MORPHINE HYPERALGESIA: CONTRIBUTION OF OPIOID RECEPTORS, DOSING REGIMEN, AND GONADAL HORMONES

by

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Abstract

MORPHINE HYPERALGESIA: CONTRIBUTION OF OPIOID RECEPTORS, DOSING REGIMEN, AND GONADAL HORMONES

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Hyperalgesia during morphine treatment is extensively conceptualized as a process that is mediated by opioid and NMDA receptors, and thought to be a causative factor in morphine analgesic tolerance. These assumptions were tested in mice assessed for nociceptive sensitivity on the tail-withdrawal test during continuous infusion of various morphine doses. Mice infused with the lowest morphine dose did not demonstrate any analgesia, but exhibited hyperalgesia that dissipated after 6 days. In contrast, the highest morphine dose elicited 2 days of analgesia followed by 10 days of hyperalgesia before resolution to baseline by Day 12. Acute exposure to NMDA receptor antagonists resulted in transient hyperalgesic reversal during both infusion paradigms, implicating NMDA receptor involvement in the maintenance of hyperalgesic states. Cross adaptation studies between the 2 doses and M3G, a primary morphine metabolite, implicated M3G in the

mediation of low-dose hyperalgesia only. The lack of cross adaptation between the 2 infusion doses themselves, suggested their distinct mechanistic mediation.

Evidence for a relationship between analgesic tolerance and hyperalgesia was not found, as ED_{50} values derived from analgesia dose-response curves yielded significant differences between hyperalgesia and tolerance including magnitude, onset, and adaptation. Studies conducted under continuous opioid receptor blockade and with triple opioid receptor knockout mice, further suggested that prior analgesia is not a prerequisite for hyperalgesia, and that morphine hyperalgesia is a non-opioid receptor mediated process.

Although sex differences in nociception and several morphine related measures have previously been described, little is known about the impact of gender on morphine hyperalgesia. This dissertation thus also compared nociception in male and female mice during morphine infusion. In contrast to males, females did not adapt to hyperalgesia during low dose infusion, and hyperalgesia during high dose infusion was refractory to NMDA receptor antagonism. Ovariectomy, but not ovariectomy followed by estrogen infusion, abolished these qualitative sex differences.

The data indicate multiple mechanisms of morphine hyperalgesia, with utilization being at least partly dependent upon female sex hormones. Some of the conclusions arising from these findings are outside of current conceptualizations, yielding new insights into the clinical utility and physiological circuitry underlying morphine analgesic therapy.

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INTRODUCTION

Pain has been defined as an unpleasant sensory or emotional experience arising from actual or impending tissue damage (Kandel, Schwartz, & Jessell, 2000). From a technical perspective, the term 'pain' is inappropriate when dealing with animals or other organisms where it is difficult to ascertain 'pleasantness' or emotionality. In these instances, the term nociception is often used to connote activation of nociceptive neurons in response to noxious stimuli. Following receptor activation, afferent nociceptive impulses are subject to modification by multiple physiological systems at every level of the neuraxis. The net effect of these modulatory inputs vary widely, as some function to suppress incoming nociceptive signals (analgesia) while others are of a more pronociceptive nature, serving to either oppose analgesia (a process known as antianalgesia), increase basal nociceptive sensitivity to noxious stimuli (hyperalgesia), or stimulate the perception of pain in response to neutral stimuli (allodynia). Thus, the perception of pain represents a cumulative effect resulting from the balance of nociceptor activation and multiple modulatory factors.

Among the various analgesic agents, opioids such as morphine have long been recognized as the most efficacious form of treatment for moderate to severe pain (Mao, Sung, Ji, & Lim, 2002; Andersen, Christrup, & Sjogren, 2003). However, despite its initial efficacy, repeated opioid exposure inevitably causes reductions in its analgesic potency, a phenomenon known as morphine tolerance (Mao, Price, & Mayer, 1995; Elliott, Kest, Man, Kao, & Inturrisi, 1995). While morphine tolerance has been studied

for decades, cogent mechanistic interpretations of opioid tolerance still remain elusive (Mayer, Mao, & Price, 1995; Gutstein, 1996; Borgland, 2001; Kieffer & Evans, 2002; Mao et al., 2002). There have also been growing numbers of reports documenting paradoxical reactions in chronic pain patients (Arner, Rawal, & Gustafsson, 1988; Katz, Cohen, Schmid, Chan, & Wowk, 2003; Angst, Koppert, Pahl, Clark, & Schmelz, 2003), wherein prolonged opioid use was linked to dramatic increases in pain sensitivity (i.e. hyperalgesia). This has led some researchers to propose that tolerance may in fact be a reflection of opioid induced hyperalgesia (OIH), whereby increasingly larger doses of opioid analgesics are required to offset enhanced nociceptive sensitivity (Vanderah et al., 2001a; Simonnet & Rivat, 2003). In addition to tolerance, non clinical opioid use is also associated with an increased the risk of experiencing severe side effects such as dependence, addiction, drug abuse and CNS depression (Xie et al., 2005; Mercadante & Arcuri, 2005).

The specific aims of the current series of studies will address the nature of OIH through an exploration of the organismic and pharmacological variables that govern its expression as well as an attempt to identify some of the underlying physiological mechanisms. The goal of study 1 was to evaluate a murine model of OIH. The tail withdrawal test, a commonly used measure of thermal nociception was used to determine basal nociceptive thresholds and subsequent variations over time. Mice were subjected to continuous morphine infusion of 3 doses, and daily tail withdrawal latencies were assessed to determine nociceptive thresholds. On selected days during the infusion paradigms, dose response analyses of morphine analgesia were also conducted to determine the

relationship between tolerance and hyperalgesia. An exploration of the mechanisms underlying OIH was then conducted to identify underlying physiological and pharmacological circuitry. This included broad based opioid antagonists, NMDA antagonists, and morphine metabolites. Following the discovery of 2 distinct, dose-dependent OIH paradigms, the goal of study 2 was to directly assess the role of opioid receptors in the elicitation and maintenance of hyperalgesic states through a series of experiments conducted with a strain of knockout mice that are lacking in all 3 opioid receptors. When the knockout animals were found to exhibit OIH in response to continuous infusion of 2 different opioid doses, they were subjected to NMDA antagonists, opioid antagonists, and morphine metabolites to ascertain the mechanisms underlying this hyperalgesia. Having established a consistent hyperalgesic paradigm in males in studies 1 and 2, the goal of Study 3 was to investigate gender differences in morphine hyperalgesia, and once they were found to be present, to focus on the activation versus organizational effects of gonadal hormones in mediating hyperalgesic states.

In order to provide a context for the current series of studies, the next section will provide a review of the relevant literature and previous findings. The topics covered will include:

1) Overview of pain physiology
2) Opioid pharmacology
    a. anatomical distribution of opioid receptors
    b. opioid receptor modulation of nociception
    c. role of metabolic processes in opioid pharmacology

3) Studies in opioid induced hyperalgesia
    a. human studies
    b. animal studies
4) Mechanisms of opioid induced hyperalgesia
    a. opioid receptor involvement
    b. cellular sensitization/ disinhibition
    c. NMDA
    d. pronociceptive opioid metabolites
5) Sex Differences
    a. Ovarian hormones
    b. Sex differences in nociception
    c. modulatory effects of gonadal hormones on nociception
    d. sex differences in opioid modulation of nociception

The final section of the introduction will provide the rationale for the specific studies and methods that comprise the current dissertation.

A. Pain Physiology

Regardless of nociceptive modality, physiological pain perception or nociception begins when specialized receptors known as free nerve endings become activated by chemical, mechanical or thermal stimulation. These receptors are specialized for the detection and transduction of pain, and play no role in somatosensation. Nociceptors can be further subdivided into two distinct functional groups – mechanical nociceptors are only responsive to intense mechanical stimulation, while polymodal nociceptors can be activated by a variety of noxious stimuli. These cells vary little in their design across different cutaneous regions, are essentially the same across many mammalian species (Zigmond, Bloom, Landis, Roberts, & Squire, 1998). An important feature of the axon terminals of nociceptors is that they possess no protective sheaths and are thus highly sensitive to chemicals produced or released at the site of injury (such as topical analgesics).

The density of nociceptors varies widely throughout the body. Their cell bodies are located outside the spinal cord in the dorsal root ganglion (DRG). Axons arising from these cells join together to form two distinct fiber tracts. Acute, fast pain (such as from stepping on a tack) travels via thinly myelinated Aδ fibers and synapses in lamina I and IV of the dorsal horn, while longer-lasting, throbbing pain (such as from a bee sting) is carried by unmyelinated, C fibers which synapse in lamina II and III of the dorsal horn. Second and third order neurons then cross over through the anterior commisure and ascend contralaterally via the anterior spinothalamic tract, terminating in various brain regions including medulla, thalamus, basal ganglia, and somatosensory cortex, before

descending projection neurons are activated. Nociceptor activation can also trigger numerous local reactions including stimulation of interneurons leading to reflexive responses within the spinal cord, and the release of chemical neurotransmitters such as Substance P (SP), neurotrophic factors, and glutamate (Urban & Gebhart, 1999).

Nociceptive processing is highly complex, and involves the recruitment of multiple systems. For example, impulse propagation from the dorsal horn to the brainstem is highly regulated by mechanisms that can be activated both endogenously and exogenously. In fact, even before reaching the dorsal horn, primary afferent inputs are subject to: inhibitory and excitatory medullary efferents (Vanderah, Ossipov, Lai, Malan, Jr., & Porreca, 2001a; Fine, 2004; White, 2004), GABA inhibition (Bernardi, Valtschanoff, Weinberg, Schmidt, & Rustioni, 1995; Dickenson, Chapman, & Green, 1997), and nitrous oxide activation (Kitto, Haley, & Wilcox, 1992; Mao et al., 1995). The net effect of these modulatory inputs vary widely, as some function to suppress incoming nociceptive signals (analgesia) while others are of a more pronociceptive nature, serving to oppose analgesia (a process known as antianalgesia), increase basal nociceptive sensitivity to noxious stimuli (hyperalgesia), or stimulate the perception of pain in response to neutral stimuli (allodynia). Thus, the perception of pain represents a cumulative effect resulting from the balance of nociceptor activation and multiple modulatory factors.

B. Opioid Pharmacology

i. Opioid Receptors

Although the opium poppy plant was first cultivated in lower Mesopotamia in 3400 B.C., it wasn't until 1803 when the active component of opium was purified by acid hydrolysis and labeled morphine (Hollman, 2005). Over the next 2 hundred years, various other compounds were synthesized from the opium poppy including heroin and codeine. However, not much was known about opioid receptor pharmacology until 1973, when it was discovered that tritiated naloxone, a powerful opiate antagonist, specifically binds to opiate receptors in the mammalian brain and guinea pig ileum (Pert & Snyder, 1973). These findings pointed toward the existence of endogenous pain relieving mechanisms, which achieved their effects through binding with these opioid receptors. Technically, the term opiate applies to substances derived from the opium poppy or their synthetic analogue, while the term opioid refers to all substances (both endogenous and exogenous) that are not opium derivatives but still bind with opioid receptors. However, both terms are often used interchangeably within the literature, and are generally referred to as opioids. In addition to exogenous opioid substances, 3 classes of endogenous opioid peptides have also been discovered: endorphins, enkephalins, and dynorphins.

Following the discovery of an endogenous opioid system, multiple opioid receptors subtypes were proposed, but advances in technology and molecular cloning ultimately led to the identification of only 3 distinct opioid receptor subtypes: mu, delta, and kappa

(Mansour, Fox, Akil, & Watson, 1995; Massotte & Kieffer, 1998). The spinal distribution of these receptors was originally revealed through autoradiographic methods which showed distinct distributions for each of the various receptor subtypes, with high concentrations of μ receptors in the outer laminae of spinal dorsal horns, δ receptors being more diffusely distributed within the dorsal horns, and κ receptors localized to the outer laminae of the lumbosacral dorsal horn (Quirion, Zajac, Morgat, & Roques, 1983; Besse, Lombard, & Besson, 1991; Ossipov et al., 2004). Subsequent immunohistological studies confirmed the aforementioned differential distribution but provided greater resolution, showing spinal expression of delta receptors primarily confined to the small-diameter, nociceptive, Aδ and C fiber tracts (Ji et al., 1995; Arvidsson et al., 1995).

ii. Opioid receptor modulation of nociception

Spinal opioid receptors play an important role in mediating opioid analgesia. They are presumed to exert their effects by directly inhibiting ascending nociceptive inputs, thereby limiting the organism's sensitivity to noxious stimuli. There has been much empirical evidence to support this hypothesis. Studies employing electrophysiological recordings from isolated nociceptors revealed that spinal opioid receptor activation predictably inhibited C fiber calcium channels (Taddese, Nah, & McCleskey, 1995; Cata, Weng, Chen, & Dougherty, 2006), which are 1 of the primary afferent pathways for pain perception. Furthermore, it has been shown that functional blockade of spinal opioid receptors means leads to a marked reduction in the analgesic potency of opioids (Hara et al., 1999).

However, opioid receptor modulation of nociception is not limited to effects mediated by the spinal cord, but also occurs via descending projections from supraspinal loci. Indeed, numerous brain regions have been shown to express rather high concentrations of opioid receptors including the frontal cortex, nucleus accumbens, hippocampus, thalamus, hypothalamus, rostral ventromedial medulla (RVM) and the periaqueductal gray (PAG) (Quirion et al., 1983; Mansour et al., 1995; Kalyuzhny, Arvidsson, Wu, & Wessendorf, 1996). Although it is only recently that immunohistological studies were able to confirm high levels of μ receptor expression in PAG and RVM (Mansour et al., 1995), their role as regions involved the modulation of nociception has long been suspected. Many of the early models of CNS pain suppression (Kalyuzhny et al., 1996; Vanderah et al., 2001b) proposed that endogenous opioid peptides bind to receptors within the PAG. From the PAG, projections are sent via the RVM, a region of the medulla that includes the nucleus reticularis gigantocellularis pars alpha, the serotonin-rich nucleus raphe magnus (NRM), as well as the nucleus paragigantocellularis lateralis (Fields, Heinricher, & Mason, 1991). The RVM in turn, sends impulses through the dorsolateral funiculus (DLF) down to the dorsal horn of the spinal cord, where they inhibit incoming nociceptive transmissions (Vanegas & Schaible, 2004). Exogenous opioid peptides are thought to take advantage of this preexisting circuitry, exerting their effects by binding with either spinal or supraspinal sites and triggering the endogenously wired cascade.

The current view of the RVM's role in nociceptive modulation has been greatly informed by electrophysiological studies which have led to the identification of 2 distinct neuronal

populations within the RVM, "on" and "off" cells (Fields, Malick, & Burstein, 1995). Off-cells are typically active, but demonstrate reductions in their firing rate immediately before the host organism performs a behavior to avoid a noxious stimulus. On-cells, by contrast, exhibit an increase in their firing rates immediately before the initiation of a pain avoidance response. Subsequent research has shown correlations between off-cell activity and antinociceptive activity, suggesting that they are the source of descending inhibition in opioid mediated analgesia (Fields, 2000). Conversely, on-cell activity has been associated with enhanced nociceptive responses, as manipulations that increase nociceptive behavioral responses similarly increase on-cell firing rates (Neubert, Kincaid, & Heinricher, 2004). Furthermore, inhibition of RVM on-cell activity through lidocaine injections reverses experimentally induced hyperalgesia (Vanderah et al., 2001b). Studies such as these highlight the importance of supraspinal contributions to nociceptive functioning, and suggest that demonstration of altered pain states (analgesia, hyperalgesia, allodynia…) brought about either through injury or pharmacology are likely representing the synergistic effects of both spinal and supraspinal structures.

iii. Opioid metabolites

The role of opioids in modulating nociceptive functioning is dependent both upon the administered substance and its metabolic derivatives. Upon introduction into the body, many opioids may initially exert an effect, only to be metabolized by enzymes into compounds that, relative to its precursor may: a) augment its effects, b) oppose its effects, c) be the primary active pathway, d) be relatively inert. For example, codeine achieves

its analgesic and euphoric effects through metabolic activity involving O-demethylation to morphine (Findlay, Jones, Butz, & Welch, 1978; Sindrup & Brosen, 1995), and is physiologically inert when the metabolic process is prevented (Desmeules, Gascon, Dayer, & Magistris, 1991). In contrast, O-desmethyltramadol, tramadol's active metabolite, has been shown to activate μ receptors much more potently than its parent compound (Paar, Poche, Gerloff, & Dengler, 1997; Gillen, Haurand, Kobelt, & Wnendt, 2000), but is not critical to tramadol's efficacy. Hydromorphone, on the other hand, is glucoronidated into hydromorphone-3-glucoronide (H3G), a neuroexcitant that opposes the analgesic effects of hydromorphone (Lotsch, 2005).

Due to its widespread clinical use, there have been many studies analyzing the pharmacologic activity of morphine and its metabolites. Morphine initiates its analgesic effects by binding to the μ receptor, but then undergoes glucoronidation in the liver by glucoronic acid (Bodenham, Quinn, & Park, 1989; Ouellet & Pollack, 1997). Roughly 15% of systemically available morphine is converted into morphine-6-β-glucoronide (M6G), a highly potent μ receptor agonist with profound analgesic properties (Paul, Standifer, Inturrisi, & Pasternak, 1989; Christrup, 1997; Faura, Olaso, & Horga, 1997; Skarke, Geisslinger, & Lotsch, 2005), while more than 50% is metabolized into morphine-3-β-glucoronide (M3G) a metabolite with virtually no opioid affinity or analgesic potency (Yaksh, Harty, & Onofrio, 1986; Christensen & Jorgensen, 1987; Gong, Hedner, Bjorkman, & Hedner, 1992; Bartlett, Dodd, & Smith, 1994b; Milne, Nation, & Somogyi, 1996). Functionally, glucoronidation may also allow for longer physiological effects, as it has been shown that during prolonged morphine therapy, the

plasma concentrations of both M3G and M6G far exceed that of morphine (Christrup, 1997). Moreover, individual differences in human morphine responsiveness have been attributed to a single nucleotide polymorphism of the μ receptor causing a diminished sensitivity to M6G, but not to morphine (Antonilli, Petecchia, Caprioli, Badiani, & Nencini, 2005). Thus morphine's effect on nociception may be more dependent upon its metabolite activity than its own properties per se.

