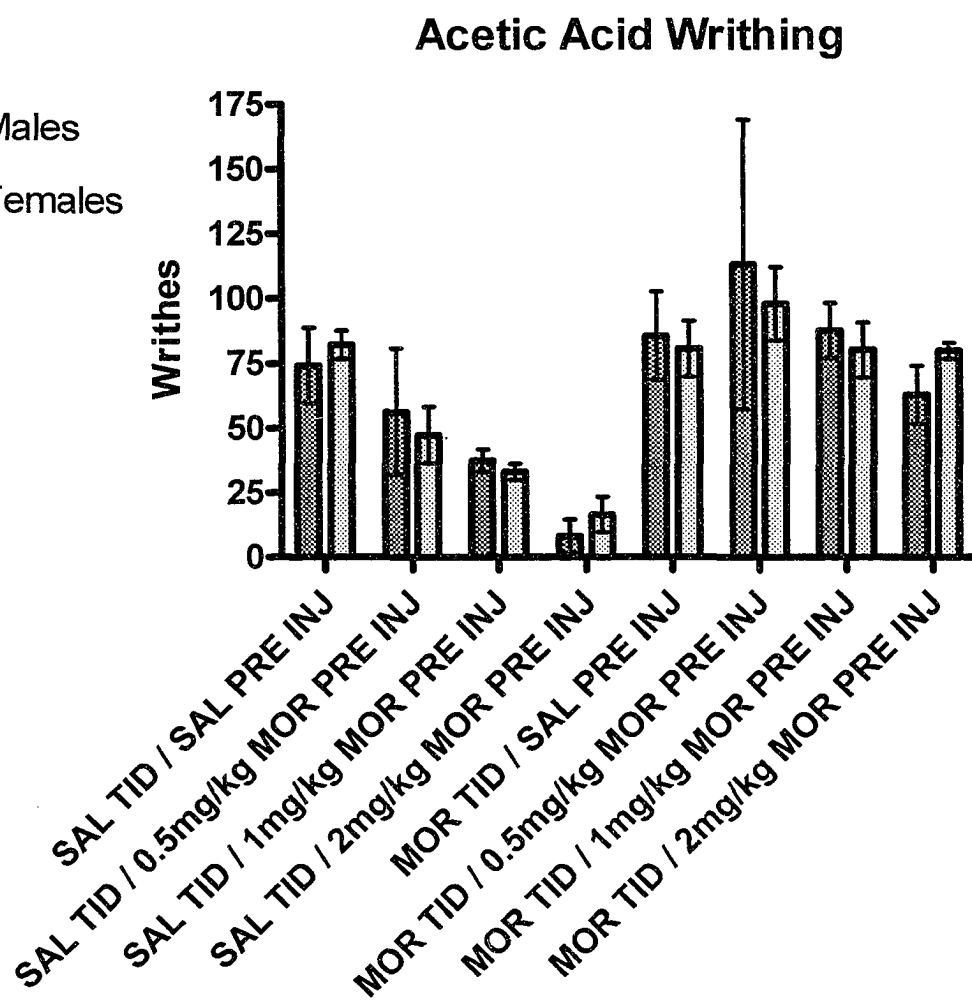
RESULTS:

Analysis of variance showed the data to be statistically significant for treatment effect ($p=.0001$). There was no main effect of sex ($p=.8264$), and no interaction effect ($p=.6638$). However, pairwise testing demonstrated that while morphine was able to attenuate writhing responses in a dose-dependent manner, it failed to produce observable analgesia in any of the groups chronically exposed to morphine (even at the dose of 2mg/kg, which was able to almost completely block the writing response in

naïve animals). There was no evidence greater of variability in writhing responses in females than males, with standard deviations being similar for most conditions. The only treatment condition in which possible evidence of a sex difference emerged was at the 0.5mg/kg dose of morphine in the morphine tolerant group, which paradoxically lead to a statistically significant INCREASE in writhing in males ($p < .01$), but not females.

Figure 6: Writhing responses in male and female mice at each morphine dose and pretreatment condition.

Figure 6.



DISCUSSION:

Results of the current study indicated a loss of analgesic efficacy of morphine in both sexes, such that the frequency of writhing responses did not decrease below baseline in animals rendered tolerant to morphine, despite these doses meaningfully reducing writhing in animals who had received matched saline control injections. There was no main effect of sex, and no interaction effect of sex by condition. The lack of an effect of sex stands in contrast to findings of sex differences in tolerance observed on the tail withdrawal assay, despite the identical method of tolerance induction being utilized in the present study. This discrepancy is not surprising however, as literature describing sex differences in morphine analgesia similarly has found that the presence and direction of sex differences is frequently test specific (see Craft, 2003, for review). However, it is also possible that the extremely large variability in responses to the acetic acid writhing response preclude the identification of the contribution of the animal's sex (if any) through the use of an ANOVA. Interestingly, in animals subjected to three days of morphine dosing, visual inspection of the data shows a trend toward a 2mg/kg dose of morphine leading to some suppression of writhing in males (but not females). Apart from the fact that this finding was not significant, several other factors argue against accepting this trend as possible evidence of a tendency toward greater tolerance in females than males on this assay. First, because further experimentation with higher doses of morphine in the tolerant animal was not conducted, it is not clear to what extent this trend would continue (or if it was an isolated finding at this dose). Secondly, even in the morphine naïve animal (those who had received three days of saline

injections), there was a trend toward higher analgesic responses in males at higher morphine doses.

While this study's use of such a restricted range of morphine doses may have prevented identification of sex differences which may have emerged if higher doses were utilized, results of the present study do shed some light on some current debates in the field of tolerance research. The possibility that tolerance to morphine's analgesia effects is the result of an evolving opioid-induced pain rather than loss of analgesic efficacy per se has been suggested in the literature (see Simonnet and Rivat, 2003 for review). The findings of the current study, however, reveal almost identical levels of writhing in animals subjected to chronic morphine and those subjected to chronic saline, indicating that, at least on the acetic acid writhing assay, notable hyperalgesia was NOT elicited through repeated morphine dosing. When saline treated controls and morphine treated animals were given a low dose morphine pre-injection (0.5mg/kg), an interesting phenomenon emerges. Rather than the morphine dose decreasing writhing, it actually resulted in a trend toward increased writhing (which was statistically significant male mice when compared with baseline controls). This finding may parallel reports of bimodal excitatory/inhibitory effects of mu agonists/antagonists in the mouse dorsal root ganglion, with low dose mu agonist such as morphine exhibiting excitatory effects *in vivo*, rather than the inhibitory effects seen with higher doses (Cren and Shain, 1994). It is possible that through the course of three days of morphine administration, this chronic exposure to high doses (inhibitory) doses, through the production of a

constitutively active state (see Chavkin et al., 2001), leads to changes in the efficiency of activation of excitatory and inhibitory G proteins, and as such, allowed the low dose of morphine to exert the properties of an antagonist. While the specific mechanisms behind the results seen have yet to be elucidated, results of the current experiment suggest that the trend toward increased analgesic tolerance in female versus male CD-1 mice following subcutaneous morphine administration may extend beyond the tail withdrawal assay to other modalities of pain, such as the chemical/visceral pain induced in the present study.

SPECIFIC AIM FIVE

Sex differences in thermoregulation after acute and chronic morphine administration

INTRODUCTION:

Analgesia is one of many physiological effects of morphine on the body. Morphine also produces a number of other effects, such as respiratory depression, hyperlocomotion, and hypothermia. As sex differences in morphine's analgesic effects have been well documented in the literature, research has also shown sex differences in many of the other physiological effects of morphine. For example, Dahan et al. (1998) showed that in young, healthy adults, morphine induced greater respiratory depression in females than in males. Further, females of various mouse strains have been shown to display greater sensitivity to acute morphine administration relative to their male counterparts (de-la-Cruz et al., 1987; Quock et al., 1985). The degree to which sex differences are also observable in tolerance to the various effects of morphine has yet to be fully explored.

Belknap et al. (1998) conducted a comprehensive study of the effect of morphine on locomotor activity and thermal responses in 15 inbred mouse strains, and found large strain differences indicating a substantial degree of genetic determination of these traits. For example, one strain was insensitive to the hypothermic effects of all doses employed (C3H/HEJ), while others showed marked hypothermia and some hypothermia at the same dose. Pharmacokinetic differences were unrelated to both measures of

morphine sensitivity in Belknap's study. Interestingly, while estimates of genetic correlations were rather high within each measure, genetic correlations between activity and thermal responses were not significant at any dose, indicating that these two traits are largely genetically independent. Only male mice were used in these studies, preventing analysis of sex differences in either of these non-analgesic effects of morphine.

The current study examines the thermoregulatory response to morphine, after both acute and chronic administration, to determine and report differences, if any, as a function of the animal's sex. This study was published in Neuroscience Letters, Volume 291 (see Kest, Adler, and Hopkins, 2000).

METHODS:

The following study was completed in accordance with approved protocols of the College of Staten Island/ CUNY Institutional Animal Care and Use Committee.

Subjects and Drugs:

Naïve adult male and female CD-1 mice (25-35 grams) 6-8 weeks of age were used in this study. All mice were housed 5 to a cage with same sex/strain mates in the College of Staten Island Animal Facility. Mice were provided with free access to food (Purina chow) and water in a temperature controlled ($22 \pm 2^{\circ}\text{C}$). There was a 12 hour light/ 12

hour dark cycle (lights on at 7 am). All testing was performed following an acclimation period of at least one week after each animal's arrival and at 7-9 weeks of age.