C. Prevalence of Opioid Induced Hyperalgesia

Although the WHO still recommends morphine as an essential drug for the treatment of chronic pain (Sakurada, Komatsu, & Sakurada, 2005), many clinicians and researchers have begun to seek alternative therapeutic methods due to morphine's paradoxical, pain enhancing properties (Mao, Price, & Mayer, 1994; Attal et al., 2002; Fine, 2004; Angst & Clark, 2006). This next section will review findings from the literature detailing OIH as a consequence of opioid therapy in both humans and animals.

i. Human Studies:

For over a century, there have been clinical reports associating heightened pain sensitivity with opioid treatment. As far back as 1880, Rossbach wrote, "When dependence on opioids finally becomes an illness of itself, opposite effects like restlessness, sleep disturbance, hyperesthesia, neuralgia and irritability become manifest" (Angst & Clark, 2006). In fact, according to the most recent DSM-IV criteria, pain symptoms are an important criterion for establishing a diagnosis of opioid withdrawal. Unfortunately, human studies of OIH have revealed it to be a prominent condition, with most of the studies being conducted in 1 of 3 settings: (1) former opioid addicts maintained on methadone, (2) patients recovering from surgery, and (3) healthy human volunteers exposed to painful stimuli.

Because of the strong dependence and severe withdrawal that often develops in opiate addicts, abusers in recovery are often prescribed methadone. Methadone is a synthetic opioid that shows a strong affinity for the μ receptor but does not elicit the analgesic and euphoric properties of 'classical' mu receptor agonists like morphine and heroin. There have been numerous clinical studies of OIH comparing the pain sensitivity of these methadone maintenance patients (as assessed through cold pressor tests, mechanical pressure or electrical stimulation) to either former addicts not on methadone maintenance therapy or healthy controls (Doverty et al., 2001; Compton, Charuvastra, & Ling, 2001; White, 2004; Pud, Cohen, Lawental, & Eisenberg, 2005). These studies have shown that methadone treatment significantly decreases pain thresholds on the cold pressor test (an indication of hyperalgesia), but has little effect on subjects' responses to electrical and mechanical stimulation. These findings suggest: (a) that continued opioid (mu) receptor activation can lead to heightened pain sensitivity, and (b) even when present, the magnitude and quality of OIH vary by pain modality even within the same organism.

Evidence for OIH has also been found in clinical settings involving prophylactic opioid treatments. In an effort to reduce post-operative pain, a number of studies have been conducted looking at the effect of initiating opioid treatment either pre or peri operatively, before the patient had experienced any distress. Although the studies have substantial methodological differences, there was a general consensus that patients who had undergone the pretreatment tend to report greater post-operative pain than patients who had undergone similar procedures without prior pharmacotherapy (Sandkuhler & Ruscheweyh, 2005; Angst & Clark, 2006). This was shown with many classical mu

receptor agonists including morphine (Eisenach, 2000), remifentanil (Guignard et al., 2000), and fentanyl (Chia, Liu, Wang, Kuo, & Ho, 1999). Furthermore, many of these pretreatment studies also reported a persistent post-operative hyperalgesia that was relatively resistant to further treatment, pointing to possible non-opioid underlying mechanisms.

Similar findings were reported in a study where women undergoing caesarian section under spinal anesthesia showed up to 63% greater opioid consumption post-operatively relative to those who received intrathecal saline (Cooper et al., 1997). There have also been many reports of sustained opioid treatment leading to long-lasting hyperalgesia, persisting even after cessation of opioid treatment (Guignard et al., 2000; Gardell et al., 2002; Gustorff, Kozek-Langenecker, & Kress, 2003; Mercadante, Ferrera, Villari, & Arcuri, 2003). Arner (Arner et al., 1988) reviewed the findings from a survey of over 750 patients who received an average of 5 months of intravenous morphine, and noted many cases of "abnormal pain" including allodynia, hyperesthesias (increased sensitivity to sensory stimuli such as light touch or brush), and hyperalgesia. The elicitation and persistence of hyperalgesia in these studies, as well as the increased post-operative opioid consumption suggest a strong link between opioid use and development of hyperalgesia.

Due to ethical restraints, there have been relatively few controlled studies of OIH in healthy human volunteers. However, a series of experiments was conducted looking at the effects of short-term remifentanil (a fast-acting μ receptor agonist) infusion on an area of skin that had been rendered hyperalgesic prior to opioid treatment (Petersen, Jones,

Segredo, Dahl, & Rowbotham, 2001; Angst et al., 2003; Hood, Curry, & Eisenach, 2003; Koppert et al., 2003). Evidence of OIH was found as early as 30 minutes post-injection, persisting for up to 4 hours post treatment. Moreover, the magnitude of the observed effect was directly related to remifentanil dosage, with increases in the amount of duration of remifentanil administered being correlated with elevations in the magnitude and duration of subsequent hyperalgesia.

ii. Animal Studies

Over the past 30 years, there has been an increasingly growing interest in OIH and its underlying mechanisms. Angst and colleagues (Angst & Clark, 2006) summarized the findings from over 100 publications on OIH in animals, which demonstrated increased pain sensitivity induced by opioid exposure on measures of thermal, chemical, mechanical or electrical pain. Despite wide variations in methodology and animal species under study, the authors were surprised at the relatively circumscribed liability of the various pain pathways mediating OIH. Similar to findings from human studies, manipulations that produce profound OIH on 1 pain assay may show no hyperalgesic liabilities on other assays, suggesting that OIH is drug and modality specific in animals as well.

Overall, the literature characterizing the onset and resolution of OIH can be subdivided into 2 groups based on the duration of opioid exposure. Acute paradigms typically involve the systemic administration of a single opioid dose, and the resulting changes in

behavioral responses. Such studies consistently demonstrate a dose-dependent, biphasic role for morphine and other μ receptor agonists whereby intense antinociceptive effects are followed by periods of enhanced pain sensitivity typically lasting 3 – 4 hours (Ding & Bayer, 1993; Larcher, Laulin, Celerier, Le, & Simonnet, 1998; Laulin, Larcher, Celerier, Le, & Simonnet, 1998; Celerier, Laulin, Larcher, Le, & Simonnet, 1999; Crain & Shen, 2001; Borgland, 2001; Xu, Colpaert, & Wiesenfeld-Hallin, 2003), and occasionally greater than 5 days (Celerier et al., 2000; Rivat et al., 2002). Furthermore, in healthy, pain-free rats, subcutaneous morphine injections elicited pain behaviors at dermatomes corresponding to the injection site, including biting and scratching (Yaksh et al., 1986). Thus despite their analgesic prowess, opioid drugs seem to possess pain-enhancing properties that can be activated after a single opioid exposure and become more manifest after the initial analgesic effects have worn off.

However, the bulk of studies investigating OIH employed more prolonged administration paradigms, where animals were administered opioids for 3 – 14 days through a variety of routes including: multiple daily injections, subcutaneous pellet or pump implantation, or intrathecal catheters. Here too, biphasic responses were often noted, with analgesic phases generally lasting 1 to 3 days gradually being followed by hyperalgesic periods (Laulin, Celerier, Larcher, Le, & Simonnet, 1999; Li, Angst, & Clark, 2001a; Li, Angst, & Clark, 2001b; Gardell et al., 2002; Bie & Pan, 2003; Ossipov, Lai, Vanderah, & Porreca, 2003; Kest, Palmese, Juni, Chesler, & Mogil, 2004; Coutaux, Adam, Willer, & Le, 2005). Furthermore, the finding of hyperalgesia in animal studies employing uninterrupted opioid delivery (Celerier et al., 2000; Celerier, Laulin, Corcuff, Le, &

Simonnet, 2001; Kest et al., 2002; Simonnet & Rivat, 2003; Ossipov, Lai, King, Vanderah, & Porreca, 2005), proves that pain hypersensitivity is not simply a consequence of opioid withdrawal, but rather a direct consequence of opioid use. There is thus a growing body of literature highlighting a dual-role for morphine and other opioids, wherein they produce simultaneous activation of 2 opposing mechanisms; a strong, but short-lasting pain inhibitory phase, and a weaker, but longer-lasting pronociceptive period. The initial hyperalgesic effect is often not-observed because it is masked by concomitant analgesia, and thus can only be evident after 'analgesic washout'. However, studies demonstrating heightened nociceptive responses following either sub-analgesic doses of morphine (Vierck, costa-Rua, Nelligan, Tester, & Mauderli, 2002) or with CXBK mice that show reduced analgesic liabilities (Li et al., 2001a) suggest that hyperalgesia may be a direct consequence of opioid exposure that is independent of prior antinociceptive processes.

D. Mechanisms of Opioid Induced Hyperalgesia

i. Opioid receptor involvement

The precise mechanism underlying the hyperalgesic liabilities of morphine and other opioids is not presently known, but it has long been presumed that opioid receptors play a major role in the development, maintenance and resolution of hyperalgesic states. Some of the earliest work in this area was conducted by Stanley Crain who argued that the bimodal properties of morphine and many other opioids are caused by the dual activation of pertussis toxin-sensitive inhibitory (G_i/G_o coupled) and cholera toxin-sensitive excitatory (G_s coupled) opioid receptors matrices (Crain & Shen, 2001). While high doses of NTX will completely block both forms of receptor matrices and induce withdrawal behaviors, Crain demonstrated that ultra-low doses (in the pico-femtomolar range) are sufficient to inhibit just the excitatory connections, leading to enhanced analgesic efficacy and significant attenuation of tolerance and hyperalgesia; thereby suggesting that OIH is induced by the excitatory actions of G_s-coupled μ receptor. Meanwhile, others have attributed OIH to either an opioid-mediated opponent process to analgesia (Celerier et al., 2000; Simonnet & Rivat, 2003; Celerier, Gonzalez, Maldonado, Cabanero, & Puig, 2006; Gardell et al., 2006), or as a systems level adaptive response to prolonged opioid exposure (Ossipov et al., 2004). The basic notion underlying these theories is that the nociceptive hypersensitivity often observed after opioid administration is regulated by opioid receptors, and related to the preceding analgesic period.

Although the opioid-mediated view of OIH has been predominant in the literature for over 30 years (Tilson, Rech, & Stolman, 1973; VonVoigtlander & Lewis, 1983), there has been a growing body of evidence suggesting that despite its initiation by opioids, OIH may be regulated by non-opioid mediated process as well (LaBella, Pinsky, & Havlicek, 1979; Sankaran, Goldfine, Deveney, Wong, & Williams, 1980; Dalsgaard et al., 1982; Yaksh et al., 1986; Smith, Watt, & Cramond, 1990; Wang & Han, 1990; Gong et al., 1992; Smith & Smith, 1995; Devillers, Boisserie, Laulin, Larcher, & Simonnet, 1995; Laulin et al., 1998; Celerier et al., 1999; Kest, Palmese, Hopkins, Adler, & Juni, 2001; Gardell et al., 2002). For instance, it has been reported that coadministration of naloxone with morphine effectively blocks analgesia, while enabling latent hyperalgesia to become visible much sooner (Larcher et al., 1998). Despite the ability of low doses of NTX to antagonize excitatory G_s coupled reactions *in vitro* (Crain & Shen, 1995; Crain & Shen, 2000; Wang, Friedman, Olmstead, & Burns, 2005), numerous clinical studies have failed to demonstrate similar effects *in vivo*. Adjuvant therapies combining ultra-low doses NTX doses with opioid analgesics have frequently failed to show significant reductions in postoperative morphine consumption or subsequent hyperalgesia (Devillers et al., 1995; Cepeda, Africano, Manrique, Fragoso, & Carr, 2002; Koppert et al., 2005; Terner, Barrett, Lomas, Negus, & Picker, 2006), although there was some indication of diminished nausea (Cepeda et al., 2002; Cepeda, Alvarez, Morales, & Carr, 2004). Finally, prior analgesia has been shown to be unrelated and not necessary for subsequent OIH development (Berge, Fasmer, & Hole, 1983; Herrero, Laird, & Lopez-Garcia, 2000; Carroll, Angst, & Clark, 2004).

ii. cellular sensitization/disinhibition

Cellular explanations for the genesis of OIH primarily describe either neuronal sensitization or disinhibition. Central sensitization refers to enhanced firing rates in the CNS in response to noxious input, and is thought to be crucial for the development of hyperalgesia. There have indeed been many reports correlating elevations in CNS activity heightened pain expression (McMahon, Lewin, & Wall, 1993; Neugebauer, Chen, & Willis, 1999; Suzuki, Morcuende, Webber, Hunt, & Dickenson, 2002; Alloui et al., 2003). Similar findings have also been reported following prolonged morphine delivery, which has been shown to produce corresponding increases in the production and release of excitatory peptides (Calcitonin-Gene Related Peptide (CGRP) and SP) within the DRG (Ma, Zheng, Kar, & Quirion, 2000; Gardell et al., 2002; King et al., 2005), as well as elevated c-fos expression in the dorsal horns of the spinal cord (Rohde, Detweiler, & Basbaum, 1996; Rohde, Detweiler, & Basbaum, 1997; Li & Clark, 2002). Nevertheless, because many of the CNS projections are inhibitory, elevated CNS neuronal firing rates may in fact promote stronger feedback inhibition and antinociception, not hyperalgesia (Sandkuhler & Ruscheweyh, 2005).

While mechanisms involving sensitization and central disinhibition are likely to be involved in OIH, the evidence to date is somewhat inconclusive and demands further clarification. Some studies have pointed to the "wind-up" response of wide-dynamic range neurons as a possible indication of peripheral sensitization (Hanai, 1998; Staud, Vierck, Robinson, & Price, 2005; Cata et al., 2006). This reaction is characterized by an

enhanced firing rate in nociceptive neurons following electrical stimulation. These heightened response patterns lead to increased activation of neuronal populations in lamina I projection neurons which are NMDA dependent, and highly sensitive to SP (Ikeda, Heinke, Ruscheweyh, & Sandkuhler, 2003). Although no direct evidence has yet been presented linking windup responses to opioid use *in vivo*, it has been proposed that they may contribute to the hyper-responsiveness to sensory stimuli seen in OIH and other forms of hyperalgesia (Herrero et al., 2000; Melzack, Coderre, Katz, & Vaccarino, 2001; Schulte, Sollevi, & Segerdahl, 2004). However, these windup responses are not unique to nociceptive neurons, as they are a common feature of many sensory neurons to electrical stimulation (Woolf, 1996). Furthermore, as C-fibers generally discharge at relatively low frequencies (Lewin, Lu, & Park, 2004), the physiological relevance of these enhanced firing rates have yet to be determined.

Proponents of a central disinhibition theory of OIH primarily focus on the inhibitory neurotransmitter γ-aminobutyric acid (GABA), and its relation to opioid activation and nociceptive functioning. Supraspinal GABA's inhibition of ascending dorsal horn nociceptive inputs has been repeatedly shown to be 1 of the primary pathways involved in centralized pain inhibition (Hara et al., 1999; Mao, 2002; Wollemann & Benyhe, 2004; Ammon-Treiber & Hollt, 2005; Chen, Chen, & Pan, 2005; Dunbar, Karamian, Yeatman, & Zhang, 2006). According to this view, disruptions in the GABA delivery system remove its dampening effect on nociceptive neurons, resulting in heightened excitation and hyperalgesia. Indeed, many pronociceptive substances have been demonstrated to elicit their excitatory effects through this system of disinhibition (Fields et al., 1991;

Moran & Smith, 2002). There have also been reports of pharmacological blockade of spinal GABAergic neurons leading to severe hyperalgesia and allodynia (Dickenson et al., 1997). In addition, reductions of GABA inhibition in the spinal dorsal horn have been shown during hyperalgesic states following inflammation or nerve ligation (Chen & Huang, 1992; Coull et al., 2003).

While GABA clearly plays a role in pain expression, establishing a direct link between GABA dysfunction and OIH in particular is not possible at this time. Spinal (Hara et al., 1999) and supraspinal (Ammon-Treiber & Hollt, 2005; Dunbar et al., 2006) GABA-opioid interactions have been implicated in the elicitation of heightened pain states following various paradigms including those featuring tolerance, withdrawal, or inflammation (Gustorff et al., 2003; Hack, Vaughan, & Christie, 2003; Bagley, Gerke, Vaughan, Hack, & Christie, 2005; Dunbar et al., 2006; Maeda, Lisi, Vance, & Sluka, 2007). Thus physiological changes in the GABA pathway such as down-regulation of GABA-ergic receptor subunits (Ammon-Treiber & Hollt, 2005; Chen et al., 2005) or elevated glutamate release (Ibuki, Marsala, Masuyama, & Yaksh, 2003), may indeed be modulating nociceptive processes, but cannot be linked definitively to OIH.

More recently, a group of neurons have been identified in lamina I of the dorsal horn that is necessary for the development of thermal hyperalgesia in neuropathic pain states (Nichols et al., 1999). These neurons are rich in the NK1 receptor (which binds to SP) and project primarily to supraspinal sites. Repeated electrical stimulation of dorsal root afferents has been shown to increase the synaptic strength (LTP) between primary

afferent C-Fibers and these projection neurons, but not with other neurons in lamina I (Ikeda et al., 2003). This LTP induction requires co-activation of NMDA receptors and is seen by some as the primary cellular mechanism underlying central sensitization in hyperalgesic states (Sandkuhler & Ruscheweyh, 2005). There has been much written about the role of NMDA receptor activity in OIH and the next section will summarize some of the most recent findings.

iii. NMDA receptor involvement in OIH

N-methyl-D-aspartate (NMDA) receptors have been shown to play a pivotal role in the development and maintenance of centralized hyperactive states underlying behavioral manifestation of hyperalgesia, allodynia, and spontaneous pain (Celerier et al., 1999; Holtman, Jr., Jing, & Wala, 2003; Jorum, Warncke, & Stubhaug, 2003; Raghavendra, Tanga, & DeLeo, 2004). It has been reported that drug-naïve animals will exhibit hyperalgesic behaviors in response to spinally administered glutamate or NMDA agonists (Mao et al., 1995). Conversely, administration of various NMDA antagonists has been shown to prevent or reduce opioid induced hyperalgesia in both animals and humans (Bartlett, Cramond, & Smith, 1994a; Elliott et al., 1995; Celerier et al., 1999; Holtman, Jr. et al., 2003; Raghavendra et al., 2004).

Investigations into the intracellular mechanisms underlying this heightened nociceptive sensitivity have focused on the role of protein kinase C (PKC) in modulating NMDA receptor functioning. *In-vitro* studies have demonstrated that mu receptor agonists

increase the NMDA-mediated glutamate response via a PKC facilitated removal of the magnesium blockade (Chen & Huang, 1992). This magnesium removal enhances the reactivity of the receptor, leading to subsequent increases in intracellular Ca^{2+} concentration which further stimulate PKC activity, completing a positive feedback loop with the net result of improved synaptic efficiency and NMDA reactivity (Mao et al., 1994; Willis, 2001; Chaban et al., 2004). Further confirmation for the prominence of PKC has come from studies demonstrating correlations between increased spinal cord PKC activity and heightened morphine tolerance (Mao et al., 1995) and reports of attenuated morphine tolerance in mice lacking the PKC gamma gene (Zeitz, Malmberg, Gilbert, & Basbaum, 2001), or in normal mice following administration of PKC translocation blockers (Mayer et al., 1995), or PKC antagonists (Javed, Dewey, Smith, & Smith, 2004).

Although it's clear that NMDA receptors play an important role in OIH, the locations and ligands of these opioid-NMDA interactions have yet to be clearly identified. Some have proposed a critical role for supraspinal NMDA activity, since blockade of medullary NMDA receptors has been shown to attenuate both somatic and visceral hyperalgesia (Urban & Gebhart, 1999). Similarly, anatomical studies involving bilateral lesions or lidocaine injections into the RVM resulted in attenuation of OIH and restored previously masked morphine analgesia (Vanderah et al., 2001a; Vanderah et al., 2001b). However, spinal contributions are also significant as intrathecal coadministration of MK-801(a potent NMDA antagonist) with morphine effectively prevented the elicitation of OIH (Mao et al., 1994). Furthermore, animals receiving morphine systemically showed a

reversal of OIH and a reinstatement of analgesia following subcutaneous administration of Mk-801. Thus while there is evidence suggesting important roles for both spinal and supraspinal NMDA receptors in the modulation of opioid-induced hyperalgesic states, the precise pathway by which opioid receptor agonists and their derivatives interact with NMDA receptors still remains to be discovered.

iv. pronociceptive opioid metabolites

Ever since the World Health Organization's 1986 recommendation of morphine as thedrug of choice for the treatment of moderate to sever cancer pain, there has been an exponential increase in global morphine consumption leading to more prescriptions being filled, and increasingly larger doses being prescribed (Hemstapat, Monteith, Smith, & Smith, 2003). However this has also led to more frequent reports of debilitating neuroexcitatory effects including allodynia, myoclonus and seizure activity (Smith, 2000). While the underlying mechanisms for these negative sequelae are not yet known, there is much indirect evidence implicating the 3-glucoronide opioid metabolites. For instance, although high doses of morphine have occasionally been shown to initiate some excitatory effects (LaBella et al., 1979), systemic M3G administration has proven it to be at least 10-fold more potent as a neuro-excitant (Bartlett et al., 1994b), eliciting a wide range of negative sequelae including myoclonus, allodynia, seizures and hyperalgesia (Bartlett et al., 1994a; Wright, Mather, & Smith, 2001). Moreover, cancer patients experiencing adverse reactions to chronic morphine administration (oral and subcutaneous) have been shown to possess elevated M3G:morphine ratios ranging from

3:1 to 10:1 (Wolff, Samuelsson, & Hedner, 1996; Smith, Wright, Williams, Stuart, & Cramond, 1999). Interestingly, i.c.v. morphine administration rarely produces neuroexcitatory effects in humans, and has been shown to elicit 50-fold increases in morphine CSF concentration and significantly lower ventricular M3G levels (Wolff et al., 1996). Thus, preliminary evidence seems to suggest that morphine's primary metabolite is relatively devoid of analgesic activity and functions in opposition to its parent compound, frequently producing neuroexcitatory effects.

While the M3G binding sites have yet to be identified, there is some indications that it may be acting through the NMDA receptor (Bartlett et al., 1994a). This is consistent with multiple reports of both *in vitro* and *in vivo* M3G activity being relatively unaffected by naloxone (Halliday, Bartlett, Colditz, & Smith, 1999; Moran & Smith, 2002; Hemstapat et al., 2003), further confirming its non-opioidergic properties. Likewise, cultured hippocampal neurons lose their M3G reactivity when treated with glutamate antagonists (a critical component of NMDA receptor functioning) with activation being reinstated upon removal of the antagonist (Hemstapat et al., 2003), lending credence to possible M3G - NMDA collusion. By administering a series of antagonists, Hemstapat was able to further demonstrate that many of the excitatory effects of M3G involve indirect activation of NMDA and AMPA/kainate receptor complexes, but was unable to identify the neurotransmitter(s) involved in mediating this process (Hemstapat et al., 2003).

E. Sex Differences

i. ovarian hormones

The testes and ovaries are the primary sites for the production of gonadal steroids. Androgens including testosterone and dihydrotestosterone are the principal products of the testes. In contrast, two types of steroid hormones are produced in the ovaries: estrogens (e.g., estradiol, estriol, estrone) and progestins (e.g., progesterone). In addition to being produced by the testes, androgens are also manufactured by the adrenal cortex and are released in response to adrenocorticotropic hormone. However, the two systems are not exclusive. The ovaries also make testosterone since testosterone is a precursor to estradiol. Likewise, since estradiol is also a metabolite of testosterone, the testes also end up producing some estrogens.

The process by which estradiol is converted to testosterone is called aromatization and is greatly facilitated by the enzyme aromatase. This means that any tissues containing aromatase can convert testosterone to estrogen and thus make use of estrogen through estrogen receptors. For example, over 50% of the estrogen in women is produced by transformation in the liver, kidneys, bowel, lungs, adipose tissue, and CNS (Brody et al., 1987). Following menopause, ovarian production of estrogens dramatically decreases, with responsibility shifting to the adrenal cortex via aromatization of androgens to estradiol, which is stored in peripheral fat tissue.

While estrogens and androgens are present in both sexes, relatively few studies have reported testosterone and estradiol blood concentrations in experimental subjects at the time of testing. However such investigation can often yield surprising results. For example, in a series of studies reported by Aloisi *et al.* (Aloisi & Ceccarelli, 2000; Aloisi, Ceccarelli, & Fiorenzani, 2003) it was shown that plasma estrogen levels are quite high in Wistar male rats and increase after gonadectomy, and that plasma estradiol levels in male Sprague–Dawley rats are as high as in females in diestrous. Moreover, they also showed that circulating hormonal levels are not just static, but increases in testosterone and estradiol plasma levels were demonstrated in both sexes following noxious stimulation.

For decades, estrogen was only thought of as a "sex hormone", playing a fundamental role in regulating some behavioral and physiological effects, but with little effect on the CNS (Li & Shen, 2005). However, more recent studies have shown that estrogen also has significant distribution in brain regions such as the hypothalamus, hippocampus and cerebellum (Malyala, Zhang, Bryant, Kelly, & Ronnekleiv, 2007; Belcher, 2007; Hajszan, Milner, & Leranth, 2007). In fact, in both males and females, estrogen is produced locally in neural tissue from precursor androgens (Labrie, Belanger, Cusan, Gomez, & Candas, 1997). Within the CNS, estrogen has been linked to a variety of functions including: dendritic and axonal growth and differentiation in the developing brain, modulating neurotransmitter production and release, dendritic arborization, and synaptogenesis (MacLusky & Naftolin, 1981; Toran-Allerand, 1996). Estrogen also appears to confer a neuroprotective effect, as estrogen depletion caused by ovariectomy or natural menopause caused declines in declarative memory and motor coordination, an

effect that was prevented by estrogen replacement therapy (Kimura, 1992; Sherwin & Tulandi, 1996). This neuroprotective effect was also demonstrated on a cellular level against cytotoxicity caused by glutamate, free radicals, and beta amyloid protein (Singer, Rogers, Strickland, & Dorsa, 1996; Behl et al., 1997; Xu et al., 1998).

ii. sex differences in nociception

There is a rapidly expanding body of literature demonstrating evidence for gender differences in pain and its relief (Berkley, Zalcman, & Simon, 2006). Although there is a great degree of variation between studies, there seems to growing support for the notion that the burden of pain is often greater and more variable in women than men (Riley, III, Robinson, Wise, Myers, & Fillingim, 1998; Sarton et al., 2000; Fillingim & Ness, 2000). In addition, some painful disorders such as temporomandibular disorder (TMD) and fibromyalgia manifest quite differently in women and men (Staud et al., 2005; Cairns, 2007), possibly reflecting gender-specific physiological mechanisms of nociception. It is thus not surprising that significant sex differences have also been demonstrated in response to pharmacological treatments (Fillingim & Maixner, 1996; Kest, Sarton, & Dahan, 2000c; Craft, Mogil, & Aloisi, 2004). Although relatively few studies thus far have examined sex differences in morphine induced hyperalgesia, there is a great deal of literature discussing sex differences in nociception and analgesia.

It has long been assumed that men and women differ in their sensitivity and thresholds for pain. Indeed, many studies have shown that gender can be an important factor

affecting both pain perception and the efficacy of pain cessation therapies (Arjune & Bodnar, 1989; Unruh, 1996; Riley, III et al., 1998) For example, there have been reports of gender differences in basal pain thresholds among healthy human volunteers (Fillingim & Maixner, 1996) gender differences in response to opioid analgesics (Kest et al., 2000c), and differential gender distributions in perceived pain (i.e. men > women, men = women, women > men) between clinical conditions (Unruh, 1996). These types of findings highlight the importance of understanding the role of gender in mediating nociception, specifically in regards to its liability for opioid induced hyperalgesia.

There have been numerous studies indicating gender differences in pain perception among healthy humans. Some of these reports described heightened nociceptive sensitivity in females (i.e. lower pain thresholds) relative to males on measures of electrical stimulation of the peroneal nerve (Mylius, Kunz, Schepelmann, & Lautenbacher, 2005), or to repeated tactile stimulation (Bragdon et al., 2002; Chang et al., 2006). Yet others failed to show differences in basal sensitivities, but reported that relative to healthy males, healthy females show a greater proportion of cerebral deactivation in response to mild foot stimulation or rectal distension (Berman et al., 2006; Moulton, Keaser, Gullapalli, Maitra, & Greenspan, 2006). These authors posited that the activation differences observed on fMRI may be reflecting higher basal cerebral blood flow volumes in females which make mild attenuations in signal intensity more apparent. Having greater cerebral blood flow rates may also predispose females to be more sensitive to minor alterations in blood chemistry following a noxious event, although this assumption has yet to be confirmed. Even in studies where basal thresholds are

equivalent, females may be more prone to central sensitization under certain experimental conditions than males (Bragdon et al., 2002; Staud, Robinson, Vierck, Jr., & Price, 2003; Chang et al., 2006).

However, the human sex difference literature is confusing, as many studies report conflicting results, and often utilize different experimental paradigms. When sex differences have been reported, they frequently show that females exhibit lower nociceptive thresholds to identify a stimulus as painful, are less tolerant to continued exposure to noxious stimuli, may report higher ratings of intensity for a given painful stimulus, and show greater variability in their response to therapeutic interventions (Bodnar, Romero, & Kramer, 1988; Berkley et al., 2006). Despite their prevalence, sex differences are often small and inconsistently observed, and are subject to influence by age, hormonal level, nutrition, and cultural factors (Berkley, 1997).

The mechanisms underlying sex differences in nociception are poorly understood. Investigations into the density of opioid receptors in the rat brain did not reveal any sex differences in μ or δ opioid receptor populations (Kepler, Kest, Kiefel, Cooper, & Bodnar, 1989). Similar investigations in mice yielded conflicting results, as one study failed to find any difference (Candido et al., 1992), while a second study reported increased levels in males (Mogil et al., 1994).

iii. modulatory effects of gonadal hormones on nociception

A variety of mechanisms have been suggested to account for sex differences in nociception. In humans, numerous psychosocial factors such as gender role (Dubreuil D.L. & Kohn P.M., 1986; Levine & De Simone, 1991), anxiety (Keogh, Hamid, Hamid, & Ellery, 2004; Elklit & Jones, 2006) and beliefs regarding one's ability to tolerate pain (Fillingim, Keefe, Light, Booker, & Maixner, 1996), have all been identified as possible factors accounting for enhanced nociceptive sensitivity in females. Others have pointed to more biological factors including blood pressure (Bragdon, Light, Girdler, & Maixner, 1997; Myers, Robinson, Riley, III, & Sheffield, 2001; Campbell, Hughes, Girdler, Maixner, & Sherwood, 2004), and genetic background (Mogil, 1999; Kim et al., 2004). However, the most fundamental and frequently cited distinction between males and females in nociception and analgesia involves the contributions of gonadal hormones (Kepler et al., 1989; Aloisi & Ceccarelli, 2000; Stoffel, Ulibarri, & Craft, 2003; Craft et al., 2004).

It has been proposed that sexually dimorphic organizational and/or activational effects of gonadal hormones may be responsible for sex differences in nociception and drug pharmacokinetics (Kest, Palmese, & Hopkins, 2000b; Craft, 2003; Cairns, 2007). Indeed, there have been many reports of changes in behavioral responses of animals and humans to noxious stimuli at different points in the estrous cycle (Bradshaw, Rimmerman, Krey, & Walker, 2006; Puri et al., 2006; Bernal, Morgan, & Craft, 2007). Likewise, many investigators have shown that artificial manipulation of gonadal hormones through

ovariectomy, gonadectomy, or hormone replacement therapies can lead to significant changes in nociceptive thresholds and pharmacological sensitivities (Claiborne, Nag, & Mokha, 2006; Nag & Mokha, 2006; Kuba et al., 2006). However, Fillingim and colleagues (Fillingim & Ness, 2000), reviewed more than 50 such studies and noted that the results are often inconsistent, perhaps due to methodological differences including: pain modality (thermal, electrical, visceral), the nature of the pain response used as a dependent measure (reflex, behavioral excitation, vocalization, rating scale, amount of self-administered analgesic), as well as the duration of noxious stimulus exposure. Nevertheless, they report that the general trend within the literature seems to suggest that elevated estrogen levels are associated with enhanced responses to painful stimuli and reduced analgesic responses to stress and pharmacological agents.

Although the mechanisms underlying gonadal hormone modulation of nociception still remain at large, there have been a number of studies suggesting it involves afferent nerve sensitization. More than 70 years ago (Herren, 1933), it was reported that differences in 2-point discrimination and basal pain thresholds correlated with changes in the human menstrual cycle. Likewise, estrogen treatments in animals increase the receptive fields of trigeminal (Bereiter, Stanford, & Barker, 1980) and pudendal (Komisaruk, Adler, & Hutchison, 1972) nerves. Changes in the sensitivity of visceral and somatic afferents to locally administered anesthetics have also been shown to be associated with hormonal elevations during pregnancy (Butterworth, Walker, & Lysak, 1990; Douglas & Bicknell, 1993; Beric, 1994; Eogan, O'Brien, Carolan, Fynes, & O'Herlihy, 2004).

In addition to direct activation, gonadal hormones also play a role in peripheral sensitization by altering the response properties of "silent" afferents that are not involved directly in the initial transduction process. Rather, they are activated during sensitizing processes such as inflammation, ischemia, or sympathetic activation. For example, it has been shown that afferents arising from the uterus that are normally insensitive to mechanical stimulation, demonstrate increased firing rates in response to mechanical stimulation during peak phases of the estrous cycle (Akaishi, Robbins, Sakuma, & Sato, 1988; Robbins, Sato, Hotta, & Berkley, 1990; Berkley, Robbins, & Sato, 1993).