Morphine sulfate was obtained from the National Institute of Drug Abuse (Rockville, MD), and stored by Dr. Ben Kest of the College of Staten Island. Morphine was dissolved in 0.9% physiological saline for all injections, which delivered via the subcutaneous route in a volume of 10ml/kg. All testing was done near mid-photo phase (between 10am and 2 pm) to minimize circadian fluctuations in morphine sensitivity. All animals were provided at least one hour acclimation period following transfer from the vivarium to the laboratory.

Tolerance induction:

Mice in morphine treatment groups were injected three times per day (tid) for three consecutive days, with increasing doses of morphine delivered each day. Specifically, day one doses were 20mg/kg TID, day two doses were 40mg/kg TID, and day three doses were 40mg/kg TID. Mice in saline control groups received saline injections TID for the three-day period. The dosing schedule used in the current experiment was identical to that used in Experiment 1.

Measurement of temperature:

Colorectal temperature was measured by inserting a Thermistor probe connected to a digital thermometer (VWR Scientific Digital Thermometer 500; San Francisco CA)

2.5cm into the rectum. The probe was removed following stabilization and recording of the temperature reading (± 0.1 °C). Baseline temperatures were recorded at 10am, prior to the initiation of any injections. Temperature changes following morphine administration were assessed 30 minutes after a 24mg/kg morphine injection.

Statistical Analysis:

Baseline data for males and females on Day 1 were compared using independent t-tests. A two way (sex x treatment) repeated measures ANOVA was used to compare baseline and pre-and post-drug injection colonic temperatures, followed by post hoc t-tests where appropriate. An alpha level of 0.05 was used for all comparisons.

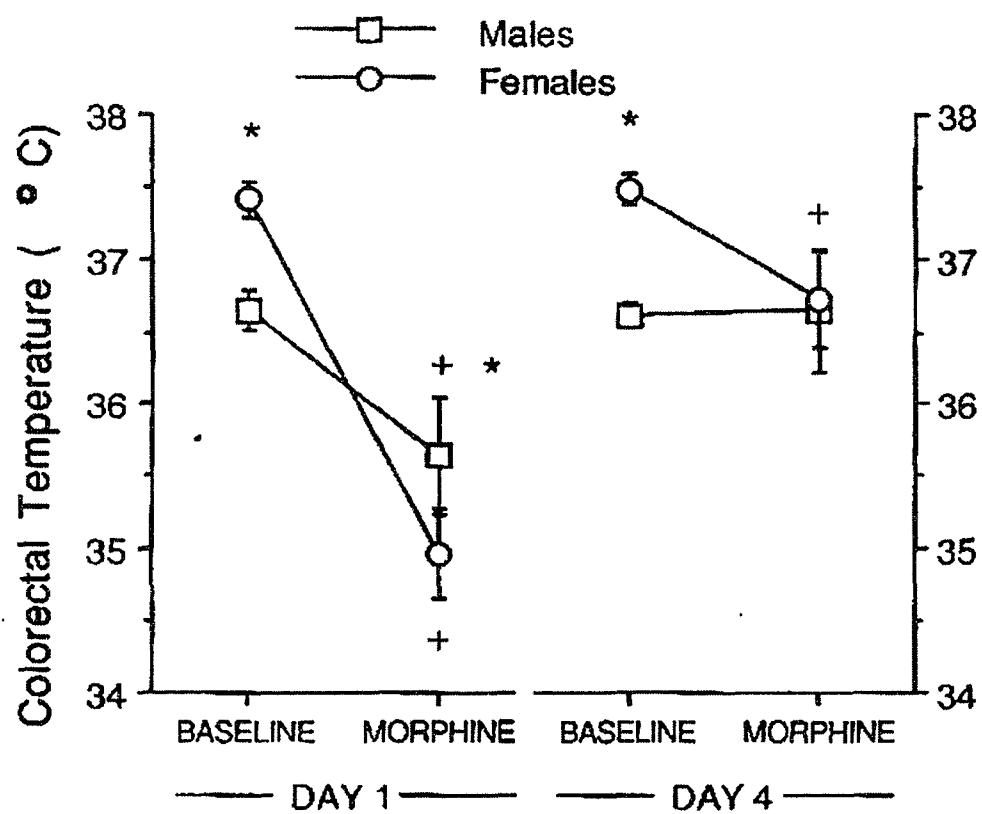
RESULTS:

Females displayed significantly higher baseline temperature readings than males on Day 1 (Fig. 7), although both sexes fell solidly within the characterized normal range for mice (36.5-38 degrees Celsius). Injection of morphine (24mg/kg) produced significant hypothermia in both sexes, however, this effect was significantly greater (2.5 fold) in females (Fig. 7). Although females still displayed higher baseline temperature readings relative to males on Day 4, values in both sexes were almost identical to those obtained on Day 1, indicating that chronic morphine injections did not alter baseline thermoregulation (Fig. 7). Significant reductions in morphine hypothermia were observed for both sexes on Day 4 relative to Day 1, indicative of tolerance. However, whereas morphine hypothermia was completely abolished in males, significant

hypothermia was still evident in females (Fig. 7). Acute and chronic saline injections in control mice did not alter baseline temperature readings on Day 1 or Day 4, discounting the possible confound of injection stress on this measure.

Figure 7. Effect of morphine on colorectal temperature.

Figure 7.



DISCUSSION:

The present data demonstrate significant sex differences in baseline colorectal temperature. This finding, while unexpected, was consistent with that reported by Selman et al. (2001), who reported higher body temperatures for females than males of two selectively bred mouse strains (high and low food intake strains) under various ambient temperature conditions. The etiology of this sex difference is unclear, although the possible impact of estrus phase is suggested (estrus was not specifically monitored or controlled for in the present study). Our results also identified significant hypothermia following acute morphine administration in both male and female mice, although the degree of hypothermia was larger in females. While a search of the literature failed to find other work done to date examining gender differences in the thermoregulatory response to morphine in mice, de la Cruz (1987) reported a greater hypothermic response in female rats than males following an administration of a combination of morphine (20mg/kg) and haloperidol (5mg/kg). Further, in a study examining the impact of the psychological stress of an open field test in guinea pigs, Dymond et al. (1999) similarly identified a gender-specific thermoregulatory response, with female guinea pigs showing significant hypothermia. Further, Schlenker et al. (2002) recently reported other gender specific metabolic (and ventilatory) responses in female mice under certain conditions. In light of some of these described phenomena, the finding of greater morphine induced hypothermia in females appears consistent. After sustained morphine exposure, however, in contrast to Specific Aim One's finding of greater tolerance to the analgesic effects of morphine in females than in males, the

current study suggests that male CD-1 mice became more tolerant to morphine's hypothermic effects, with a total loss of morphine's ability to elicit this response subsequent to three days of morphine treatment (delivered in the identical manner as in Specific Aim one). The findings as such suggest a dissociation in the development of tolerance to morphine's analgesic and hypothermic effects, with the direction of sex differences being different for these two effects.

The data may be of consequence in the study of other sex-dependent measures of morphine in mice, particularly analgesia, tolerance, and dependence. Almost all studies comparing male and female mice for morphine analgesia have used tests of thermal noxious stimuli [i.e. tail withdrawal and hot-plate tests (Kest et al., 2000)]. A clear consensus has yet to emerge regarding the mechanisms underlying morphine analgesic sex differences in mice, but several lines of evidence, including sex differences in analgesia following ICV morphine administration (Kest et al., 1999) suggest sex differences in morphine pharmacodynamic variables. Thus, it is possible that sex differences in morphine thermoregulatory responses in mice may contribute in part to sex differences in morphine analgesia. Indeed, morphine-induced reductions in colorectal body temperature in mice display a significant negative correlation with increased analgesic effect on the tail-withdrawal test (Lichtman et al., 1993), although indirect evidence suggests no correlation on the hot-plate test (Belknap et al., 1989; Belknap et al., 1998). In Specific Aim One, using a chronic morphine injection protocol identical to that employed in this study, females displayed greater analgesic

tolerance than males on the tail withdrawal test. What is not obvious is how the greater hypothermia in female mice, which would presumably increase the analgesic effect on this thermal nociceptive assay, would contribute to their greater analgesic tolerance. It is entirely possible that core body temperature reduction induces compensatory increases in peripheral tissue temperature in the service of thermoregulation. This action would increase tail skin temperature, which has been demonstrated by some (Hole and Tjolsen, 1993) but not others (Lichtman et al., 1993) to decrease withdrawal latencies. Thus, greater hypothermia in females relative to males following chronic morphine administration, as presently reported, would lead to quicker response and the appearance of greater analgesic tolerance. However, as mentioned in the background section, the study by Lichtman et al. (1993) has shown that changes in colonic temperature in mice failed to significantly correlate with changes in skin temperature. Finally, it has been demonstrated that hypothermia may significantly contribute to several components of morphine withdrawal in mice (Belknap, 1989), and that there are dose-dependent sex differences in withdrawal behavior in a mouse model of acute morphine dependence (Palmese et al., 1999). Whether the present sex differences in morphine hypothermia contribute, at least in part, to sex differences in morphine analgesia, tolerance, and/or dependence, warrants further study. Importantly, however, the current experiment has clarified that sex differences in tolerance to morphine's effects, like those observed in the immediate effects of morphine, are dependent upon the specific effect / response examined.