Gonadal hormones have also been shown to influence CNS nociceptive pathways. For instance, changes in sex hormones have been shown to alter production rates of substance P (Debeljuk, Villanua, & Bartke, 1992; Duval, Lenoir, Moussaoui, Garret, & Kerdelhue, 1996; Gautreau, Duval, & Kerdelhue, 1997), GABA (Gimeno, Fernandez-Pardal, Viggiano, Pezzot, & Gimeno, 1986; Wilson, 1992; Blurton-Jones & Tuszynski, 2006), glutamate (Kumar et al., 2005), as well as other neurotransmitters (Smith, 1994). There are also reciprocal interactions between gonadal hormones and endogenous opioid activity. For example, luteinizing hormone (LH), a substance that is highly regulated by the sex hormones estrogen and progesterone, has been shown to attenuate opioid receptor activation induced inhibition of cAMP in the brain (Rapkin et al., 1996; Ratka & Simpkins, 1997). This opioid-estrogen connection has also been demonstrated through neuroanatomical studies showing colocalization of endogenous opioid peptides and estrogen in hypothalamic nuclei (Morrell, McGinty, & Pfaff, 1985), and estrogen mediated increases in μ receptor mRNA levels in the ventromedial hypothalamus

(Quinones-Jenab, Jenab, Ogawa, Inturrisi, & Pfaff, 1997). These studies suggest possible sites for hormone-nociceptive interactions, through which gonadal hormones could affect basal nociceptive thresholds either by altering endogenous pain suppression and or by mediating responses to pharmacological agents through changes in opioid receptor density and/or sensitivity.

iv. sex differences in opioid modulation of nociception

Numerous animal studies have demonstrated sex differences in responses to opioid drugs. However the findings from these reports offer some barriers to interpretation due to wide variations in experimental conditions such as: nociceptive assays, animal species/strains, pharmacological agents, injections sites, injection volumes, and time course. For example, it has been demonstrated that central administration of morphine into the RVM (Boyer, Morgan, & Craft, 1998), PAG (Krzanowska & Bodnar, 1999), or lateral ventricles (Kepler et al., 1989), produces greater analgesia in males than females. Yet it has also been reported that administration of identical doses of a κ opioid receptor agonist into the RVM of male and female rats elicits analgesia in females but opposes analgesia in males (Tershner, Mitchell, & Fields, 2000). Meanwhile, intracerebroventricularly (icv) administered DAMGO, a highly selective mu agonist, revealed sex differences in analgesia on the tail-flick test (males > females), but no differences on shock-jump tests (Kepler et al., 1991).

To further complicate matters, even within the same experimental paradigm, sex differences often vary as a function of drug receptor type. In a recent study of mechanical nociception (Barrett, Smith, & Picker, 2002), male rats were shown to have higher initial nociceptive thresholds (they could tolerate longer periods of stimulation before limb withdrawal) and demonstrated greater analgesic responses consistently across a broad spectrum of μ receptor agonists. However when using this same paradigm with a wide range of κ receptor agonists, sex differences were not always apparent. Even with agonists that show strong affinities for only 1 particular receptor subtype (i.e. morphine, DAMGO, and fentanyl that are all μ selective) sex differences are inconsistent (Mogil, Chesler, Wilson, Juraska, & Sternberg, 2000; Kest et al., 2000b). Studies such as these highlight some of the idiosyncrasies of the sex difference literature, that even when present, sex differences are often specific to the nociceptive modality and pharmacological agent under study. For example, a meta-analysis of sex-differences in the in the rodent literature found that both the direction of the sex difference (i.e. male > female vs. female > male) and the magnitude were highly variable, strikingly dependent upon genotype and the nociceptive assay employed (Mogil et al., 2000). In their paper, they reviewed the results of 23 studies employing rodent tail withdrawal from a noxious thermal stimulus as the dependent measure. 12 studies failed to find any significant sex difference, 8 studies reported increased sensitivity in males (in rats), while 2 reported increased sensitivity in females (in mice).

Methodological differences notwithstanding, the majority of animal studies investigating sex differences in morphine analgesia have reported that males typically show a greater

analgesic response to morphine than females following systemic (Mogil et al., 2000; Kest et al., 2000c; Baker & Ratka, 2002) and central routes of administration (Kepler et al., 1989; Kepler et al., 1991; Krzanowska & Bodnar, 1999). This heightened sensitivity has been manifested as either enhanced analgesia (i.e. relative increases in nociceptive response latencies) or increased potencies (i.e. lower ED_{50} values) across a wide range of nociceptive modalities including thermal (Kepler et al., 1989; Mao et al., 1995; Cicero, Nock, & Meyer, 1997), chemical (Berkley et al., 2006), mechanical (Barrett et al., 2002), somatic (Berkley, 1997), and visceral (Cicero, Nock, & Meyer, 1996) assays.

F. Rationale

Given the prominence of morphine and other opioids in the clinical treatment of pain, identifying the physiological process underlying opioid induced hyperalgesia and other unintended sequelae of prolonged opioid exposure remains an important area of study. The goal of this dissertation is to highlight the mechanisms underlying OIH, specifically in regards to the relative contributions of opioid receptor activity and gonadal hormones. The next section will provide some general background regarding the rationale for the experimental methods chosen, followed by a description of the specific studies conducted and hypotheses regarding expected findings.

Issues and Decisions Regarding the Use of Morphine and oxymorphone

Opioids are widely used analgesics, and to date, morphine is still the most highly efficacious and widely used treatment for moderate to severe pain (Inturrisi, 2002). It is for this reason that morphine was chosen for the bulk of the experiments described in this thesis. For Specific Aim 2, the nociceptive qualities of oxymorphone were also assessed due to recent reports of oxymorphone induced hyperalgesia that was opioid receptor dependent (Gardell et al., 2006). Although much of the currently available literature regarding OIH contains descriptions of studies with acute morphine dosing, differences in effects during continuous opioid administration may be more relevant clinically, as opioids such as morphine and oxymorphone are often administered to alleviate chronic pain. Obtaining an understanding of the hyperalgesic liabilities during continuous

morphine exposure can thus provide a substantial and important scientific contribution to the field.

Issues and Decisions Regarding the Use of Mice

Hyperalgesia, like many other behaviors, is difficult to study in humans, because of the likely contribution of multiple genetic and environmental factors. Animal models possess an obvious advantage in this respect, for they allow for much greater control of relevant variables (i.e. age, genotype, prior drug exposure, cultural issues…). Using intact animals that have not been subject to prior surgical alterations or inflammatory agents also precludes the possibility of chronic pain leading to alterations in nociceptive processing. Animal studies also control for random pain escalation, by allowing daily comparisons to premorbid basal nociceptive thresholds, a process that would be difficult to conduct in humans.

For this dissertation, mice were chosen as subjects because of the large body of literature documenting pain related traits in the mouse, the high degree of genetic homology with human beings, and the ready availability of inbred mouse strains. Although inbred strains were not used in this study to enhance generalization, they are extremely useful for QTL analyses and other forms of genetic identification paradigms that could not feasibly be conducted with outbred strains due to excessive variability. Finally, post-mortem analysis allows for more precise measurements of nociceptive physiology than would be feasible in humans. As such, using mice for the current series of studies not

only provides for a better understanding of contributory factors than would be possible in humans, but also more readily allows for ongoing research through follow-up studies.

Issues and Decisions Regarding Route of Drug Administration

The studies described in this dissertation primarily featured systemically administered morphine through either subcutaneous injections or continuous infusion paradigms. Systemic administration results in widespread distribution along the neuraxis and periphery, leading to activation at spinal, supraspinal and peripheral levels. As opposed to more focal administration paradigms (i.e. intrathecal or icv), systemic exposure allows for natural distribution gradients throughout the organism. Although systemic exposure provides less anatomical localization than other methods, the resulting data is the most clinically relevant, as systemic administration is the most common opioid delivery route in humans.

Issues and Decisions Regarding Assessment of Nociception

In order to study hyperalgesia, tolerance, and analgesia, decisions had to be made regarding whether to make assessments within each animal (experimental values versus pretreatment) as opposed to between subject data (non treated controls versus experimental subjects). Although within subject comparisons have more statistical power, many of the manipulations featured in this dissertation (i.e. ovariectomy, morphine infusion) would confound future comparisons if subjected to repeated measure designs. Thus the proposed sets of studies described within this dissertation will be

comprised of a mixture of these paradigms, as necessitated by the large number of issues that need to considered to minimize potential confounds.

Multiple assays have been developed for use with both animals and humans to aid in the assessment of nociception. These measures typically employ vastly different techniques and tap into distinct modalities such as thermal pain, mechanical pressure, noxious chemicals or nerve injury. Due to the wide variety of available measures, the fundamental relationship between disparate noxious stimuli must be clearly understood. For example is abdominal constriction pain more similar to inflammatory pain or mechanical pressure? Are they mediated by similar physiological mechanisms and thus subject to similar intervention, or do they represent different systems that function independently?

This critical question was addressed by Mogil and colleagues (1999), who used multivariate analysis of the responses of 11 inbred mouse strains on 12 common measures of nociception to identify 3 clusters of pain tests which seem to share common genetic substrates and presumably underlying physiology (Mogil et al., 1999a; Mogil et al., 1999b). The 3 clusters were: 'thermal nociception' (Hargreaves' test, hotplate test, tail-immersion withdrawal test, and autotomy), ''chemical nociception'' (acetic acid abdominal constriction, magnesium sulfate abdominal constriction, and both the acute- and tonic-phases of the formalin test), and ''mechanical hypersensitivity'' (von Frey test, carrageenan thermal hypersensitivity, peripheral nerve injury, and mechanical hypersensitivity). His findings argue for a multiaxial approach to the study of pain,

whereby stimulus modality and genetic background should play primary roles, while other factors such as the site or duration of noxious stimuli, neuropathy, or inflammation seem to be of limited clinical relevance.

Studies of nociception are further limited by the specificity of the nociceptive process under investigation. For example, some of the most common techniques for studying pain sensitivity include measures of behavioral changes following exposure to high temperatures, nerve ligation, mechanical compression, or inflammation. Not only do these measures differ in modality, but they also show little commonality in their response to pharmacologic intervention (Lai, Ossipov, Vanderah, Malan, Jr., & Porreca, 2001) and may even be modulated by different genes (Mogil, 1999). Thus, pharmacologic interventions that lead to an organism becoming highly sensitive to 1 measure of thermal pain for example, may have little bearing on its responses to noxious mechanical or inflammatory stimuli. This makes comparisons between studies especially difficult, as it demands congruence of both pharmacological agents and the nociceptive modality. To address these concerns, nearly all of the studies within this dissertation utilized the same pharmacological substance (morphine) and nociceptive assay (warm water tail withdrawal).

The tail withdrawal assay: For the experiments discussed in this dissertation, latency of tail-withdrawal from a water bath was used as the dependent measure to indicate nociceptive sensitivity. The tail withdrawal assay is a well-known measure of nociceptive sensitivity based on reflexive limb withdrawal from a noxious stimulus.

Although first described using rats exposed to a focused light beam (D'Amour & Smith, 1941), various modifications of this procedure have been utilized over the years to objectively assess animal's sensitivity to noxious stimulation. A modified version of the test which uses warm water (Jannssen, Niemegeers, & Dony, 1963) was chosen because it is minimally invasive, and has been shown to produce stable baselines even after repeated assessments (Wilson & Mogil, 2001).

The basic mechanism involves the heat of a hot-water bath activating nociceptors in the distal half of the animal's tail, which transduce the impending damage into a train of action potentials which are transmitted along the axons of the nociceptors to cell bodies located in the dorsal root ganglion (Yeomans & Proudfit, 1996). Neurons within the DRG synapse in the dorsal horn of the spinal cord on local interneurons and on projection neurons which send afferents primarily to the brain stem, thalamus and hypothalamus (Hanai, 1998). Local neurons in the dorsal horn process incoming nociceptive impulses leading to activation of the autonomic nervous system and motor neurons mediating local withdrawal reflexes (Chen et al., 2005). Regulation of the tail withdrawal can therefore occur via modulation of either peripheral or central mechanisms (McCormack, Prather, & Chapleo, 1998).

Issues Regarding Hyperalgesia and Morphine Tolerance

Based on the background information provided, it is clear that morphine induced hyperalgesia is an increasingly prevalent phenomenon with significant clinical implications. However, many of the studies exploring the relationship between opioids

and nociception often fail to account for the effects of opioid tolerance on pain perception. Tolerance is a functional depiction, used to describe the situation wherein administration of a previously effective drug dose now produces a diminished effect. While the mechanisms underlying this potency reduction may be quite complex, the determination of tolerance is based on the rather simple observation of reduced drug efficacy. Thus, by definition, tolerance is nonspecific, as it is merely a description of an observable phenomenon without regard to cause. This severely limits its utility as a diagnostic feature, as a multitude of altogether different mechanisms can give rise to the same functional tolerance.

For example, in a clinical population, when a patient requires increasingly higher doses of morphine to achieve the same initial analgesic effect, the patient is said to be experiencing drug tolerance. However, this 'tolerance' may be due to any, or some combination, of the following possibilities: 1) a worsening of the patient's injury or disease progression leading to increased pain, thus requiring more morphine; 2) an endogenously activated system opposing morphine analgesia, thus requiring larger doses for effective pain suppression (Mao et al., 1994; Larcher et al., 1998); 3) a loss in morphine's analgesic potency, caused by downregulation, internalization, or alterations in the binding properties of the opioid receptors, within the context of unaltered basal pain (Bernstein & Welch, 1998; Borgland, 2001; Kieffer & Evans, 2002); 4) a progressive enhancement in the patient's basal pain thresholds (hyperalgesia) in the presence of preserved analgesic potency (Laulin et al., 1999; Crain & Shen, 2000; Gardell et al., 2006). Hence, even seemingly identical cases of functional tolerance may be mediated

through divergent physiological mechanisms. It is rather surprising that so many researchers have taken tolerance at 'face value' attributing it to reductions in opioid receptor sensitivity or availability (Bernstein & Welch, 1998; Borgland, 2001; Stafford, Gomes, Shen, & Yoburn, 2001; Vanderah et al., 2001a; Kieffer & Evans, 2002) as opposed to more dynamic processes.

Opioid Induced Hyperalgesia vs. Withdrawal Induced Hyperalgesia

Studies of opioid induced nociception must also be careful to distinguish OIH from withdrawal induced hyperalgesia (WIH). WIH is a well documented phenomenon involving nociceptive hypersensitivity in opioid dependent organisms following cessation of opioid treatment, or administration of opioid antagonists. It has been studied for decades and has often been interpreted as an indication of opioid dependence severity (Kayan, Woods, & Mitchell, 1971; Tilson et al., 1973; VonVoigtlander & Lewis, 1983; Bederson, Fields, & Barbaro, 1990; Gutstein, 1996; Compton, Athanasos, & Elashoff, 2003; Angst et al., 2003). However, unlike manifestations of OIH which tend to be limited to exaggerated nociceptive responses, WIH is associated with a host of more generalized responses including temperature dysregulation (Chen, Geller, DeRiel, Liu-Chen, & Adler, 1996; Pinelli, Trivulzio, & Spezia, 1998; Houshyar, Cooper, & Woods, 2001), and appetitive changes (Schoenbaum, Martin, & Roane, 1989; Kanof, Aronson, & Ness, 1993; Pinkofsky et al., 2005).

Although the mechanisms underlying OIH and WIH are not fully understood, it is clear from the literature that they represent physiologically divergent processes and are subject

to different manipulations (Harris, Hanes, & Gewirtz, 2004; Dunbar, Karamian, & Zhang, 2006). For example, many of the investigations into WIH have employed naloxone, a broad-based opioid antagonist that shows a high affinity for all 3 receptor subtypes and can induce immediate withdrawal behaviors. Following treatment with opiates such as morphine or alfentanil, naloxone administration produces significant increases in nociceptive-related slow ventral root potentials above preopiate control values (Feng & Kendig, 1996), and elevates the firing rates of the 'hyperalgesic' on-cells in the RVM relative to pre opiate firing rates (Bederson et al., 1990). Naloxone not only reverses morphine's depressive effects on dorsal horn firing rates, but also increases firing rates above the control level, indicating a hyperresponsiveness of these spinal cord neurons to noxious stimuli (Le, Guilbaud, Jurna, & Besson, 1976). In addition, naloxone administration in non-opioid treated animals produces no discernable nociceptive effects (Kest et al., 2002) thus further suggesting that WIH is strictly an adaptive response to opioid removal. There is thus substantial support for the notion that the nociceptive hypersensitivity associated with WIH is not actively mediated by opioid receptors, but rather is an adaptive response consisting of heightened pain sensitivity following blockade of the opioid receptor system in dependent organisms.

Relatively few studies of OIH have taken WIH into account. Numerous investigations of OIH have been conducted using repeated injection paradigms, whereby basal nociceptive measures are compared to values obtained after an extended period of multiple opioid exposures (Laulin et al., 1999; Yuan, Han, Chang, & Han, 1999; Celerier et al., 2000). Nociceptive hypersensitivity arising from such treatments, may in fact be demonstrating

WIH, not OIH, as the inter-injection interval is sufficient to produce "mini-withdrawal" episodes (Gutstein, 1996), which can elicit hyperalgesia. To properly study OIH, 1 must therefore look at studies employing continuous exposure paradigms which by definition, are immune to Gutstein's criticisms of WIH, and thus provide a better model for studying OIH. It is for this reason that the studies described in this dissertation used morphine infusion pumps, to ensure uninterrupted opioid delivery that would not be subject to spontaneous withdrawal. As withdrawal has also been demonstrated following acute morphine exposure (Yaksh et al., 1986; Kest et al., 2001), there were concerns that WIH may develop on Day 0 if morphine infusion and opioid blockade were initiated simultaneously. Therefore for all studies, NTX pellets were always implanted at least 24 hours before beginning morphine infusion to ensure complete opioid blockade prior to opioid exposure, thereby precluding the possibility of WIH.

Rationale for Specific Aims of the Present Dissertation

As is clear from the literature reviewed above, opioid induced hyperalgesia is a growing clinical problem which seems to be poorly understood. Although it has often been documented in organisms with pre-existing pain conditions, there have been relatively few studies documenting opioid induced hyperalgesia in naïve, healthy subjects, looking at the relative contributions of analgesia, tolerance, opioid receptors, and gonadal hormones. It is hoped that the present dissertation will provide a significant contribution to our understanding of this debilitating phenomenon and lead to insights reducing its occurrence in the future.

The goal of Specific Aim 1 was to determine the relative contributions of analgesia, tolerance, and morphine dose to opioid induced hyperalgesia. In light of the criticism directed at paradigms using repeated injections (Gutstein, 1996), we first set out to establish a paradigm of OIH in naïve CD1 mice using osmotic pumps that maintain a constant infusion rate. This setup also minimizes the handling of animals which has previously been to shown mediate behavioral responses to pain (Nakama-Kitamura, 2002). The tail withdrawal test was thus an ideal measure to use for this study, due to its non-invasive nature, short recovery period, and immunity to practice effects.

Once a reliable model of OIH had been established, dose response studies were conducted at several points throughout a 14 day exposure period, allowing for comparisons of analgesia, tolerance, and hyperalgesia both within and between subjects. As the NMDA receptor system has been implicated in the literature to be mediating hyperalgesia, animals were exposed to small doses of NMDA antagonists during hyperalgesic phases to assess NMDA receptor involvement. Although this paradigm allowed for separation of analgesia and hyperalgesia based on severity, it was insufficient to determine if analgesia and/or opioid receptor activity is necessary for subsequent hyperalgesia. Thus the study was repeated with pre-implantation of naloxone pellets to block any analgesia or opioid receptor activity, and see its effects upon the elicitation of hyperalgesia. Once 2 different hyperalgesic paradigms had been established, cross adaptation studies were conducted to assess if tolerance to the effects of 1 dose would prevent the hyperalgesic expression associated with the other dose.

The final aspect of Specific Aim 1 involved identifying possible non-opioid mechanisms that may be underlying OIH. Although the literature on this topic is relatively sparse, M3G has been identified as a pronociceptive opioid metabolite with little opioid receptor affinity. It was thus hypothesized to be the most likely candidate underlying the hyperalgesic phases that were not affected by opioid receptor blockade. This was addressed directly through cross adaptation comparisons. It was hypothesized that if M3G was indeed responsible for the hyperalgesia, then tolerance to M3G should diminish subsequent hyperalgesia. Likewise, if resolution of hyperalgesia under continuous morphine infusion was presumed to be reflecting tolerance to M3G, then subsequent exposure to M3G should not elicit acute hyperalgesia. Thus, the first cross adaptation experiment involved making animals tolerant to M3G's effects prior to morphine infusion; while the second experiment involved acute M3G exposure following resolution of OIH.

The goal of Specific Aim 2 was to determine the hyperalgesic liabilities of triple opioid receptor knockout mice that are devoid of all 3 opioid receptors. To date there have been no published studies documenting such OIH in animals that are devoid of opioid receptors. This represents a slight refinement over Specific Aim 1, as it could be argued that OIH in the presence of NTX may be resulting from minimal opioid receptor activity. Furthermore, a recent study postulated that the pronociceptive actions of sustained opiate administration requires specific interaction with opiate receptors and is unlikely to be the result of accumulation of metabolic products (Gardell et al., 2006). Thus the knockout animals were subject to similar manipulations as in Specific Aim 1, to

firmly establish the role of opioid receptors in the mediation of hyperalgesic states. Using knockout mice also allowed for exploration of the hypothesis that NMDA antagonism may be reversing hyperalgesia through utilization of opioid antinociceptive circuitry, which never developed in the mutant mice.

Having established a reliable paradigm of opioid induced hyperalgesia, the goal of Specific Aim Three was to determine sex differences in morphine hyperalgesia specifically with regards to effects of gonadal hormones. Although much has been written on the topic of sex differences in nociception and analgesia, there have been relatively few reports of gender contributions to opioid induced hyperalgesia. Thus, female CD1 mice were subjected to similar paradigms that had produced divergent effects in Specific Aims 1 and 2. Following discovery of significant sex differences, follow up studies using mice that had undergone ovariectomy and/or hormone replacement treatments were conducted to assess for activational vs. organizational effects of gonadal hormones.

GENERAL METHODS

Approval

All procedures were approved by the Queens College/City University of New York Institutional Animal Care and Use Committee and conform to guidelines of the International Association for the Study of Pain.

Subjects:

All of the experiments described in this dissertation were performed using mice. Unless otherwise noted, all mice were obtained from either The Jackson Laboratory (Bar Harbor, Maine) or Charles River Laboratories (Kingston, NY). For all experiments, mice were housed 4 or 5 per cage with same strain and sex. They were given free access to food (Purina chow) and water. They were housed in a temperature controlled facility (21° Celsius) maintained on 12 hour light-dark cycle (lights on from 7AM – 7PM).

Unless otherwise noted, all of the mice used in this dissertation were of the CD1 strain. This strain was chosen because it is a standard, wildtype, outbred strain and thus obtained results are likely to be more generalizable to other strains and humans than studies done with inbred strains.

Drugs:

Morphine – an opioid analgesic, was obtained from the National Institute of Drug Abuse (Rockville, MD) and stored by Dr. Benjamin Kest in secure facilities in the College of

Staten Island and in Queens College. Morphine was dissolved in 0.9% physiological saline for all injections and infusion paradigms.

Naltrexone – a broad based opioid antagonist, and naltrexone placebo were obtained from the National Institute of Drug Abuse (Rockville, MD) in the form of 30 mg pellets and stored in a secure facility in Queens College.

MK-801 - a non-competitive NMDA antagonist, was obtained from Sigma-Aldrich (St. Louis, MO) and stored in a secure facility in Queens College

LY235959 - a competitive NMDA antagonist, was obtained from Tocris Bioscience (Ellisville, MO) and stored in a secure facility in Queens College.

Estrodial Benzoate – a synthetic sex hormone, was obtained from was Sigma-Aldrich (St. Louis, MO) and stored in a secure facility in Queens College

Oxymorphone - a synthetic opioid analgesic, was obtained as a gift from Dr. C. E. Inturrisi, a professor at Cornell University and stored in a secured facility at Queens College.

Drug Delivery Mechanisms:

Continuous infusion - Infusion doses are expressed throughout the dissertation as cumulative dose in a 24 h period. Continuous drug or saline (vehicle control) infusion

was achieved using osmotic pumps (Model 2001; Alzet, Mountain View, CA) with a flow rate of 1 μl/h. Under oxygen/isofluorane inhalant anesthesia, pumps were implanted through a small dorsal midline incision and closed with stainless steel surgical staples. To ensure maximum reliability, pumps were replaced every 7 days.

Injection paradigms - Throughout this dissertation, drug injection paradigms involved subcutaneous bolus injections of the chemical substance dissolved in 0.9% saline solution (unless otherwise stated). The volume of drug administered was based on the animal's weight in kilograms, according to the formula of 10ml drug solution per kg of mouse weight.

Pellet Implantation – NTX and placebo pellets were wrapped in a sterile nylon mesh and subcutaneously implanted into the nape of the neck under oxygen/isofluorane inhalant anesthesia, through a small incision and closed with stainless steel surgical staples.

Hormone Replacement Therapy – Following at least a 2 week recovery period post ovariectomy, mice were implanted with sterile silastic tubing containing estradiol benzoate (EB) dissolved in sesame oil (SO) via a small dorsal midline incision that was subsequently sealed with stainless steel surgical staples according to the procedure described by P. E. Cohen (Cohen & Milligan, 1993). This procedure has previously been shown to be an efficacious method of hormonal regulation (Milligan & Finn, 1997), capable of sustaining pregnancy in previously ovariectomized mice.

Surgical Procedures:

Ovariectomy – During surgery, mice subject to ovariectomy (OVX) received a single dorsal midline incision. The fallopian tubes were then exposed and ligated with surgical silk proximal to the ovaries before removing the distal ends (including the ovaries and surrounding ovarian fat). Control mice received sham surgery (SHAM), involving externalization and very gentle manipulation of the ovaries for 20-30 seconds, after which the ovaries were returned. Incisions were closed using surgical silk sutures (3-0) and mice were allowed a 2 week recovery period with unrestricted access to food and water, before being subject to experimental protocols. This is a common surgical procedure and has previously been shown to disrupt estrus cycles and effectively prevent estrogen expression (Cohen & Milligan, 1993).

Nociceptive Assays:

All testing was performed following an acclimation period of at least 1 week to the local vivarium, and was conducted when the mice were between 6 and 9 weeks of age. On the days they were being tested, mice were allowed to acclimate to the testing laboratory for at least 1 hour before any procedures were performed. Unless otherwise noted, all experiments were conducted near mid-photophase to reduce circadian effects on nociception (Kavaliers and Hirst, 1983).

Warm water tail withdrawal:

Studies described in this dissertation were performed with a water temperature of 47.3° C, since in pilot studies baseline latencies of 9–10 seconds were consistently obtained,

thus minimizing the possibility of floor effects during hyperalgesia. Nociception was always assessed prior to any surgical procedure. Animals with tails that were visibly injured or otherwise deformed or diseased were excluded from the study, given their likely effect upon peripheral pain processing in the tail. Likewise, animals that showed obvious signs of disease, motor impairment, or failed to exhibit the tail withdrawal response during baseline behavioral assessments (withdrawal latencies exceeding 30s), were not included for further analysis.

Animals that were going to be subject to this assay were brought into the testing facility at least 1 hour before any testing was performed to afford them time to acclimate to the room conditions. Each mouse was then wrapped snugly in a terry-cloth pouch so that only its tail protruded. The animal was then lowered so that the distal third of its tail was immersed in a water bath maintained at 47.3° Celsius ± 0.2° by an immersion circulator pump (Fisher Isotemp Model 71). Latency between water immersion and reflexive withdrawal of the tail was measured twice to the nearest hundredth of a second, with each determination separated by at least 30 seconds to ensure adequate recovery time between assessments. The 2 measures were then averaged. A 30s cutoff latency was employed to prevent possible tissue damage. Ambient room temperature for all assessments trials was at 22-23 °C, as it has been documented that changes in tail skin temperature can affect tail withdrawal latencies (Tjolsen & Hole, 1993).

Dose–response measures – A method for assessing analgesic potency by means of a dose-response approach (Elliott et al., 1995; Kest et al., 2002). Naïve mice are assayed

for basal withdrawal sensitivity (using the tail-withdrawal method described above) and then, starting with 2 mg/kg, given a series of acute morphine subcutaneous injections of increasing (~0.25 log units) magnitude. Latencies are reassessed 30 min after each injection. This sequence of injection and testing continued until mice were analgesic. This method has previously been demonstrated to be a reliable method of tolerance induction (Kest et al., 2001), as it allows for evaluations of analgesic potency based on the amount of drug needed to meet analgesic criterion. Analgesia was operationally defined as a doubling of each subject's withdrawal latency relative to baseline measures to ensure that the analgesic criterion was consistent despite individual fluctuations in baseline withdrawal latencies. All mice (even those who were already analgesic) received the same cumulative morphine dose by the culmination of the induction period to ensure that the level of drug exposures was consistent among all subjects.

Data analysis

Acute morphine analgesia was expressed as % maximum possible effect (% MPE), a commonly used measure (Nemmani, Grisel, Stowe, Smith-Carliss, & Mogil, 2004), using the formula [post-morphine latency − baseline latency] / [cut-off latency − baseline latency] × 100). These % MPE are then analyzed together with the obtained withdrawal latencies using the BLISS-21 computer program (Umans & Inturrisi, 1981), which considers non-overlapping 95% confidence intervals (CI) as significantly different.

Morphine Tolerance was assessed by comparing ED_{50} potency estimates (effective dose for 50% of the animals to achieve a doubling of the baseline tail withdrawal latencies)

derived from cumulative dose–response curves as previously described (Elliott et al., 1995; Kest et al., 2002). ED_{50} values and associated 95% confidence intervals (CI) were calculated for all dose response data according to the method of (Murray, Gmerek, Cowan, & Tallarida, 1981). ED_{50} values without overlapping CI's were considered significant.

Tail withdrawal Latencies - Withdrawal latencies were analyzed using 1 and 2-way ANOVA's followed by Tukey-Kramer post hoc comparisons. When sex differences in BL values were detected, latencies were presented as difference scores (post-infusion latency – BL latency) to facilitate comparison. For all comparisons, differences that exceeded $\alpha = 0.05$ were considered significant.

SPECIFIC AIM ONE:

Morphine hyperalgesia is unrelated to opioid receptor activity, analgesia or tolerance

INTRODUCTION:

As previously described, morphine analgesic treatment is complicated by the development of unwanted side effects such as hyperalgesia and tolerance. Since both hyperalgesia and tolerance are consequences of morphine administration, it is logical to assume that they are somewhat related. For example, tolerance and opioid-induced hyperalgesia display overlapping neuroanatomical (Vanderah et al., 2001a) and neurochemical (Mao, 2002; Xie et al., 2005) substrates, and are sensitive to similar pharmacological interventions such as NMDA receptor blockade (Elliott et al., 1995; Plesan, Hoffmann, Xu, & Wiesenfeld-Hallin, 1999; Li et al., 2001a). There are even indications of genetic commonality, as the magnitude of morphine analgesic tolerance and hyperalgesia have been shown to be highly correlated in 11 inbred mouse strains (Kest et al., 2002). These associations have prompted some to identify hyperalgesia as a causative factor in morphine analgesic tolerance (Shen & Crain, 2001; Simonnet & Rivat, 2003; Xu et al., 2003; Ossipov et al., 2004), wherein increasing morphine doses are required to maintain previous analgesic states to offset subsequent enhancement of nociceptive sensitivity.

The present study tests several assumptions regarding the relationship between morphine analgesia, hyperalgesia, and tolerance in mice. Whether hyperalgesia is indeed a

causative factor in morphine tolerance was evaluated by comparing analgesic potencies and nociceptive sensitivities at various stages of morphine infusion. The relationship between hyperalgesia and morphine analgesia was also considered by assessing tail withdrawal latencies in mice implanted with NTX pellets (which effectively prevent analgesic expression) prior to morphine infusion. The finding of hyperalgesia which is refractory to analgesic blockade afforded the opportunity to explore the mechanisms by which NMDA receptor antagonists attenuate morphine hyperalgesia (Bartlett et al., 1994a; Celerier et al., 1999). Specifically, it allowed for assessing whether NMDA receptors mediate hyperalgesia directly via interactions with nociceptors, or rather if hyperalgesic suppression is simply the consequence of NMDA antagonists potentiating concurrent morphine analgesia (Kozela, Danysz, & Popik, 2001; Nemmani et al., 2004). Finally, the discovery of significant differences in hyperalgesic onset between high and low morphine infusion doses (which were particularly salient during NTX blockade of morphine analgesia) suggested that each dose may utilize somewhat distinct hyperalgesic mechanisms. This possibility was assessed directly through cross-adaptation experiments between the morphine doses themselves and between the morphine doses and the pronociceptive morphine metabolite M3G.

Another important feature of this study is the use of morphine infusion pumps as the primary drug delivery systems. Although morphine induced hyperalgesia has previously been demonstrated following both repeated injection (Ammon-Treiber & Hollt, 2005; Suzuki, Porreca, & Dickenson, 2006) and continuous infusion (Vanderah et al., 2001a; Xie et al., 2005) paradigms, it has been argued that under discontinuous morphine

delivery (i.e. multiple injections) hyperalgesia may be due in part, or exacerbated by, 'mini withdrawal' episodes (Gutstein, 1996; Angst et al., 2003; Ossipov et al., 2004). This hyperalgesia is exhibited when morphine occupation of opioid receptors is greatly reduced, such as might occur between long morphine inter-injection intervals, or following administration of an opioid antagonist, and has been shown to be mechanistically distinct from opioid induced hyperalgesia (Harris et al., 2004; Dunbar et al., 2006). Utilizing morphine infusion pumps precludes the possibility of spontaneous withdrawal by providing uninterrupted, constant morphine exposure.

METHODS:

Subjects

Adult male CD-1 mice (Charles Rivers, Kingston, NY), 6 – 8 weeks of age, were housed 4 to a cage with same-sex littermates and were maintained on a 12 hour light / 12 hour dark cycle in a climate-controlled room (22 °C ± 2°C) with free access to food and tap water. Each subject was used once. For all conditions, $n \geq 7$.

Tail-withdrawal assay

The warm-water tail-withdrawal test, as described above in the Genrral Methods section, was chosen for its stability in the context of repeated testing (Elliott et al., 1995; Kest et al., 2002; Nemmani et al., 2004).

Drug Delivery

Infusion doses are expressed throughout as cumulative dose in a 24 h period. Continuous subcutaneous morphine, M3G, or saline (vehicle control) infusion was achieved using

osmotic pumps. NTX (30 mg) or placebo control pellets were subcutaneously implanted 24 h prior to pump implantation. Acute subcutaneous morphine, MK-801 (a non-competitive NMDA antagonist), and M3G doses were dissolved in saline and injected in a volume of 10 ml/kg.

Morphine analgesia dose–response studies

Tolerance was assessed by comparing ED_{50} potency estimates, derived from cumulative dose–response curves as previously described.

Data analysis

Tail withdrawal latencies and % MPE values are presented as mean (±SEM) and were analyzed using 1 or 2-way ANOVA followed by the Tukey–Kramer test post-hoc. For both tests, $\alpha = 0.05$.