SPECIFIC AIM SIX

Sex differences in morphine tolerance: strain survey study

INTRODUCTION:

Studies comparing rodent strains have demonstrated the important contribution of genetic background to individual differences in the development and magnitude of tolerance (Oliverio and Castellano, 1974; Ho et al., 1977, Gwynn and Domino, 1984; Mas et al., 2000). These studies however were based on data obtained from just two or three strains (e.g., Sprague Dawley and Wistar rats; C57/BL/6, BALB/c, and DBA/2J inbred mice), typically compared two at a time, thereby limiting the analysis of variability in tolerance liability as well as precluding the ability to perform a correlation analysis with other pain-related phenotypes that may be predictive of tolerance itself. Furthermore, there was substantial variability in the methodology of these experiments, including the duration of morphine treatment, the nociceptive assay utilized, and/or parameters within even a given nociceptive assay. In almost all studies, only single morphine doses were tested after tolerance induction, precluding the generation of dose-response curves and the subsequent calculation of potency estimates upon which comparisons can be made. The present study attempts a more comprehensive and systematic analysis of the genetic contribution to inter-individual differences in morphine analgesic tolerance. The morphine tolerance liability of 11 inbred mouse strains was determined by assessing the loss of relative analgesic potency through comparisons of ED₅₀ estimates obtained before and after chronic morphine treatment. By comparing many strains in a single study under identical experimental protocols, the

intended strain survey hopes to facilitate comparison between strains studied previously as well as those as yet untested. It also allows for the correlation of tolerance with other phenotypes such as baseline nociceptive sensitivity and morphine analgesia both before and after chronic morphine treatment. Finally, AD₅₀ data was also collected and analyzed in this study, in order to evaluate whether specific methods of quantifying analgesia can impact on the specific results found. Further, the study will examine these data for both male and female mice of each strain, with the goal of identifying the degree to which sex differences in morphine analgesic tolerance exist, and may vary according to genotype.

The findings of this study were published in Pharmacology, Biochemistry, and Behavior, Volume 73 (Kest, Hopkins, Palmese, Adler, and Mogil, 2002).

METHODS:

Subjects and Drugs

Male and female adult mice (n=18-20 mice per sex per strain) of the following inbred strains were obtained from The Jackson Laboratory: 129P3, A, AKR, BALB/c, C3H/He, C57BL/6, CBA, DBA/2, LP, SJL, and SWR (all “J” substrains). All mice were housed 4/5 to a cage with same sex/strain mates in the College of Staten Island Animal Facility. Mice were provided with free access to food (Purina chow) and water in a temperature controlled environment (22 degrees Celsius). There was a 12 hour light / 12 hour dark cycle (lights on at 7 am). All testing was performed following

an acclimation period of at least one week after arrival and at 7-9 weeks of age.

Morphine sulfate was obtained from the National Institute of Drug Abuse (Rockville, MD), and stored by Dr. Ben Kest of the College of Staten Island. Morphine was dissolved in 0.9% physiological saline for all injections, which were delivered via the subcutaneous route in a volume of 10ml/kg.

Nociceptive Assay

All testing was conducted near mid-photophase (10:00-15:00 hours) to reduce the circadian effects on nociceptive and analgesic sensitivity (Kavaliers and Hirst, 1993).

Although the large numbers of mice used required testing to continue over several weeks, both strain and sex were completely counterbalanced so that an equal number of mice per strain per sex were tested together. Following a 1 hour habituation to the testing room, mice were assessed for baseline nociceptive sensitivity on the 49 °C tail-withdrawal test. In this assay of acute, thermal nociception, the mouse is gently restrained and the distal half of its tail is immersed in water maintained at $49 \pm 0.2^{\circ}\text{C}$ by an immersion circulator pump (Fisher Isotemp Model 71). Latency to reflexive withdrawal of the tail was measured twice to the nearest 0.1 second. The two determinations were later averaged. The tail withdrawal test was chosen because of its stability even after repeated exposures to noxious water temperatures (D'amour and Smith, 1941). Animals doubling their baseline latencies were considered "responders" for the purpose of calculating ED₅₀ values. A cutoff latency of 15 seconds was

employed to prevent the possibility of tissue damage (and was used to calculate the additional measure of AD₅₀ values/%MPE).

Dose Response Data and Tolerance Induction:

To greatly reduce the number of mice required for these studies, morphine analgesic potency was determined using cumulative dose-response curves. Immediately following baseline latency assessment, subjects were injected with an initial 1.0mg/kg dose of morphine, followed in succession with increasing (~ 0.25 log) doses (2.0, 3.6, 6.5, 11.7, and 21 mg/kg). These doses have been demonstrated to provide reliable potency estimates in a variety of mouse strains (Kest et al., 1995; 2000). To ensure that all mice received the same cumulative dose of drug on Day 1, all mice were injected with identical morphine doses. In the event that any mouse did not cut off at the highest dose of 21mg/kg, repeated administrations of the 21mg/kg dose occurred until up to three times or until cut-off was achieved. Tail withdrawal latencies were retested and recorded 30 minutes after each dose, and the subsequent dose of morphine injected immediately thereafter. Baseline latencies and dose-response data were collected prior to (Day 1) and after (Day 4) tolerance induction.

Immediately following dose-response studies on Day 1, subjects were injected with morphine three times daily (TID) for three days according to an escalating daily dosage schedule (10mg/kg TID, 20mg/kg TID, and 40mg/kg TID on days 1, 2, and 3 respectively). To assess for possible repeated handling/injecting effects, control mice of

each strain received an equal number of saline injections throughout the three days, except on Day 4 when morphine dose-response curves were collected in them similarly to those mice undergoing tolerance induction.

Analgesia at each dose was expressed through the calculation of ED₅₀ values. In addition, the percentage of the maximum possible analgesic effect (%MPE) as calculated by the formula: %analgesia = [(post morphine latency – baseline latency)/(cut-off latency-baseline latency)] x 100 was also obtained. (The use of this transformed dependent measure takes into account the cut-off latency and the individual baseline latency for each subject, so that any variation in baseline values between test Days 1 and 4 will not bias the quantification of analgesia obtained each day.)

Data analysis:

A two way ANOVA was used to examine the main effects of strain and sex, and their interaction, on tail withdrawal baseline latencies. Subjecting Days 1 and 4 latencies to repeated measures ANOVA allowed assessment of changes in baseline latencies after chronic morphine treatment. ED₅₀ values and associated 95% confidence intervals (CI) were calculated for dose response data. In addition, AD₅₀ values / %MPE, using the method of Tallarida and Murray (1981) were also analyzed. Values with no overlapping CI's were considered significantly different.

Estimates of narrow-sense trait heritability were calculated by comparing the ANOVA-derived between strain variance to the total variance: $h^2 = \frac{V_a}{V_a + V_e}$; where V_a is the allelic (within-strain) variance. For baseline nociception, raw Day 1 tail-withdrawal latencies for each mouse were used. The estimation of heritability of Days and 4 analgesic sensitivity and tolerance was more complicated, since these measures are usually calculated based on group data, and individual data is required for heritability estimation. For the purpose of determining heritability of analgesia and tolerance, the lowest cumulative morphine dose producing $\geq 50\%$ analgesia was used as the estimated AD_{50} value, and tolerance in each mouse was estimated as the ratio of the Day 4 AD_{50} to the Day 1 AD_{50} .

To assess genetic codetermination between phenotypes, baseline withdrawal latencies and morphine AD_{50} strain means obtained prior to and following chronic morphine treatment, and their subsequent differences, were ranked from smallest to highest according to effect size. Correlation coefficients were then obtained using Spearman's r followed by Bonferroni correction for multiple comparisons. In all statistical tests, a threshold alpha criterion level of .05 was employed.

RESULTS:

Baseline withdrawal latencies:

There was a significant main effect of sex and strain ($p < .001$ for each) on baseline tail-withdrawal latencies, but no significant interaction ($p = .40$). Therefore, baseline tail-

withdrawal latencies obtained before (Day 1) and after (Day 4) chronic morphine treatment are presented for all strains (collapsed across sex) and sex (collapsed across strains) in Table 5. Withdrawal latencies varied significantly between strains on both Days 1 and 4 ($p < .001$). Heritabilities of Days 1 and 4 baseline latencies were estimated as $h^2 = 0.36$ and 0.35 , respectively, which are very similar to those obtained previously for this trait (Mogil et al., 1999a).

Analysis of baseline tail withdrawal latencies showed a significant main effect of the time of testing (Day 1 versus Day 4) in morphine treated mice only ($p < .001$). There was a significant interaction effect of this repeated measure with strain ($p < .001$). Pairwise comparisons using Student's *t*-test revealed a significant decrease in baseline latencies on Day 4 relative to Day 1 in an overwhelming majority of the strains (Table 5). Exceptions were noted for mice of the 129P3 and A strains, for whom no changes in Day 4 withdrawal latencies were observed, and for SWR mice, in which Day 4 latencies were significantly increased. Considering strain means, the correlation between baseline nociceptive sensitivity on Days 1 and 4 approached significance ($r = .69$), but there was no correlation between Day 1 latency and latency alterations from Days 1 to 4 ($r = 0.35$) (see Table 8). There was no significant alteration in baseline withdrawal latencies obtained on Day 4 relative to Day 1 in saline-treated control mice of any strain, suggesting that alterations in morphine-treated mice were likely due to chronic drug administration and not possible confounding factors such as repeated handling, injection, or testing.

Table 5. Nociceptive sensitivity before and after chronic morphine treatment in 11 inbred mouse strains.

^aMean latency (s)±S.E.M. to withdraw distal half of tail from 49 °C water.