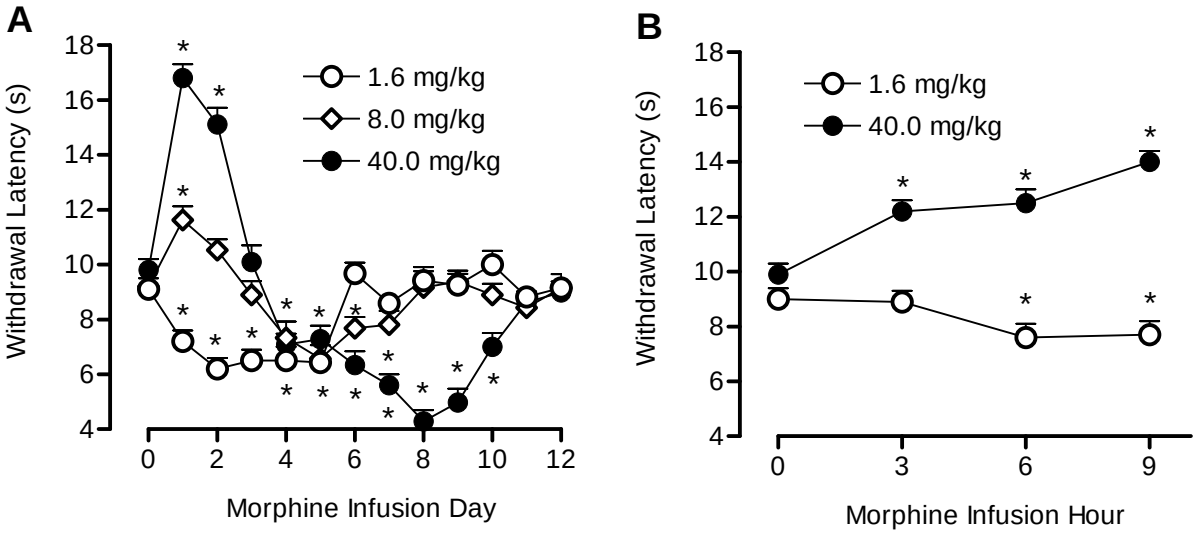
RESULTS:

Morphine hyperalgesia - dose and time responses:

To assess the hyperalgesic magnitude and duration of various morphine infusion doses, mice were implanted with pumps containing saline or 1.6, 8.0, and 40.0 mg/kg morphine. Latencies of saline-treated control mice did not vary from those obtained prior to the start of infusion (baseline (BL); Day 0) across 14 days of repeated daily testing (data not shown), demonstrating no effect of repeated measurement on nociception. Continuous infusion of both 8.0 and 40.0 mg/kg morphine resulted in an initial dose-dependent increase of withdrawal latencies relative to their respective BL latencies, illustrating an initial analgesic effect of about 2 days of duration (Figure 1A) followed by dose-

dependent hyperalgesia starting on Day 4. In contrast, 1.6 mg/kg morphine infusion produced no detectable initial analgesic effect (Figure 1A). Instead, significantly decreased tail-withdrawal latencies (i.e. hyperalgesia) were already evident 6 hours after the start of morphine infusion (Figure 1B), and continued unabated until Day 6 (Figure 1A). Likewise, the initial analgesic phase elicited by the 40.0 mg/kg morphine dose was already visible 3 hours after the start of infusion (Figure 1B). During continuous morphine delivery of all doses, latencies eventually returned to baseline values (Day 6 for 1.6 mg/kg; Day 8 for 8.0 mg/kg; Day 11 for 40.0 mg/kg; Figure 1A), demonstrating hyperalgesic adaptation.

Figure 1. Effect of continuos morphine infusion on tail-withdrawal latencies.



(A) For all 3 morphine doses, latencies were assessed immediately before (BL; Day 0), and once daily, every 24 hours after the start of subcutaneous morphine infusion. Latencies of saline-treated control mice did not vary from BL values at any time (data not shown). (B) Latencies on Day 0 were assessed immediately before (BL; hour 0), and every 3 hours, after starting subcutaneous morphine infusion. Data represent mean ± SEM latencies. (*) Significant time-dependent and dose-dependent increases and decreases in latency to tail withdrawal relative to BL values, indicative of analgesia and hyperalgesia, respectively, are indicated ($p < 0.05$; Tukey–Kramer post-hoc test).

Morphine hyperalgesia and analgesic tolerance

In this and all subsequent studies, only 1.6 and 40 mg/kg morphine doses were tested since they displayed the most divergent behavioral responses. To assess the relationship between morphine hyperalgesia and analgesic tolerance, analgesia dose–response studies were conducted both during the hyperalgesic phase and following hyperalgesic adaptation. For mice infused with 1.6 mg/kg morphine, these corresponded to Days 3 and 6. Animals infused with 40.0 mg/kg morphine were assessed on Days 6 and 12, correspondeing to date of hyperalgesic onset and adaptation. Control groups infused with saline instead of morphine were run concurrently with the morphine groups. The control groups were tested on Days 3, 6, and 12. They did not differ in respect to baseline withdrawal latencies, subsequent morphine analgesia dose–response curves, or derived ED_{50} values, and were thus pooled into a single saline control group (Table 1 and Figure 2).

For the 1.6 mg/kg morphine group, the data show a double dissociation between hyperalgesia and morphine tolerance. On Day 3, although their tail withdrawal latencies were significantly decreased relative to saline controls (i.e. hyperalgesia), their morphine analgesia dose–response curves (Figure 2) and resultant ED_{50} estimates were similar to the control group and not suggestive of tolerance (Table 1). Conversely, on Day 6, the prior morphine exposure caused a rightward shift in the analgesic dose–response curve relative to saline-treated controls (Fig. 2), reflecting a significant, 2.5-fold decrease in morphine potency (demonstrating tolerance: Table 1), despite withdrawal latencies that were indistinguishable from saline controls (i.e. no hyperalgesia:Table 1).

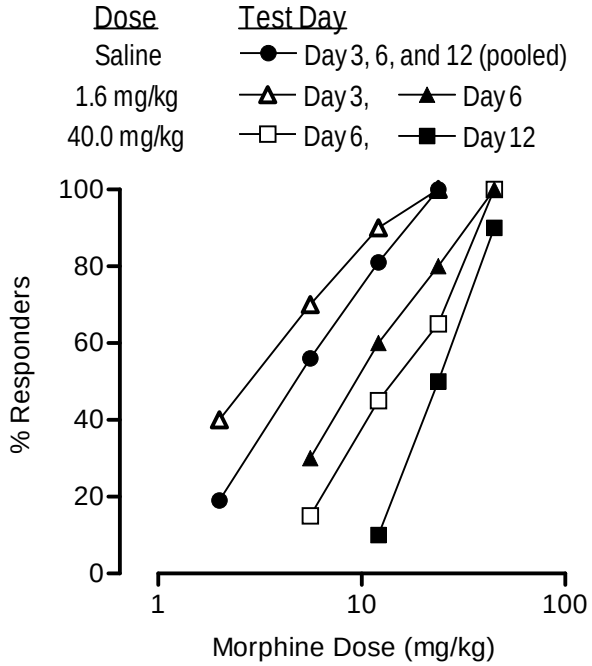
Mice infused with 40.0 mg/kg morphine, exhibited an even larger rightward shift of the analgesic dose–response curve relative to controls on Day 6 (Fig. 2), representing a significant 4.0-fold reduction in morphine analgesic potency (i.e. tolerance) concomitant with significant reductions in tail withdrawal latencies (Table 1). Relative to saline controls, rightward shifts in the analgesic dose–response curve were even more evident on Day 12 (Fig. 2), where a significant, 5.1-fold reduction in morphine potency estimates was revealed (Table 1), despite withdrawal latencies that were comparable to baseline and control values (Table 1). Thus despite demonstrating tolerance at both time points, the greatest tolerance during high-dose infusion was manifested following hyperalgesic adaptation, when latencies had returned to baseline.

Table 1. Morphine hyperlagesia and morphine analgesic tolerance during continuous morphine infusion.

Dose/24 h	Test Day	Latency	ED_{50} (mg/kg)	95% CI (ED_{50})
Saline	–	9.5 ± 0.4	3.5	(2.6–4.5)
1.6 mg/kg	3	5.9 ± 0.5*	3.1	(1.9–4.9)
1.6 mg/kg	6	9.6 ± 0.3	8.8^{+}	$(5.5–11.1)^{+}$
40.0 mg/kg	6	5.0 ± 0.5*	14.1^{+}	$(10.6–18.6)^{+}$
40.0 mg/kg	12	9.1 ± 0.3	17.8^{+}	$(12.7–26.5)^{+}$

The latency values represent mean ± SEM tail withdrawal latencies immediately prior to dose–response initiation.
(*) significant reductions ($p < .05$) in withdrawal latencies relative to basline
(+) significant reductions in analgesic potency estimates relative to baseline

Figure 2 - Morphine analgesic potency during morphine infusion



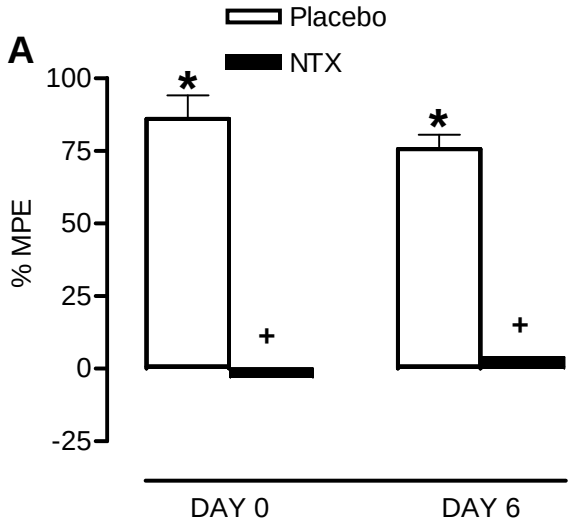
Morphine dose–response curves were obtained by injecting acute morphine doses during infusion of 1.6 mg/kg (Days 3 and 6) and 40.0 mg/kg (Days 6 and 12) morphine doses. Dose–response data for mice treated concurrently with saline and tested on Days 3, 6, and 12 did not differ and were thus pooled into a single control group. Symbols represent the percentage of analgesic responders 30 min following each cumulative morphine dose. ED_{50} values derived from the curves were used to compare groups as noted in Table 1.

Effect of opioid receptor blockade on morphine hyperalgesia

To confirm the functional blockade of opioid receptors by NTX, mice were implanted with NTX or placebo pellets on Day −1. On Days 0 and 6 (24 hours and 7 days later, respectively), they were tested for nociception before, and 30 min after, a single acute morphine (10 mg/kg) injection. As indicated by % M.P.E. values in Figure 3, acute morphine injection produced significant analgesia on Days 0 and 6 in placebo pelleted mice, but analgesia was completely abolished in mice implanted with NTX pellets.

Subsequently, we used this NTX treatment protocol to block analgesia prior to, and/or concurrent with, hyperalgesia during morphine infusion. Importantly, naïve mice implanted with NTX pellets alone did not show any deviation from baseline withdrawal latencies during repeated testing over 14 days (data not shown).

Figure 3 Blockade of morphine analgesia through NTX implantation



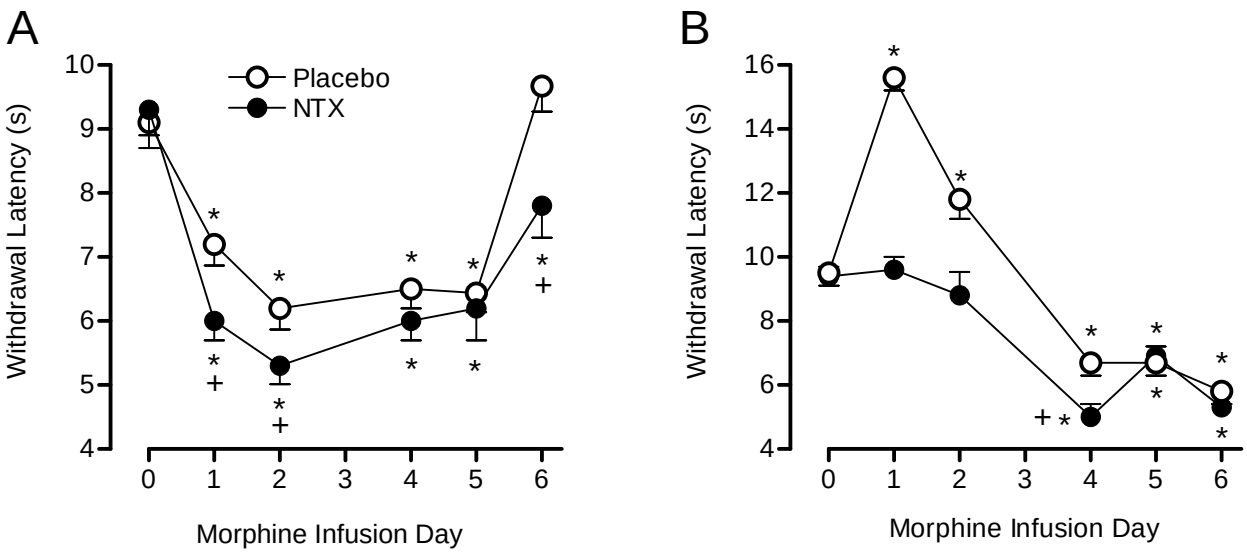
NTX (30 mg) or placebo pellets were implanted in mice on Day −1. On Day 0 and Day 6, withdrawal latencies were measured before, and 30 min after, a single morphine (10 mg/kg) injection. Values are expressed as mean (±SEM) % analgesia (see Experimental procedures for formula).

(+) significant differences ($p < 0.05$) using Tukey–Kramer post-hoc test were observed relative to corresponding values in placebo-treated mice

Results showed decreased latencies for placebo pelleted mice starting on Day 1 for the 1.6 mg/kg morphine infusion dose (Figure 4A), and on Day 4 for the 40.0 mg/kg infusion dose (Figure 4B). NTX implantation prior to low-dose infusion (Figure 4A), resulted in a significant, albeit small effect, as slightly lower withdrawal latencies were manifested throughout the 6-day hyperalgesic phase. Consistent with the hyperalgesia time–response study described above, 40 mg/kg morphine infusion elicited an initial analgesic response

on Days 1 and 2 in placebo-treated mice, which was completely abolished by prior NTX implantation, further confirming the NTX pellets antagonistic efficacy (Figure 4B). Intetestingly, despite analgesic blockade, hyperalgesia was still not manifest earlier than Day 4 (Figure 4B).

Figure 4. Effect of NTX implatation on morphine hyperalgesia



NTX (30 mg) or placebo pellets (30 mg) were implanted in mice on Day −1. Baseline latencies were assessed immediately prior to the start of (A) 1.6 mg/kg or (B) 40.0 mg/kg morphine infusion and once daily every 24 hours thereafter. Data represent mean (±SEM) latency.

(*) Significant differences ($p < 0.05$) using Tukey–Kramer post-hoc test relative to pre-morphine BL latencies.
(+)Significant differences ($p < 0.05$) using Tukey–Kramer post-hoc test relative to corresponding values in placebo-treated mice.

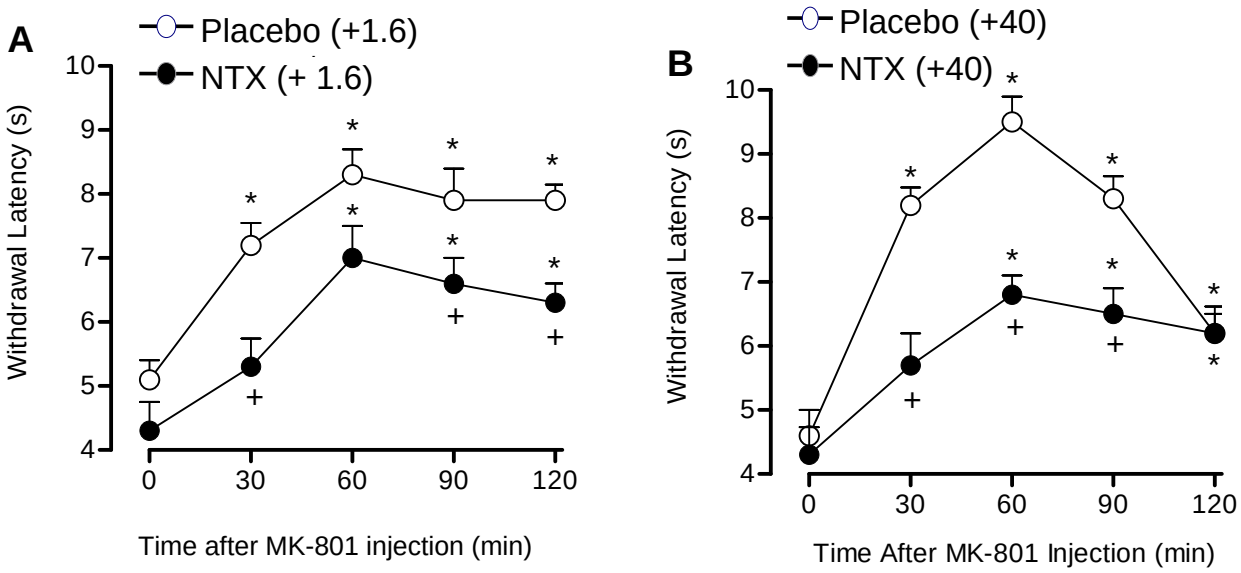
Effect of NMDA receptor blockade on morphine hyperalgesia

To determine if NMDA receptor antagonists attenuate morphine hyperalgesia by potentiating latent concurrent morphine analgesia, nociception was tested immediately

before (time 0), and every 30 min after, a single MK-801 (0.05 mg/kg) or saline injection in both NTX and placebo pelleted mice receiving morphine infusion. MK-801 injections were given on Days 2 and 6 during 1.6 (Figure 5A) and 40.0 (Figure 5B) mg/kg morphine infusion, respectively. The intervals chosen for each dose were reliably associated with hyperalgesia relative to pre-morphine (Day 0) latencies in all the studies above. Indeed, latencies obtained immediately before MK-801 injection (time 0) on the respective test days indicate a significant ($p < 0.05$) reduction from Day 0 latencies for both infusion doses (1.6 mg/kg: 9.1 ± 0.3; 40.0 mg/kg: 10.1 ± 0.5).