^bPercent reductions on Day 4 relative to Day 1 values

*▼ Significant decrease ($p<.05$) in baseline tail withdrawal latencies

*▲ Significant increase ($p<.05$) in baseline tail withdrawal latencies

[†]Significantly different in latency change relative to females

Table 5.

Strain	Baseline Latency ^a		Latency Change ^b
	Day 1	Day 4	
129P3	2.4±0.1	2.1±0.1	-13%
A	2.4±0.1	2.4±0.2	0%
AKR	2.9±0.2	2.2±0.1	-24%*▼
BALB/c	3.1±0.2	2.5±0.2	-32%*▼
C3H/He	1.8±0.1	1.5±0.1	-19%*▼
C57/BL/6	2.2±0.1	1.5±0.1	-17%*▼
CBA	1.9±0.1	1.3±0.1	-32%*▼
DBA/2	2.5±0.1	2.1±0.1	-16%*▼
LP	2.8±0.1	2.5±0.1	-11%*▼
SJL	2.2±0.1	1.8±0.1	-18%*▼
SWR	1.8±0.1	2.2±0.1	+22%*▲
Males (All)	2.5±0.1	2.1±0.1	-16% [†]
Females (All)	2.2±0.1	2.0±0.1	-9%

Morphine analgesia and tolerance assessed via ED_{50} values:

Data were analyzed using the BLISS-21 computer program, which maximizes the log-likelihood function to fit a parallel set of gaussian sigmoid curves to the dose-response data, and provides ED_{50} values, 95% confidence intervals (CI), and estimates of relative potency. Groups with non-overlapping confidence intervals are considered significantly different. The data demonstrate strain differences in morphine analgesic tolerance, as indicated by strain differences in relative potency. In addition, as shown in Table 6 and Figure 8, sex differences were observed in some strains, but not others.

Among the strains, the SWR, BALB/c, and A strains were the most tolerant, displaying an approximately 3-4-fold loss in analgesic potency. In contrast, there was no tolerance observed for the DBA/2, LP, and 129 strains.

Sex differences were also evident. The direction of these differences was genotype-dependent. Specifically, in both DBA/2 and 129 mice, significant tolerance was observed in females only. In LP mice, only male mice became tolerant. Sex differences in morphine analgesia prior to tolerance induction was observed in LP mice only (M>F).

Table 6.

Morphine analgesic tolerance by strain and sex, calculated by ED₅₀ estimates

*Significant loss of analgesic potency

†Significant sex differences

Table 6.

Strain	Day 1 ED ₅₀ (95%CI)	Day 4 ED ₅₀ (95% CI)	Magnitude of Day 1 Potency Relative to Day 4
<u>BALB/c</u>			
Males	.82 (.43 – 1.31)	3.05 (1.66 – 4.77)	3.72*
Females	.73 (.36 – 1.17)	3.26 (1.96 – 4.81)	4.42*
<u>DBA/2</u>			
Males	5.85 (4.50 – 7.48)	3.55 (2.29 – 5.28) [†]	0.60
Females	5.06 (3.87 – 6.53)	15.51 (11.02 – 21.43)	3.06*
<u>CBA</u>			
Males	2.00 (1.41-2.72)	4.77 (3.17 – 6.99)	2.39*
Females	2.24 (1.60 – 3.04)	7.16 (4.91 – 10.28)	3.20*
<u>129</u>			
Males	6.33 (4.98 – 7.97)	3.09 (2.02 – 4.52) [†]	0.49
Females	5.49 (4.30 – 6.94)	10.23 (7.06 – 14.28)	1.86*
<u>C57/BL6</u>			
Males	2.38 (1.71 – 3.24)	5.72 (3.69 – 8.64)	2.40*
Females	2.78 (2.02 – 3.77)	7.01 (4.61 – 10.50)	2.52*
<u>C3H/He</u>			
Males	2.65 (2.03 – 3.39)	5.35 (3.82 – 7.47)	2.01*
Females	3.22 (2.46 – 4.10)	7.29 (5.24 – 10.02)	2.26*
<u>AKR</u>			
Males	4.24 (3.13 – 5.58)	8.65 (5.95 – 12.34)	2.04*
Females	3.62 (2.62 – 4.81)	13.32 (9.36 – 18.71)	3.70*
<u>SWR</u>			
Males	2.21 (1.56 – 2.99)	7.35 (5.66 – 9.33)	3.33*
Females	2.19 (1.49 – 3.04)	8.45 (6.35 – 10.88)	3.89*
<u>LP</u>			
Males	2.79 (1.69 – 4.42) [†]	9.26 (5.50 – 15.55)	3.32*
Females	6.99 (4.93 – 9.91)	6.37 (3.70 – 10.38)	0.91
<u>SJL</u>			
Males	1.72 (1.21 – 2.35)	4.41 (3.07 – 6.04)	2.56*
Females	1.90 (1.24 – 2.74)	3.51 (2.20 – 5.23)	1.84*
<u>A/J</u>			
Males	1.47 (0.95 – 2.21)	5.75 (3.38 – 8.98)	3.91*
Females	0.79 (0.41 – 1.21)	2.56 (1.18 – 4.49)	3.24*

Figure 8.

Percentage of animals analgesic at each dose, reported by sex, strain, and day of testing (analgesia defined as doubling of baseline tail withdrawal latencies, as used to calculate ED50 values).

Figure 8.

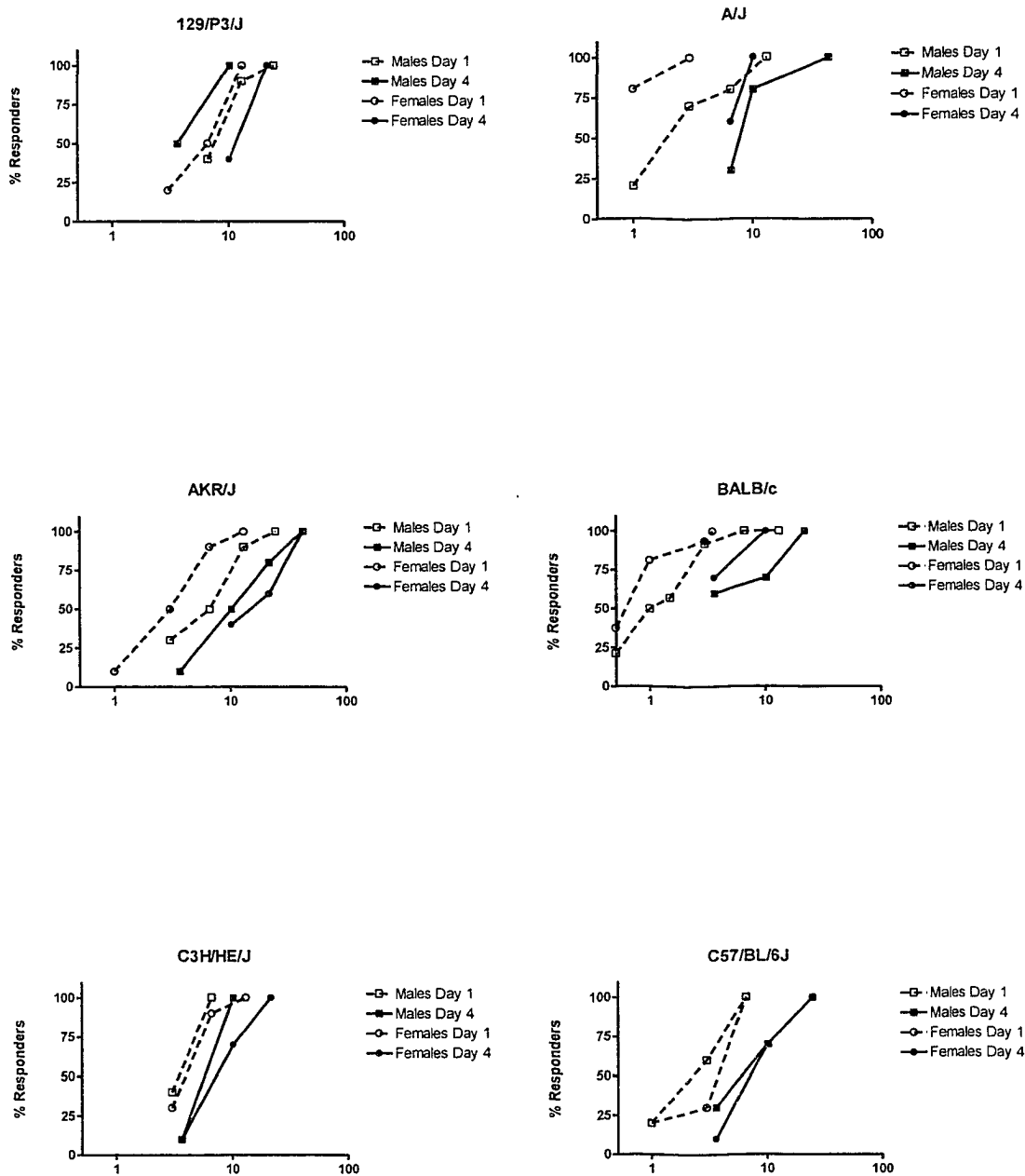
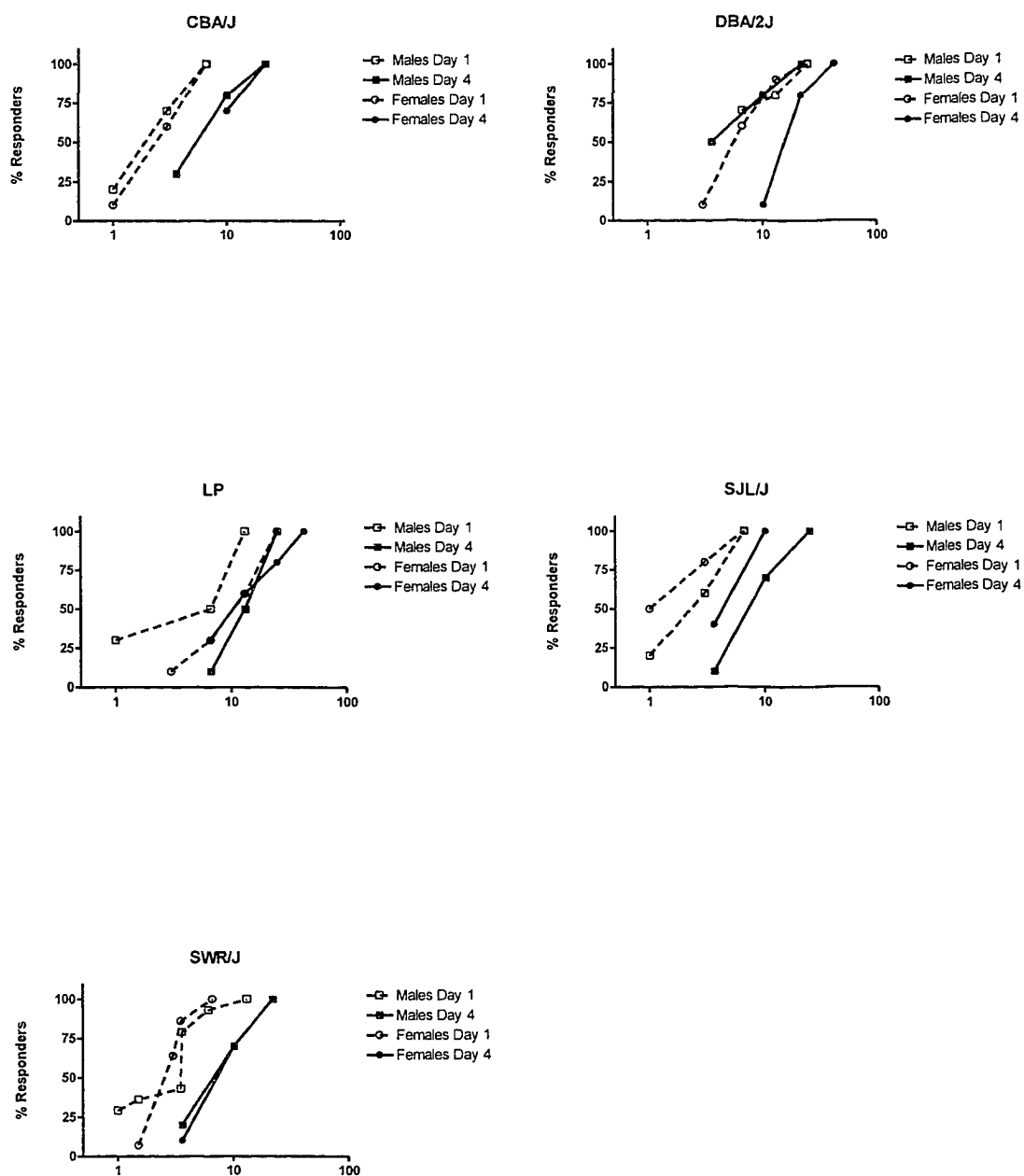


Figure 8., Continued



Morphine analgesia and tolerance assessed via AD₅₀ values:

Unlike the finding of sex differences for 3 inbred strains using ED₅₀ values, morphine analgesic potency determined using AD₅₀ estimates derived from cumulative dose-response curves did not differ between the sexes either before or after chronic morphine treatment in any strain tested. Thus, for the remaining AD₅₀ based analyses to be discussed, male and female cumulative dose-response data from Days 1 and 4 were pooled for each strain. As illustrated in Fig. 9, all strains displayed an increase in analgesia with increasing doses of morphine. However, the dose response function can be seen to differ between strains. Indeed, derived AD₅₀ values presented in Table 7 reveal significant potency differences between strains on Day 1, with a 6.7 fold difference between BALB/c (AD₅₀: 2.8, 95% CI: 2.3-3.5) and SWR (AD₅₀: 18.7, 95%CI: 15.6-22.0) mice, the most and least sensitive strains, respectively.

Heritability of Day 1 AD₅₀ values was estimated at $h^2=0.31$, which is very similar to that reported previously for this trait (Bergeson et al., 2001). Morphine analgesic dose-response curves obtained following chronic morphine treatment, on Day 4 ($h^2=0.44$), are also illustrated in Fig. 7 alongside those obtained on Day 1. Significant rightward shifts were observed for 9 of the 11 strains tested on Day 4 relative to Day 1, demonstrating a loss of morphine analgesic potency. For two strains (129P3 and LP), the dose-response functions of Days 1 and 4 were practically indistinguishable. The heritability of tolerance in these strains was estimated at $h^2=0.28$, indicating that more than one-quarter of the observed variability among strains can be attributed to the

additive effect of inherited genetic alleles. For reasons described above, this is likely an underestimate.

AD₅₀ and relative potency estimates derived from strain morphine dose-response curves are presented in Table 7. Day 4 AD₅₀ values for all strains, except 129P3 and LP, were significantly increased relative to AD₅₀ values obtained prior to chronic morphine injections on Day 1, indicative of tolerance. Even among strains where significant tolerance was observed, there was considerable interstrain variation in magnitude. Relative potency estimates in Table 1 show that potency reductions ranged from as low as approximately 2 fold in A and SJL mice, to as great as approximately 7, 8, and 11 fold losses in C57BL6/J, C3H/He, and CBA mice, respectively. Day 4 morphine AD₅₀ estimates obtained from saline-injected control mice did not differ from the Day 1 morphine AD₅₀ estimates in experimental mice of any strain, nor were any overt other behavioral differences observed at any time between these two groups. Thus, strain differences in tolerance can not be attributed to response variability to repeated handling and/or injection.

Table 7. Morphine analgesic tolerance by strain calculated by AD₅₀ estimates

Table 7.

Strain	Day 1 AD ₅₀ (95%CI)	Day 4 AD ₅₀ (95% CI)	Potency Shift
<u>BALB/c</u>	2.8 (2.3 – 3.5)	12.5 (9.5-16.5)	4.5*
<u>DBA/2</u>	13.8 (11.7-16.7)	39.6 (32.3 – 43.7)	2.9*
<u>CBA</u>	5.0 (4.4 – 5.8)	53.9 (43.1-67.4)	10.8*
<u>129/P3</u>	13.7 (11.3-16.5)	11.4 (9.9-13.2)	0.8
<u>C57/BL6</u>	6.8 (5.5-8.6)	48.7 (39.3 – 60.4)	7.2*
<u>C3H/He</u>	5.4 (4.7 – 6.3)	42.8 (35.3 – 51.8)	7.9*
<u>AKR</u>	13.0 (10.9-15.4)	42.3 (35.7-50.1)	3.3*
<u>SWR</u>	18.7 (15.6 – 22.0)	93.8 (80.2 – 109.7)	5.0*
<u>LP</u>	15.0 (11.9 – 18.8)	18.4 (14.4 – 23.5)	1.2
<u>SJL</u>	7.2 (5.6 – 9.3)	15.7 (12.5-19.7)	2.2*
<u>A/J</u>	4.4 (3.6-5.4)	8.4 (6.7 -65.4)	1.9*

AD₅₀ values are mg/kg morphine

Potency Shift = Day 4AD₅₀ / Day 1 AD₅₀

*Significant loss of analgesic potency (p<.05).

Figure 9.

AD₅₀ Data: Morphine dose–response relationships on the 49 °C tail-withdrawal assay for 11 inbred mouse strains before and after chronic morphine administration. Key to figures is contained in the first graph (top left). Morphine was injected three times daily for 3 days according to an escalating dose schedule (10, 20, and 40 mg kg on Days 1, 2, and 3, respectively). Symbols represent strain mean percentages ($\pm$ S.E.M.) of the maximum possible analgesia (% analgesia) at each cumulative dose on Days 1 and 4. Significant rightward curve shifts, indicative of analgesic tolerance, were observed for all strains except 129P3 and LP.

Figure 9.