In placebo-treated mice infused with either 1.6 (Figure 5A) or 40.0 (Figure 5B) mg/kg morphine, MK-801 injection significantly increased latencies relative to pre-injection values between 30 min and 120 min, indicating hyperalgesic reversal. However, in NTX pelleted mice, MK-801 reversed hyperalgesia starting only 60 min later, and the ensuing hyperalgesic reversal was of significantly lesser magnitude relative to that observed in placebo-treated mice (Figures 5A, 5B). Saline injected concurrently in morphine-treated control mice had no effect on latencies in the presence of either NTX or placebo pellets (data not shown). Likewise, MK-801 injections in both NTX and placebo pelleted control mice (who were not undergoing morphine infusion) yielded no discernable behavioral or nociceptive effects (data not shown).

Figure 5. Effect of NMDA receptor antagonism on morphine hyperalgesia



Mice were implanted with NTX (30 mg) or placebo pellets on Day −1. 24 hours later they were implanted with either 1.6 or 40.0 mg/kg morphine infusion pumps. Mice were assessed daily for hyperalgesia using the tail withdrawal test. Once mice were exhibiting significant hyperalgesia (Day 2 for low-dose, and Day 6 for high-dose), mice were injected subcutaneously with an acute bolus dose of the NMDA receptor antagonist, MK-801 (0.05 mg/kg). Data represent mean (±SEM) latencies immediately before, (time 0) and every 30 min after, MK-801 injection during 1.6 (**Figure A**) and 40.0 (**Figure B**) mg/kg morphine infusion. Significant differences ($p < 0.05$; Tukey–Kramer test) from time 0 (*) or corresponding placebo-treated (+) values are noted.

Hyperalgesic cross-adaptation between morphine doses

Differences in hyperalgesic onset between high and low morphine infusion doses were particularly salient when analgesia was absent during NTX blockade. This suggested that the existence of distinct, morphine dose-dependent, hyperalgesic mechanisms, that were unrelated to morphine analgesia. To verify this supposition, mice were infused with 40.0 mg/kg morphine for 14 days and, consistent with our earlier findings, demonstrated hyperalgesic adaptation by Day 13 (data not shown). Infusion pumps were then exchanged either for those containing the same (40.0 mg/kg) or the lower (1.6 mg/kg)

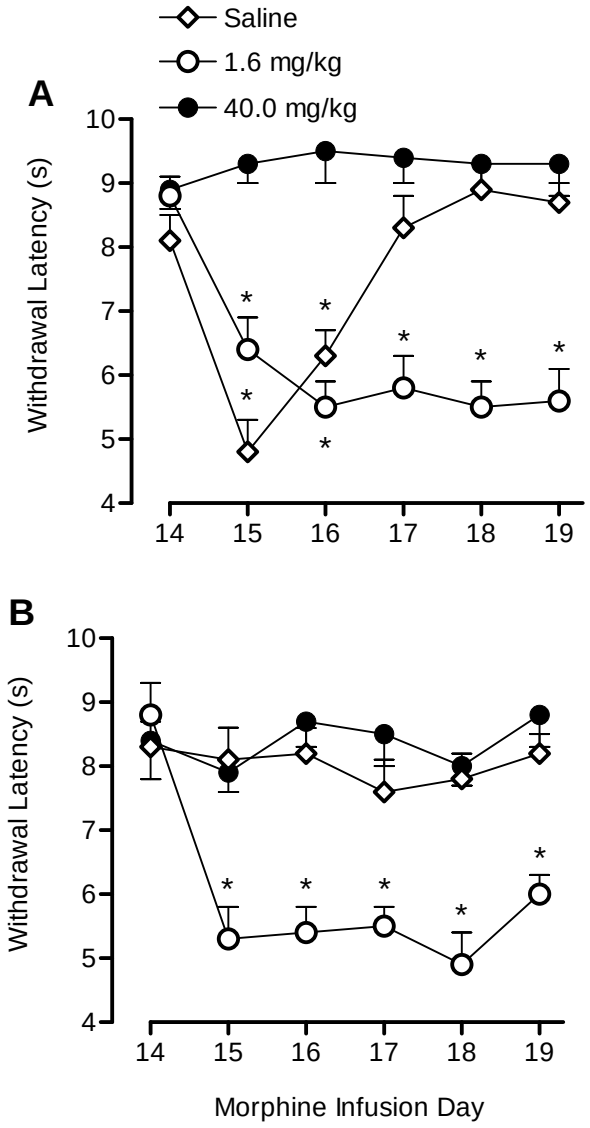
morphine dose, and nociception was tested daily over the next 5 days. Since hyperalgesia may result from morphine withdrawal in morphine-dependent mice following a drastic reduction in morphine delivery (Elliott et al., 1995), we also tested control mice subject to complete morphine abstinence by having their 40.0 mg/kg morphine pumps exchanged for those filled with saline.

Results showed that after undergoing 40 mg/kg hyperalgesic adaptation, mice infused with the lower 1.6mg/kg morphine dose displayed hyperalgesia relative to pre-exchange (Day 14) values starting 24 hours later (Day 15), and continuing for the next 5 days (Day 19; Figure 6A). Hyperalgesic adaptation followed by reinfusion of 40mg/kg morphine did not cause any change in withdrawal latencies. Control mice whose pumps were replaced with saline pumps were only hyperalgesic for the first 48 hours following pump exchange (Days 15–16; Figure 6A), but returned to baseline levels by Day 17, suggesting that the extended 5 day hyperalgesic phase during 1.6mg/kg infusion was not due to spontaneous withdrawal.

Blockade of opioid receptors has previously been shown to prevent morphine dependence and withdrawal (Bhargava et al., 1994). Therefore, to further confirm that latency reductions elicited by the lower morphine dose were not confounded by morphine withdrawal, separate groups of naive mice were implanted with NTX pellets 24 hours prior to, and throughout, 40.0 mg/kg morphine infusion. Following hyperalgesic adaptation on Day 14, their pumps were exchanged with the same dose (40.0 mg/kg), the lower morphine dose (1.6 mg/kg), or saline (controls). As expected, exchanging 40.0 mg/kg morphine filled pumps for those filled with the same (40.0 mg/kg) dose or saline

did not alter latencies relative to pre-exchange (Day 14) values (Figure 6B), indicating hyperalgesic adaptation and the absence of hyperalgesia associated with withdrawal. Conversely, exchanging the 40.0 mg/kg morphine pumps with the 1.6 mg/kg dose significantly reduced latencies relative to pre-exchange values over the subsequent 4 days (6B).

Figure 6. Hyperalgesic cross-adaptation between morphine infusion doses



(A) Mice were subject to continuous 40.0 mg/kg morphine infusion for 14 days. On Day 14, following assessment of tail withdrawal latencies, infusion pumps were exchanged for those containing morphine doses of 40.0 or 1.6 mg/kg or saline. Latencies were reassessed once daily for the next 5 days (Days 15–19).

(B) The identical protocol was conducted in mice subject to continuous opioid receptor blockade by means of NTX (30 mg) pellets implanted 24 hours prior to the start of morphine infusion.

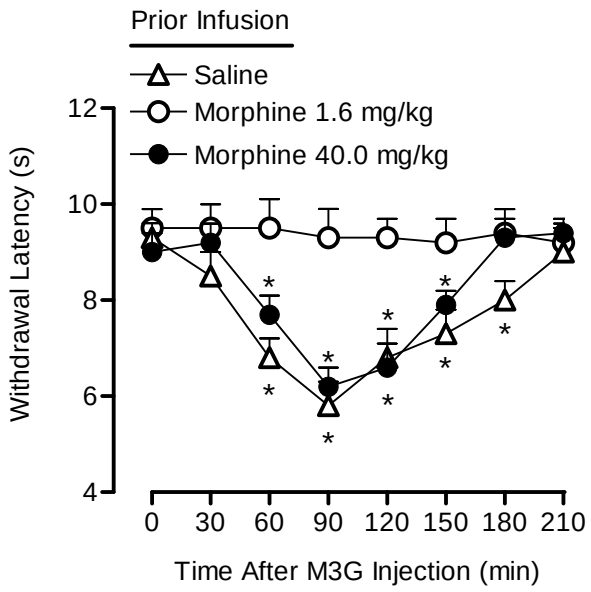
Data represent mean (±SEM) latencies.

*Indicates significant ($p < 0.05$) decreases in latency relative to values obtained prior to pump exchange on Day 14 (Tukey–Kramer test).

Hyperalgesic cross-adaptation between morphine infusion doses and M3G

To further assess whether distinct mechanisms underlie 1.6 and 40.0 mg/kg morphine hyperalgesia, the effect of M3G on nociception was tested in mice adapted to hyperalgesia of both infusion doses. Groups of mice receiving either 1.6 or 40.0 mg/kg morphine infusion were assayed daily for nociception using the tail withdrawal test. By Days 7 and 14, respectively, the mice within each of the 2 morphine infusion groups were adapted to hyperalgesia and, with pumps intact, were immediately injected with a single subcutaneous bolus dose of M3G (5 mg/kg). Nociception was then tested every 30 minutes thereafter for 210 minutes. Control groups were infused with saline for 7 or 14 days before M3G injection. In the 2 saline-treated groups (7 days and 14 days), M3G caused reductions in withdrawal latencies (i.e. hyperalgesia) of similar magnitude and duration (60–180 min) and were thus pooled into a single saline control group (Figure 7). M3G injection in mice adapted to 40.0 mg/kg morphine caused reductions in withdrawal latencies 60–150 min later that were very similar in magnitude to control mice and of only slightly shorter duration (Figure 7). In contrast, M3G had no effect on latencies at any time in mice adapted to the 1.6 mg/kg morphine dose (Figure 7). There were no overt behavioral signs after acute M3G injection.

Figure 7. Cross-adaptation between morphine hyperlageisa and M3G



Mice were subject to continuous infusion with 1.6 and 40.0 mg/kg morphine. On infusion Days 7 and 14, respectively, nociception was assessed immediately before (time 0) and for 210 minutes after a single M3G bolus (5 mg/kg) injection. 2 separate control groups infused with saline were similarly tested after M3G injections on Days 7 and 14. There was no significant difference between control groups, and their values were therefore pooled. Data are mean (±SEM) latencies.

*Indicates significant ($p < 0.05$) differences from withdrawal latencies obtained immediately prior to M3G injection (time 0).

DISCUSSION:

The major findings of Specific Aim 1 were as follows: 1) NTX pellets providing continuous opioid receptor blockade during morphine infusion blocked analgesic expression, but did not prevent hyperalgesia or reduce the ability of the NMDA receptor antagonist MK-801 to reverse hyperalgesia. 2) Acute bolus morphine injections used to generate analgesia dose–response curves in mice undergoing morphine infusion revealed that morphine tolerance is not related to hyperalgesic onset, offset, or magnitude. 3) In mice adapted to hyperalgesia during 40.0 mg/kg morphine infusion, only the lower 1.6

mg/kg morphine infusion dose restored hyperalgesia, an effect that was not attributable to spontaneous withdrawal. 4) Acute injections of M3G following hyperalgesic adaptation only produced hyperalgesia following high-dose infusion, but not after low dose infusion. These findings are discussed in greater detail below.

Non-opioid receptor mediation of hyperalgesia

In this study, implantation of NTX pellets was used to ensure opioid blockade during morphine infusion. Similar results have been reported using rats, where 30 mg NTX pellets yielded significantly elevated NTX plasma levels starting 1 hour after implantation, and sustained pharmacologically active levels of NTX for a minimum of 8 days (Yoburn, Cohen, & Inturrisi, 1986), such that there is a greater than 50-fold rightward shift in the morphine analgesia dose–response curve at that time. Also in rats, implantation of a single 10 mg NTX pellet completely blocked morphine analgesia on the hot-plate test for at least 30 days (Misra & Pontani, 1978). This was consistent with the findings reported in the study above, where in the much smaller mouse, 30 mg NTX pellets completely abolished the analgesic effect of an acute 10 mg/kg morphine injection as early as 24 hours post implantation, and remained maximally effective 7 days later. Since NTX pellets were implanted 24 hours before infusing relatively much lower morphine doses than described in the literature, it is logical to assume that functional opioid receptor blockade was in effect throughout the entire 7-day infusion course. Indeed, the initial 2-day analgesic response in placebo pelleted mice infused with the 40.0 mg/kg morphine dose was completely abolished in NTX pelleted mice. As the NTX doses employed were orders of magnitude larger than those necessary to selectively block

excitatory G_s-coupled opioid receptors that yield hyperalgesia (Crain & Shen, 2000; Crain & Shen, 2001) and, as presently demonstrated, inhibitory $G_{i/o}$-coupled opioid receptors that yield analgesia, the data demonstrate persistent morphine hyperalgesia that is independent of concurrent or prior opioid receptor activation. This finding argues for conceptualizations of morphine hyperalgesia that do not involve opponent processes (Simonnet & Rivat, 2003) or other analgesia-dependent adaptive mechanisms (Celerier et al., 2001; Ossipov et al., 2004).

As the hyperalgesia described in this study was initiated via morphine exposure, it is possible that morphine elicited hyperalgesia via non-opioid metabolites, of which M3G, the primary product of morphine biotransformation in rodents (Wahlstrom, Hammar, Lundin, & Rane, 1986; Kuo, Hanioka, Hoshikawa, Oguri, & Yoshimura, 1991), is the best characterized. It has no detectable affinity at any opioid receptor subtype (LaBella et al., 1979; Bartlett et al., 1994a; Bartlett et al., 1994b), is devoid of analgesic effect (Gong et al., 1992; Lipkowski, Carr, Langlade, Osgood, & Szyfelbein, 1994), and has been shown to produce rather potent nociceptive neuroexcitation following relatively small i. c. v. (LaBella et al., 1979; Gong et al., 1992; Bartlett et al., 1994a), intrathecal (Woolf, 1981; Yaksh et al., 1986), and systemic (Ekblom, Gardmark, & Hammarlund-Udenaes, 1993; Lipkowski et al., 1994) exposure, that is naloxone insensitive (LaBella et al., 1979; Yaksh et al., 1986). In the only previously reported mouse study, systemic injection of M3G doses between 10 and 100 mg/kg failed to elicit overt behavioral responses or hyperalgesia (Bian & Bhargava, 1996). This differs from the findings of the current study where acute 5 mg/kg systemic M3G injections in saline-infused control mice led to

increased nociception on the tail-withdrawal test in the absence of overt behavioral anomalies. It is very likely that the inability to detect M3G hyperalgesia in the previous mouse study (Bian & Bhargava, 1996) was confounded by 'floor effects', a consequence of using a very high intensity thermal stimulus which yielded baseline latencies of 2 seconds. Nonetheless, methodological limits of the present study do not allow for definitive conclusions regarding the role of M3G in morphine hyperalgesia in mice. The contribution of other morphine metabolites demonstrating negligible opiate receptor binding but potent neuroexcitatory effects (Christrup, 1997; Lotsch, 2005) must also be considered, although their effect upon thermal nociception has yet to be determined.

Relationship between morphine analgesia, hyperalgesia, and tolerance

Although the mechanisms of morphine analgesic tolerance are not well understood, prior opioid receptor activation and/or analgesia are logically requisite events (Bhargava et al., 1994; Shen & Crain, 2001). For example, opioid receptors are converted from inhibitory ($G_{i/o}$-coupled) to excitatory (G_s-coupled) mode by alterations in neuronal GM1 ganglioside concentration (Wu et al., 1998; Crain & Shen, 2000). This corresponds to a change in opioid receptor function from those that mediate analgesia to those that mediate hyperalgesia, which has been interpreted as indicative of tolerance (Crain & Shen, 2000; Crain & Shen, 2001; Shen & Crain, 2001). In support of this view, the cholera toxin-B subunit which selectively binds to GM1 ganglioside has been shown to block excitatory opioid receptor function, potentiate morphine analgesia, and prevent morphine tolerance (Shen & Crain, 2001).

In the present study, however, the lowest morphine dose produced significant hyperalgesia starting from 6 h post implantation which persisted for several days, despite the lack of prior analgesic effect. Critically, NTX implantation 24 h prior to the start of morphine infusion did not affect hyperalgesic expression. The fact that these NTX doses exceeded those necessary to block both $G_{i/o}$-coupled and G_s-coupled opioid receptors, thus precludes the supposition of morphine activity at either G-protein-coupled opioid receptor as the source of the presently described hyperalgesia. This data is substantiated by the results of analgesia dose–response curves generated with acute morphine injections in morphine-infused mice, which demonstrated both tolerance in the absence of hyperalgesia and hyperalgesia in the absence of tolerance. Instead, as would be expected, the presence and magnitude of tolerance were only related to increasing morphine treatment duration and/or dose. Therefore, these findings do not support a causative role for hyperalgesia in morphine analgesic tolerance.

These results are not necessarily contradictory to previous demonstrations of physiological and genetic commonality between morphine hyperalgesia and tolerance, since some mechanistic overlap in a drug's effects is expected. However, it should be noted that in previous studies (such as those cited above) the relationship between tolerance and hyperalgesia was investigated using only 1 morphine dose and treatment interval. As the present data demonstrates, analgesic tolerance and hyperalgesia can manifest concurrently – here, they do so on Day 6 during 40.0 mg/kg morphine infusion – but not at all treatment intervals. Clearly, future studies are needed that assess the

neurochemical, neuroanatomical, and genetic relationship between analgesic tolerance and hyperalgesia at multiple morphine treatment doses and intervals.