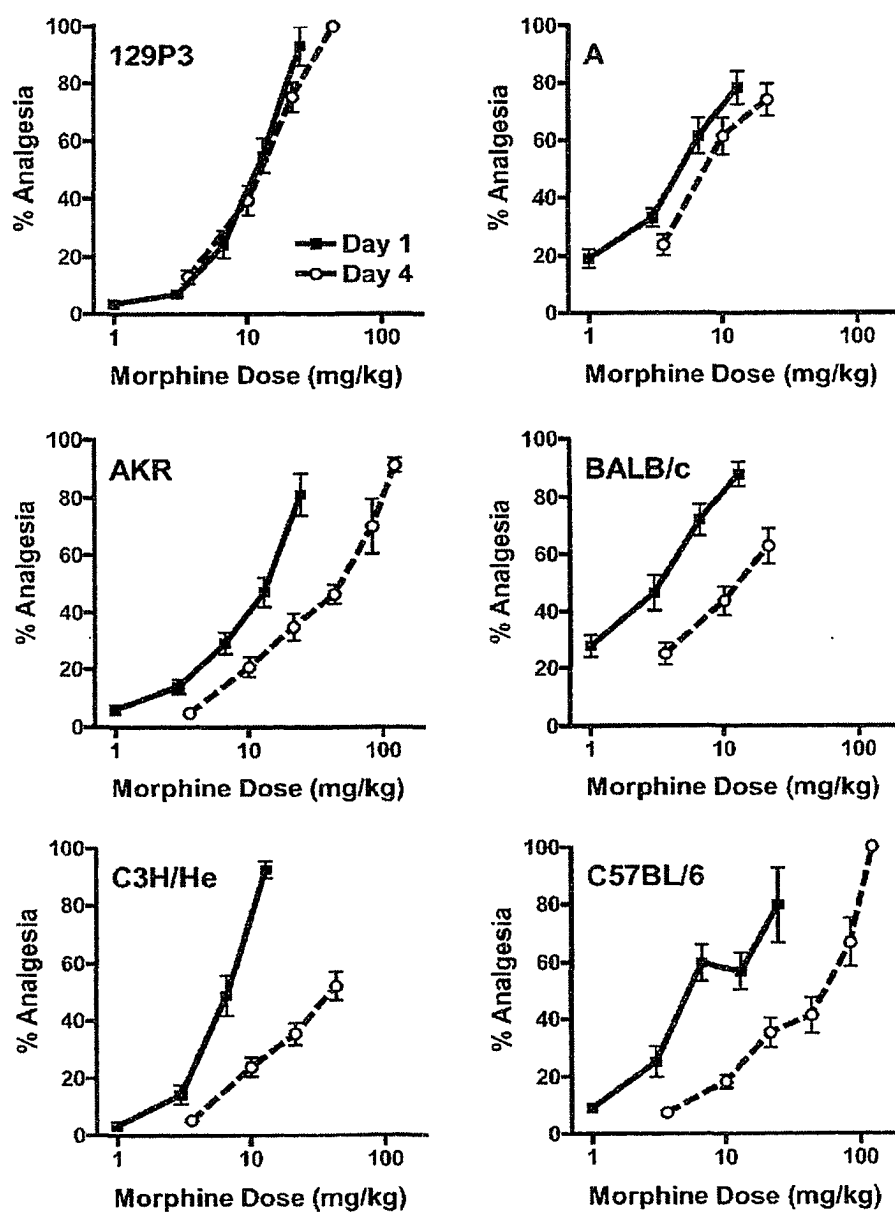
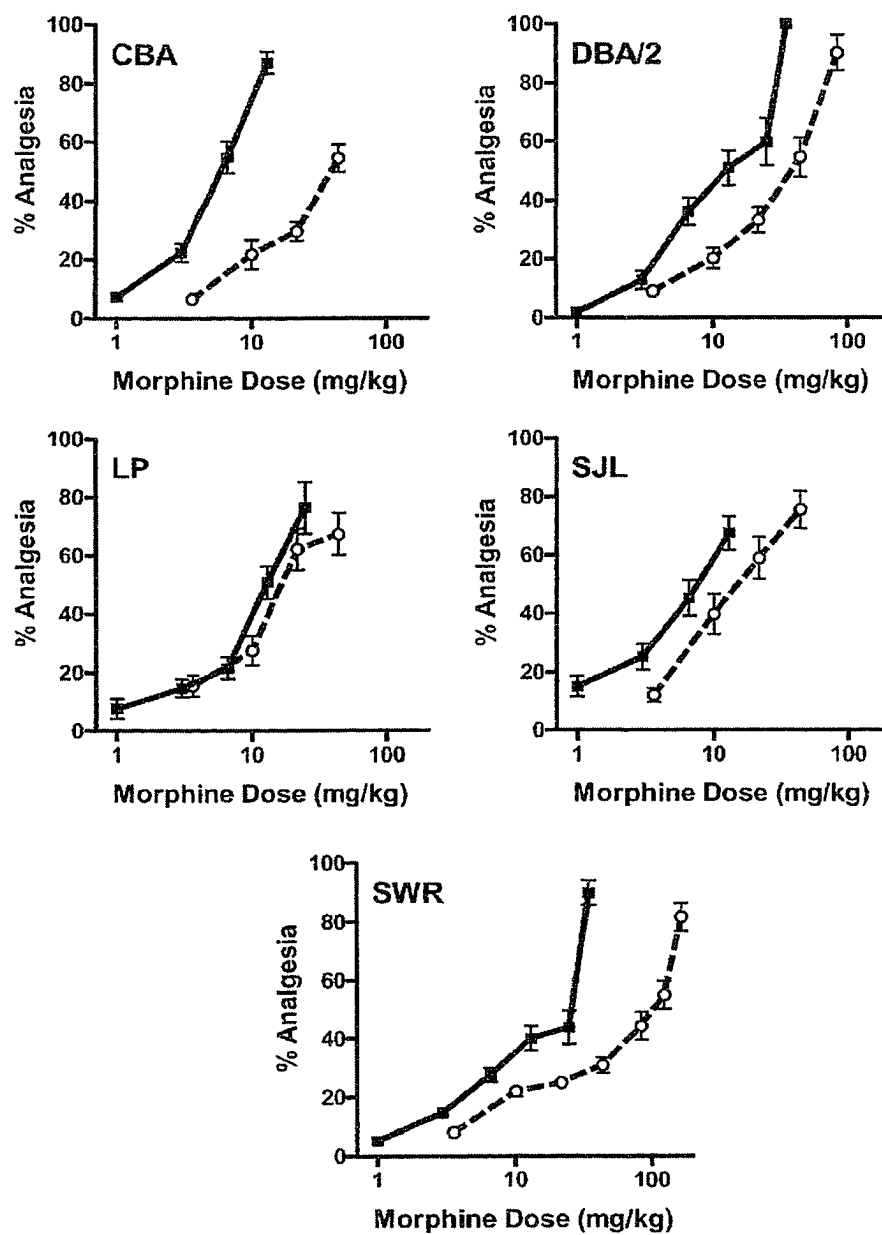


Figure 9, continued.



Correlations of baseline nociception, baseline analgesia, and tolerance:

There was no significant correlation between tolerance and baseline withdrawal latencies on Day 1 or 4, or between tolerance and initial morphine analgesic potency on Day 1. Tolerance was, however, significantly correlated with baseline latency changes resulting from chronic morphine treatment and with Day 4 AD₅₀ values (see Table 8).

Table 8.

Spearman's rank (r_s) correlation coefficients between inbred mouse strains for nociceptive sensitivity and morphine analgesia before and after chronic morphine administration.

Table 8.

	Baseline Latencies Day 1	Baseline Latencies Day 4	Latency Change	AD ₅₀ Estimates Day 1	AD ₅₀ Estimates Day 4
Baseline Latencies – Day 1	-				
Baseline Latencies – Day 4	.69	-			
Latency Change	-.35	-.38	-		
AD ₅₀ Estimates – Day 1	-.05	.14	.02	-	
AD ₅₀ Estimates – Day 4	-.54	-.51	.80*	.27	-
Potency Shift	.48	.57	-.76*	.35	-.79*

DISCUSSION:

Studies of morphine tolerance utilizing inbred strains are relatively few, making comparisons and confirmations between studies difficult. Compounding the problem is the use of the same few strains, but with comparisons typically limited to two strains in each study. Among those for whom data are available are C57/BL/6 and BALB/c mice, for which approximately equal magnitudes of tolerance were observed in the present study. Similar findings were obtained by treating subjects with both higher (Eidelberg et al., 1975) and lower (Oliverio et al., 1975) morphine doses, for, respectively, longer and shorter periods of time than those presently employed. The current study's observation of greater morphine tolerance in C57/BL/6 relative to DBA/2 mice was also complimentary with the more rapid onset of tolerance observed in the C57/BL/6 strain when an intracerebroventricular route was used to inject morphine (Frigeni et al., 1981). Although C57/BL/6 mice have been differentiated from DBA/2 mice by their greater brain, spinal cord, and plasma morphine concentrations after chronic morphine treatment (Gwynn and Domino, 1984), a plausible explanation for greater morphine concentration could be advanced for either the more or less tolerant strain. Indeed, morphine tolerance for both strains in that study was of equal magnitude. In general, attempts to ascribe magnitude of tolerance to strain differences in morphine pharmacokinetic parameters have been unsuccessful (Gwynn and Domino, 1984; Mas et al., 2000), implying the existence of differential alleles at genes playing a pharmacodynamic role in tolerance liability.

Although the studies cited above were all performed with using the hot-plate test of nociception, it is still not possible to suggest that strain differences in sensitivity to tolerance, as revealed by the tail-withdrawal test used in the present study, may be generalized to other nociceptive assays since discrepancies exist. For example, using the hot-plate test to measure analgesia, DBA/2 mice were found to be 14% more tolerant than BALB/c mice (Oliverio and Castellano, 1974), whereas the current study found BALB/c mice to approximately 50% more tolerant on the tail-withdrawal test. Also, in contrast to the current study's finding that there was an approximately 2.5 fold greater reduction in morphine potency in C57/BL/6 mice relative to DBA/2 mice, those strains displayed equal levels of tolerance on the hot plate test (Gwynn and Domino, 1984). Note, however, that in another study, C57/BL/6 and DBA/2 differences consistent with findings of the present study were observed using the hot plate test (Frigeni et al., 1981). Certainly, differences in methodology such as morphine dose, injection route and schedule, and quantification of analgesia (i.e., area under the curve vs. % analgesia vs. quantally defined responders), can contribute to variable findings between studies. Therefore, by using many strains in a single study under identical experimental protocols, the present study advances our understanding of variability associated with genotype in morphine tolerance.

Similar considerations may also underlie discrepant findings in studies comparing responses of male and female mice. For example, significant sex differences in Day 1 morphine AD₅₀ estimates were previously observed in 4 of the presently tested 11

strains, including C57/BL/6 mice (Kest et al., 1999). Although there was no overall strain x sex interaction in the present study, planned contrasts did reveal a sex difference for this particular strain. However, important differences between these studies remain, including the systemic vs. central routes of drug delivery. In contrast to the present subcutaneous delivery of morphine, intracerebroventricular microinjections of drug will not distribute along the entire neuraxis and recruit spinal and supraspinal analgesic mechanisms shown to act in synergy (Roerig and Fujimoto, 1988). There may consequently be differences in analgesic responses elicited by the two different injection routes, disallowing simple comparisons between studies, including those on sex differences. The present data also confirm sex differences in three strains, consistent with reported sex differences reported for CD-1 mice in Specific Aim 1, although only when the method used to calculate analgesia is ED₅₀ estimates, and not when the alternative AD₅₀ estimate was used. As noted above, quantification of analgesia can contribute to the variable findings between studies, and this is particularly true with regards to sex differences in morphine tolerance. Sex difference in tolerance in CD-1 mice were observed when analgesia was compared using ED₅₀ estimates based on quantal data (% responders) but not %MPE (Kest et al., 2000). Similarly, the current study revealed strain sex differences only with ED₅₀ values, and not when % MPE values rendered into AD₅₀ estimates were used.

The present data identify two strains, 129P3 and LP, that appear refractory to morphine tolerance. Previous studies have demonstrated that 129S6 mice (previously referred to

as 129/SvEv), another 129 substrain, develop little or no morphine tolerance (Kolesnikov et al., 1998; Crain and Shen, 2000). Thus, although the current study's description of 129P3 mouse insensitivity can be considered a partial replication of previous studies in the highly related 129S6 mouse, it is also a partial extension of these previous findings since 129 substrains display allelic variation at any number of genetic loci (Simpson et al., 1997). An important finding of the present study is that 129 mice are not unique in their resistance to tolerance development, since LP mice also displayed no tolerance, and other strains (e.g., A and SJL) exhibited the phenomenon only weakly. With regard to the absence of tolerance liability in 129S6 mice, previous research has yielded the following possible mechanisms. Crain and Shen (2000) have provided evidence that deficiencies in GM1 ganglioside-regulated excitatory opioid function mediate both the enhanced analgesic sensitivity and reduced tolerance to morphine. In the present study, however, Day 1 AD₅₀ estimates for 129P3 mice were among the highest of all strains tested, indicating low morphine analgesic sensitivity. It should be admitted that this finding was in contrast to another study showing relatively high sensitivity to ICV morphine (Kest et al., 1999). There is no clear explanation for these findings, other than possibly being related to the fact that the studies were conducted in different laboratories (see Crabbe et al., 1999).

Kolesnikov et al. (1998) found functional deficiencies in the NMDA excitatory amino acid receptor system of the 129S6 strain, and/or the biochemical cascade activating nitric oxide synthase consequent to its activation. The importance of this signaling

system in morphine tolerance has been previously delineated (Elliott et al., 1995).

Whether the lack of morphine tolerance development in 129P3 mice is due to similar deficiencies in NMDA/nitric oxide activation remains to be addressed. With regard to the understudied LP mice, they were similar to the 129 P3 mice in displaying low initial morphine analgesic sensitivity. However, the overall strain correlation between the initial analgesic potency of morphine (Day 1) with the loss of potency after tolerance induction was not significant, consistent with the dissociation of analgesia and tolerance in many previous reports (Eidelberg et al., 1975; Gwynn and Domino, 1984; Kolesnikov et al., 1998).

An additional finding of the present study was that 3 days of repeated morphine injection strain-dependently altered baseline nociceptive thresholds. Tail-withdrawal latencies obtained prior to dose-response studies on Day 4 for 7 of 11 strains tested were significantly decreased by 11% or more relative to pre-drug Day 1 values, and as much as 32% in C57/BL/6 and CBA mice. These increases in nociceptive sensitivity stand in contrast to the significant decrease in nociceptive sensitivity in SWR mice, whose withdrawal latencies were increased 22% and 129P3 and A mice, who were altogether refractory to change. Thus, although previous studies have reported increased nociceptive sensitivity on various assays in response to chronic morphine treatment (Mao et al., 1994; Hoffman et al., 1998; Celerier et al., 1999; Vanderah et al., 2000; Li et al., 2001), the study appears to be the first to demonstrate that the presence, magnitude, and even direction of these alterations in mice are strain dependent.

Furthermore, the lack of a significant correlation between Day 1 ($r=-.35$) baseline withdrawal latencies and their subsequent alteration by chronic morphine injection on Day 4 suggests their independent genetic mediation, and argues against the possibility that the phenomenon is confounded by Lord's (1967) paradox (i.e. the "law of initial values"). Germane to the present report, hyperalgesia following chronic opioid administration has been advanced as contributing to opioid analgesic tolerance (Mayer et al., 1999; Vanderah et al., 2001). Specifically, opioids may maintain their analgesic efficacy after repeated administration, but this analgesia is offset by a concomitant increase in pain sensitivity (Celerier et al., 1999). All strains considered, this study found a significant genetic correlation between strain rankings for changes in baseline latencies (i.e., hyperalgesia) and analgesic tolerance. Genetic correlation of these or any two traits directly implies, although does not prove, similarity of their physiological substrates (see Mogil et al., 1999b). However, further studies are needed to confirm the results of the present study for two main reasons. First, hyperalgesia can be observed 1-3 days after even a single 30mg/kg morphine injection, even in the absence of a withdrawal-precipitating antagonist (Grilly and Gowans, 1986). Thus, hyperalgesia in the present study may have resulted from one or more spontaneous "mini-withdrawal" episodes elicited by and interspersed among the lengthy inter-injection intervals during tolerance induction. Second, our 49 °C water bath for the tail-withdrawal test elicited mean withdrawal latencies of between 1.8 and 3.1 seconds, towards the lower limits of physiologically allowable responses. Thus, our ability to detect greater reductions within strains, and differences between strains, may have been prevented by a floor

effect. Exploration using sustained morphine infusion, if possible, as well as use of a milder water bath temperature (to lengthen initial response latencies), may each contribute substantially to interpretation of the findings of the present study. Even with the limitations of the methodology used, however, the findings of the present study confirm the existence of a strong genetic contribution to morphine analgesic tolerance, and suggest that for individuals of certain genotypes, the individual's sex may also play a role in morphine's ability to sustain its analgesic efficacy after repeated administration.

GENERAL DISCUSSION

The main conclusion of this work is that there are significant sex differences in morphine tolerance under some conditions, but not others. Further, when found, these differences are specific to the strain of the animal, the assay employed (acetic acid writhing versus tail withdrawal), and method of quantification of analgesia (ED_{50} versus AUC versus AD_{50}). As a result of the current series of experiments it has become clear that the presence, and even direction, of sex differences in tolerance development can vary according to the specific effect of morphine studied (for example, with female CD1 mice displaying greater tolerance to the analgesic effects of morphine, and conversely, males demonstrating greater tolerance to the hypothermic effects of morphine).

In Specific Aim One, it was demonstrated that female CD-1 mice show a greater rightward shift in morphine ED_{50} values on the tail withdrawal nociceptive assay than male mice. Further, the magnitude of this shift increased when the morphine treatment phase was extended from 3 to 7 days, although the relative proportion remained the same. However, when the two sexes were compared on the analgesic effects of a single dose morphine challenge before and after chronic treatment, no significant sex differences were observed. The findings of this study highlight the extent to which sex differences reported in the literature (for tolerance, or other drug effects) may reflect the specific methods used.

Specific Aim Two used intracerebroventricular injection of morphine during the tolerance induction period and subsequent cumulative dose response studies, to determine the extent to which sex differences in CD-1 mice in morphine tolerance were mediated through central mechanisms. While mice were made tolerant to the repeated injections, sex differences in the extent of tolerance produced were not observed. As such, Specific Aim Three explored the possibility that spinal mechanisms may underlie the sex differences observed earlier with systemic injections. Indeed, sex differences mirroring those found with systemic injection were found, suggesting that spinal mechanisms, at least in part, appear to underly the differences observed.

It was interesting to consider whether the sex differences observed in the analgesic tolerance of CD-1 mice after chronic systemic morphine administration would generalize to other types of nociceptive assays. Specific Aim Four used the identical tolerance induction paradigm, but employed the acetic acid writhing assay to assess analgesia (and thus analgesic tolerance). An ANOVA, while revealing a significant effect of treatment group, failed to reveal a main effect of sex, or any sex x condition interactions. The lack of a significant finding was not unexpected, given the large inter-individual variability on this measure. Some interesting trends did emerge, including the ability of low dose morphine to actually increase writhing responses in male mice after three days of morphine treatment (as compared to saline treated mice), suggesting that hyperalgesia was elicited by this low morphine dose. In addition, while

increasing doses of morphine began to have analgesic effects in morphine treated animals, this trend was not evident in female animals, and may suggest that sex differences exist in this measure as well (but were not captured given the limited dosing used). Given this limitation (which was imposed to minimize animals needed), drawing conclusions regarding the generalizability, or lack thereof, of sex differences in morphine tolerance to other nociceptive assays is difficult.

Specific Aim Five took the generalizability issue one step further, actually looking at another of morphine's effects, the production of hypothermia. Male and female mice (subjected to the identical tolerance induction paradigm as used in Specific Aims One and Four) were assessed for colonic temperature before, and after, a morphine challenge on Days 1 (prior to three days of morphine) and Day 4 (subsequently to the tolerance induction period). In contrast to findings of females undergoing greater analgesic tolerance to morphine on the tail withdrawal assay, this study found males to appear more tolerant to morphine's hypothermic effects on Day 4, with morphine fully unable to elicit a change in colonic body temperature in males after chronic treatment, despite continuing to elicit a significant change in females. Other than "providing evidence for the dissociation of these two effects", furnishing possible explanations for these disparate findings is difficult. One area that must be considered is the relative role of possible associative and non-associative factors in each of these assays. In the tail withdrawal assay, each animal is subjected to an initial, baseline assessment of nociception before the beginning of a series of test doses, followed in succession by re-

assessment on the tail withdrawal test. Animals chronically exposed to morphine for three days might be expected to have an underlying, latent hyperalgesia, which as shown in Specific Aim Four, may be unmasked with administration of a low dose of morphine. The cumulative dose response method starts with such low doses (e.g, 1 or 2mg/kg), and then proceeds upward until analgesic doses are reached. It is plausible that the baseline assessment of nociception on Day 4, followed immediately by a low dose of morphine and then, 30 minutes later, being subjected again to the circulating hot water, may result in the classical conditioning of a rapid learned response to the water bath. It is likewise plausible that these conditioning effects might be greater in female than male CD-1 mice, and as such underlie the higher doses of morphine needed to attenuate the response on Day 4 relative to male mice. In the assessment of colonic temperature in response to a morphine challenge, there does not appear to be a route for learning effects to play a role, and as such, the finding of greater tolerance in males versus females may reflect the involvement of solely non-associative factors in tolerance development. Further work would be necessary to explore these possibilities further.

Specific Aim Six examined the extent to which morphine analgesic tolerance may be dependent on the strain of animals studied, and the specific method used to calculate an analgesic response. Its findings, while of specific relevance to the topic of tolerance, provide information of interest to analgesia research in general. First and foremost, the study found significant strain differences in morphine analgesic tolerance as assessed by

the tail withdrawal test, with strains ranging from 10 fold shifts in potency from Day 1 to Day 4, to no significant changes in morphine's analgesic effects across the time period. Using ED_{50} values to calculate analgesia and assess tolerance, three of eleven strains demonstrated significant sex differences in tolerance, supporting the finding of Specific Aim One, but reminding us of the limited generalizability of findings of sex differences, as is becoming increasingly evident from analgesia research (Craft, 2003). When an alternative measure of analgesia was used (AD_{50} values), sex differences were not significant. However, extreme responding strains were identified similarly by each measure, suggesting that while sex differences may be better captured by certain methods of study, potent and meaningful findings should generalize across measures.

Specific Aim 6, in contrast to the earlier studies using CD-1 mice, found baseline latency changes in the tail withdrawal response for many strains after the three days of chronic morphine exposure. In fact, it was found that the extent of hyperalgesia (as defined by a reduction in baseline tail withdrawal latencies on Day 4 versus Day 1) was strongly and significantly correlated with both Day 4 AD_{50} values ($r=.80$) and potency shifts from Day 1 to Day 4 ($r=.76$). These findings suggest that the development of tolerance to morphine's analgesic effects and the development of morphine induced hyperalgesia are genetically correlated. In total, the studies demonstrate that pain facilitation, whether at baseline or following "unmasking" with low dose morphine, appears to be a contributing factor to the observation of behavioral tolerance.

There are several limitations to the studies presented above. As indicated from the disparate results (and an important finding in its own right), it is clear that findings of sex differences (when they occur), should be considered as limited to the specific species, strain and methods used. Applicability beyond the narrow and explicit conditions of the experiment should not be assumed unless clearly demonstrated. To date, work in this area has been quite limited, and much additional scientific effort must be exerted in order to provide a broad-based understanding of the factors involved.

From results of the subcutaneous analgesia and thermoregulation studies in CD1 mice, there emerged the suggestion of a trend related to one of the hypothesis considered at the outset of the studies. Specifically, in the study in which females appeared initially to be somewhat (although not significantly) less sensitive to morphine's effects (Specific Aim One), females were also the strain that demonstrated the most tolerance to those effects. In Specific Aim 5, where males appeared less sensitive to the initial hypothermic effects of morphine, males similarly became most tolerant, with a complete loss of morphine's ability to cause temperature changes. These findings suggested the possibility that findings of sex differences in tolerance may merely be related to a general reduced sensitivity to specific behavioral/physiological effects of the pharmacological agent in one sex or the other. However, correlational analyses conducted in Specific Aim 6 ruled this out as a possible explanation for sex differences, with no significant correlation observed between baseline analgesic effects of morphine and analgesic effects after chronic treatment, or between extent of tolerance

development and initial nociceptive sensitivity or acute morphine analgesia. In fact, of all the measures examined, changes in baseline tail withdrawal latencies after three days of morphine treatment (hyperalgesia) was one of the most strongly correlated measures with the extent of potency shift in morphine analgesia after chronic administration (tolerance). Table 9 shows relative rankings of various pain related measures in the current series of studies.

Table 9. Relative rankings of each strain on various pain-related measures (based on data obtained through studies conducted as part of Specific Aim 6).

Table 9.

Strain	Baseline Tail Withdrawal Latency (shortest to longest)	Baseline Morphine ED ₅₀ value (Lowest to highest)	Baseline AD ₅₀ value (lowest to highest)	Relative Potency Shift during tolerance induction (least to most tolerance) (AD ₅₀)	Day 4 ED ₅₀ value (lowest to highest)	Day 4 AD ₅₀ value (lowest to highest)	Baseline latency change (least to most hyperalgesic on day 4)
<u>BALB/c</u>	11	1	1	7	1	3	11
<u>DBA/2</u>	8	10	9	5	10	6	5
<u>CBA</u>	3	4	3	11	4	10	10
<u>129/P3</u>	6	11	8	1	9	2	4
<u>C57/BL6</u>	5	6	5	9	6	9	6
<u>C3H/He</u>	2	7	4	10	5	8	8
<u>AKR</u>	10	8	7	6	11	7	9
<u>SWR</u>	1	5	11	8	8	11	1
<u>LP</u>	9	9	10	2	7	5	3
<u>SJL</u>	4	3	6	4	2	4	7
<u>A/J</u>	7	2	2	3	3	1	2

One of the areas not explored in the present series of studies was the contribution of estrus phase to differences in tolerance development in females, in part because of the chronic tolerance paradigm used which presented a logistical challenge to classification of estrus phase (especially in light of the species used, and the demonstrated ability of chronic morphine treatment to alter estrus cycles). However, when it was possible to assess variability in the current studies (e.g., in acetic acid writhing responses and baseline tail withdrawal latencies) there did not appear to be greater variability in females than in males, which might argue against estrus effects playing a notable role. Given the use of ED50/AD50 values to assess tolerance development, however, statistical calculation of potential sex differences in variability in degree of tolerance development was not possible. As such, it is impossible to rule out a role for estrus effects in the development of tolerance to morphine's analgesic effects, and further study of this area may contribute meaningfully to our understanding of sex differences observed. Overcoming the challenges of controlling for estrus in chronic drug administration paradigms, while perhaps difficult, may be an important contribution of future work in this area. Interestingly, some recent work has suggested a strong correlation between acute morphine tolerance and chronic tolerance (see Borgland, 2001). As such, exploration of these factors through acute induction methods, along with classification of estrus phase, may help clarify some of the findings of the present studies.

Age-related changes in morphine's analgesic efficacy have been reported (see Bodnar, 1989 for review), and the extent to which the animal's age affects tolerance development to morphine's analgesic effects was not specifically explored in the current studies (with most animals about 8 weeks of age at the time of testing). As a basic measure of neural plasticity, it would be interesting to explore changes in the development of tolerance as a function of age, and whether sex differences are observed across the lifespan. Likewise, it would be of interest to determine the degree to which genetic differences contribute to tolerance at different ages (although study of this may be confounded by the different life spans of available inbred strains).

The studies conducted as part of the present dissertation further elucidate the contribution of sex and genotype to morphine analgesic tolerance. Marked individual differences are apparent in tolerance to the analgesic effects of morphine clinically, and the series of studies conducted have clarified some of the factors which may modulate these differences. While behavioral studies exploring contributions of sex and genotype to morphine analgesic tolerance have, to date, been limited, it is hoped that the experiments conducted as part of the present dissertation will help set the foundation for other work in this area, with the goal of achieving a better understanding the development of morphine tolerance, and as a result, improving our ability to effectively manage chronic pain.

APPENDIX**Abbreviations**

AA	acetic acid
AD ₅₀	Analgesic Dose 50
AUC	area under the curve
CI	confidence interval
°C	degrees Celsius
CNS	central nervous system
DLF	dorsal lateral funiculus
ED ₅₀	Effective Dose 50
ICV	intracerebroventricular
IP	intraperitoneal
IT	intrathecal
kg	kilogram
LC	locus coeruleus
%MPE	percent maximum possible efficacy
mg	milligrams
ml	milliliter
NMDA	<i>N</i> -methyl- <i>D</i> -aspartate
PAG	periaqueductal gray

PCA	patient controlled analgesia
QTL	quantitative trait loci
RA/VE	relative activity versus endocytosis
RVM	rostral ventral medulla
s	seconds
SIA	stress induced analgesia
TID	Latin “ter in die”; three times a day
TW	tail withdrawal

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