Distinct morphine dose-dependent hyperalgesic mechanisms

The possibility of distinct morphine dose-dependent hyperalgesic systems is suggested based on the following findings. First, 40.0 mg/kg morphine infusion resulted in hyperalgesia starting on Day 4, even when the analgesic response on Days 1–2 was abolished during NTX treatment. This contrasts with a hyperalgesic onset of just 6 h after the 1.6 mg/kg dose. If similar hyperalgesic mechanisms were utilized, we would expect the larger morphine dose to elicit hyperalgesia more rapidly or, at minimum, at the same time as the smaller dose when under conditions of opioid receptor blockade.

Second, there was no hyperalgesic cross-adaptation between morphine doses. Specifically, the 25-fold lower 1.6 mg/kg, but not the 40.0 mg/kg, morphine dose elicited hyperalgesia in mice already adapted to hyperalgesia after 14 days of 40.0 mg/kg morphine infusion, suggesting that the low dose restored hyperalgesia by utilizing a different hyperalgesic system. It is unlikely that this restoration of hyperalgesia was a consequence of withdrawal precipitated by a reduction in the morphine infusion dose, since in mice adapted to 40 mg/kg morphine for 14 days who were subsequently subjected to total abstinence (via saline infusion), hyperalgesia weakened after 24 h and completely dissipated after only 2 days. In contrast, the 1.6 mg/kg morphine replacement dose generated unremitting reductions in withdrawal latencies of significant magnitude for a minimum of 5 days, suggesting a dynamic process driven by this low morphine

infusion dose. In addition, the 1.6 mg/kg dose significantly reduced latencies in mice adapted to 40.0 mg/kg morphine hyperalgesia, even when they were subject to concurrent continuous opioid receptor blockade with NTX. Thus, hyperalgesia was not likely a consequence of withdrawal precipitated by a 25-fold lower morphine infusion dose, as NTX blockade of opioid receptors during morphine delivery prevents the development of morphine dependence and withdrawal (Bhargava et al., 1994). Indeed, this was confirmed in the present study as NTX pelleted control mice subject to morphine abstinence (via saline infusion) after 14 days of 40.0 mg/kg morphine infusion did not manifest hyperalgesia. Importantly, NTX pelleted mice adapted to 40.0 mg/kg morphine hyperalgesia after 14 days of morphine infusion, also did not exhibit a restoration of hyperalgesia after their pumps were replaced with new pumps containing the identical dose. The fact that hyperalgesia was only manifested after 1.6 mg/kg morphine infusion points toward the existence of distinct hyperalgesic mechanisms.

Third, hyperalgesic cross-adaptation with M3G was dependent on morphine infusion dose. Although acute M3G injection reduced withdrawal latencies relative to pre-injection values in saline-infused controls, it did not do so in mice adapted to 1.6 mg/kg morphine hyperalgesia. This cross-adaptation is suggestive of common active mechanisms underlying the sensitization response exhibited following M3G exposure and during 1.6 mg/kg morphine infusion. If the 1.6 and 40.0 mg/kg morphine infusion doses utilized common hyperalgesic mechanisms, 1 would expect M3G hyperalgesia to also be cross-adaptive with the 25-fold larger 40.0 mg/kg dose, especially since it is the primary metabolite. Instead, prior 40.0 mg/kg morphine hyperalgesic adaptation had no

effect on subsequent M3G induced hyperalgesia. Furthermore, the magnitude and time course of the reduction in tail withdrawal latencies in 40.0 mg/kg infusion mice observed following acute M3G injection was nearly identical to those observed in drug-naïve control mice. The differential cross-adaptation between M3G and the 2 infusion doses, thus suggest multiple morphine dose-dependent hyperalgesia systems, an intriguing possibility warranting further study.

NMDA receptors and morphine hyperalgesia

Prior NTX implantation increased subsequent hyperalgesic magnitude during infusion of both morphine doses. This likely reflects NTX blockade of latent analgesia during morphine infusion that is normally obfuscated by – and apparently acts to limit the full expression of – the larger concurrent hyperalgesic effect. Here and elsewhere (Plesan et al., 1999; Li et al., 2001a; Mao et al., 2002), NMDA antagonism attenuated morphine induced hyperalgesia. NMDA antagonists, however, have also been shown to potentiate morphine analgesia (Kozela et al., 2001; Nemmani et al., 2004). If latent morphine analgesia is concurrent with morphine hyperalgesia, it is possible that NMDA antagonists reversed morphine hyperalgesia in previous studies, at least partially, by potentiating concurrent latent analgesia. Indeed, here we demonstrate that the NMDA antagonist MK-801 is less effective in reversing hyperalgesia in NTX pelleted mice relative to placebo pelleted controls. Since NTX pellets abolished the analgesic effect of an acute 10 mg/kg morphine injection, they almost certainly did likewise to any concurrent analgesia arising from infusing small amounts of morphine. Therefore, the present demonstration of MK-801 reversal of hyperalgesia in NTX-pelleted mice provides the

first evidence of a direct role for NMDA receptors in morphine hyperalgesia that is unrelated to its effects upon concurrent morphine analgesia.

At his time, the mechanism by which morphine activates NMDA receptors in the absence of opioid synaptic activity remains to be determined. It is worth noting that the excitatory effects of M3G are attenuated by NMDA receptor blockade (Bartlett et al., 1994a; Bartlett et al., 1994b), and M3G injection in the present study caused hyperalgesia that cross-adapted with the lower morphine infusion dose. Since agonists at the benzodiazepine binding site on the GABA-A receptor complex also attenuate M3G-induced excitation (Bartlett et al., 1994a; Bartlett et al., 1994b), they likely play a role in morphine induced hyperalgesia as well, and are currently under study.

SPECIFIC AIM TWO

Opioid infusion increases nociception in opioid receptor triple knockout mice

INTRODUCTION

Despite the wide variations in experimental methodology utilized in studies of OIH, nearly all theories agree that the initiation and maintenance of hyperalgesic states is a consequence of opioid receptor-mediated mechanisms. However, the results of Specific Aim 1, where continuous opioid infusion was shown to elicit thermal hyperalgesia that was unaffected by opioid receptor blockade, is inconsistent with theories stressing ongoing opioid receptor involvement in hyperalgesia. Even high-dose infusion, which featured an analgesic phase that was mediated by opioid receptors and consequently blocked by NTX, demonstrated hyperalgesia that was refractory to opioid receptor blockade.

Recently, the role of opioid receptors in hyperalgesia was assessed directly via spinal infusion of rats for several days with oxymorphone enantiomers (Gardell et al., 2006). This led to the demonstration of analgesia followed by hyperalgesia only after exposure to the levorotatory (*L*) enantiomers, which preferentially bind to μ receptors, while infusion of the non-opioid dextrorotatory (*D*) enantiomers was without effect. Importantly, the authors note that both the *L* and *D* enantiomers produce identical glucoronidated metabolites, oxymorphone-3-glucuronide (O3G), a substance that has been shown to elicit neuroexcitatory effects very similar to those of M3G (Armstrong &

Cozza, 2003). The finding that only the *L* isomer caused thermal and tactile hypersensitivity led the authors to suggest that oxymorphone produces hyperalgesia by specifically interacting with opioid receptors, and is not a function of neuroexcitatory non-opioid metabolites. These findings contrast sharply with those observed in Specific Aim 1, where the M3G metabolite did appear to play a role in low-dose hyperalgesia, and hyperalgesia was demonstrated under conditions of opioid blockade.

Specific Aim 2 thus directly tested the assumption that prior analgesia and/or opioid receptor activation are critical to opioid hyperalgesia using opioid receptor knock-out (KO) mice lacking all 3 opioid receptor genes (Clarke et al., 2002; Cox et al., 2005). This offers some significant advantages over the previous study where opioid analgesia was abolished by concurrently treating mice with NTX. Although NTX is an effective opioid antagonist at all 3 receptor subtypes, it preferentially binds to the μ receptor (Lewanowitsch & Irvine, 2003), and studies have shown that in the absence of μ receptors, morphine demonstrates enhanced κ binding (Yamada et al., 2006). Furthermore, gene knockout also prevents the development of opioid analgesic circuitry, which may be susceptible to activation via non-traditional means. For example, naloxone has been shown to elicit analgesia in drug-naïve BALB/C mice on the formalin test via κ receptor activation (Vaccarino, Plamondon, & Melzack, 1992). Although the authors failed to find similar effects with other inbred strains or on other measures of nociception (including the tail-flick), opioid blockade via gene knockout obviously represents a much more comprehensive form of opioid blockade. In addition, the KO mice possess a recombinant inbred background most closely resembling the B6129 strain. The use of

these mice and their controls thus affords the opportunity to assess whether the previous results reported in CD-1 may be generalized to include other genotypes as well.

To confirm the functional consequence of opioid receptor KO, KO and B6129F_1 control mice were injected with acute bolus doses of morphine and oxymorphone and assayed for analgesia. Both strains were also assayed daily for nociception during continuous infusion of morphine and oxymorphone for 7 and 10 days, respectively. Finally, to address the possibility that NMDA receptors contribute to opioid hyperalgesia by interacting with opioid receptors, MK-801 was injected into hyperalgesic KO and control mice undergoing morphine and oxymorphone infusion.

METHODS

Subjects: KO mice were generated in the laboratory of Dr. Jonathan Pintar by cross-breeding mice singly deficient in the genes coding for μ, δ, and κ receptors using standard homologous recombination techniques (Clarke et al., 2002; Cox et al., 2005). Accordingly, B6129F_1 mice were bred and served as controls. The combinatorial mice are devoid of brain or spinal cord [^{3}H]naloxone receptor labeling, indicating the complete absence of any μ, δ, and κ receptor subtypes, and lack gross behavioral or anatomical alterations (Clarke et al., 2002; Cox et al., 2005). Animals were housed 4 to a cage and maintained on a 12:12 h light-dark cycle in a temperature controlled environment with unrestricted food and water and tested as adults. To eliminate any interaction of sex with nociceptive sensitivity and morphine analgesia (Kest et al., 2000b), only males were

tested. For all conditions, naïve groups of $n \geq 6$/strain were used.

Drug doses and delivery: Morphine, oxymorphone, and the non-competitive NMDA receptor antagonist MK-801, were dissolved in physiological saline (0.9% NaCl). Acute bolus doses of opioid (10 mg/kg) and MK-801 (0.05 mg/kg) were injected subcutaneously. Continuous infusion of morphine and oxymorphone at cumulative daily doses of 40.0 and 20.0 mg/kg, respectively, were delivered via osmotic pumps implanted s.c. according to the procedures described above.

Nociceptive assay: Nociception was assessed using the warm water tail-withdrawal described above. After obtaining BL latencies, mice were either injected with an acute opioid bolus dose and retested for 120 min, or implanted with opiate filled pumps and assessed every subsequent 24 h throughout the infusion period. MK-801 reversal of hyperalgesia was tested in separate infusion groups at intervals determined in the above study to cause morphine and oxymorphone hyperalgesia (Days 6 and 9, respectively) in control and KO mice. In these groups, latencies were assayed prior to infusion and MK-801 exposure to confirm manifest hyperalgesia, and for 120 min after MK-801 injection.

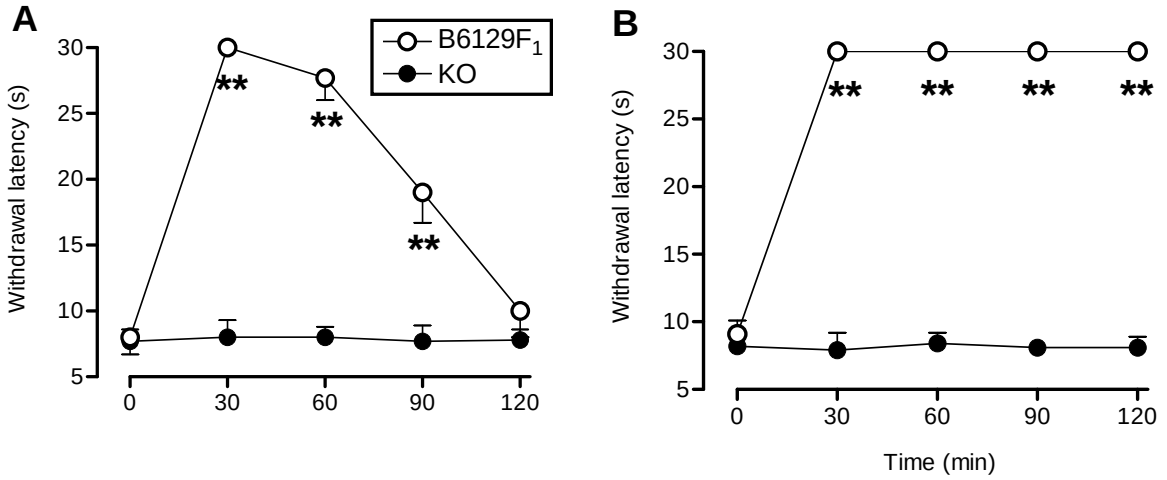
Data analysis: Withdrawal latencies were analyzed using 2-way (strain X time) repeated-measures ANOVA followed by Tukey-Kramer tests post-hoc. Differences that exceeded $\alpha = 0.05$ criterion were considered significant.

RESULTS:

Opioid analgesia is abolished in opioid receptor triple KO mice

Acute morphine and oxymorphone bolus doses produced large significant main effects of strain, time, and their interaction. The striking effect of triple opioid receptor KO on morphine and oxymorphone analgesia is shown in Figures 8A and 8B, respectively. In control mice, morphine increased withdrawal latencies approximately 2- to 3- fold from BL (time 0) values for 90 min, whereas oxymorphone elevated latencies to maximally allowable values during the entire 120 min testing period. However, both drugs were without even minimal effect on KO latencies which remained completely unchanged from BL values. There were no BL differences between KO and control mice prior to opioid injection.

Figure 8. Opioid analgesia in triple opioid receptor knock-out mice



Tail-withdrawal latencies (30 sec cut-off) were obtained immediately before (time 0) and after a 10 mg/kg bolus dose of **(A)** morphine and **(B)** oxymorphone in mice with triple opioid receptor knock-out (KO) and $B6129F_1$ controls. Data are mean (± S.E.M.) latency; ** significant analgesia relative to baseline (p <0.01).

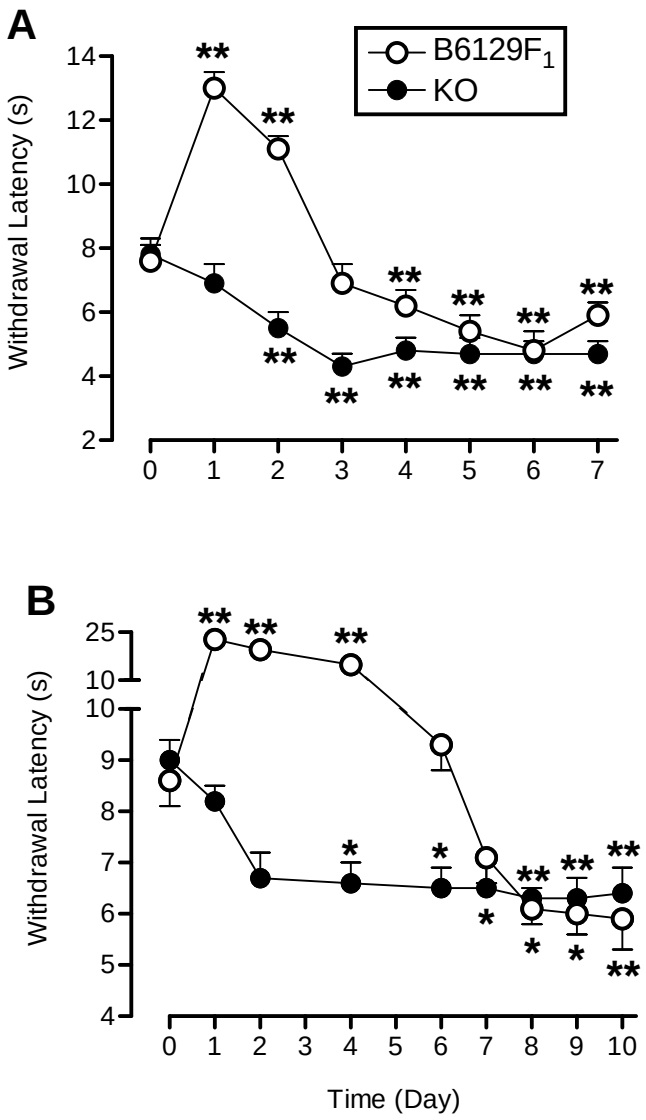
Opioid induced hyperalgesia in mice lacking opioid receptors.

Morphine infusion in control and KO mice caused significant latency alterations related to strain, time, and their interaction (for all 3 comparisons $p < 0.0001$). Control mice exhibited analgesia on the first 2 days, followed by significant hyperalgesia on Days 4-7 (Figure 9A). KO mice did not exhibit latency alterations relative to BL until Day 2, when they began to manifest persistent hyperalgesia. There was no strain difference in BL values.

Oxymorphone infusion in control and KO mice also caused significant latency alterations related to strain, time, and their interaction (for all 3 comparisons $p < 0.0001$). Relative to BL values, control mice exhibited elevated latencies for the first 4 days followed by significantly decreased values on Days 8-10 (Figure 9B), once again demonstrating analgesia in this strain followed by hyperalgesia. KO mice latencies, however, were significantly reduced by Day 4 and were not preceded by analgesia. Here too, basal nociceptive sensitivity prior to opioid infusion (BL) was equivalent between the KO and control mice.

Importantly, there was no main effect of strain, time, or their interaction, in either drug-naïve control mice or KO mice tested daily for 10 days (data not shown), indicating that tail withdrawal latencies are unaffected by daily thermal nociceptive assessments in either mouse strain.

Figure 9. Opioid induced hyperalgesia in triple opioid receptor knockout mice



Tail-withdrawal latencies were obtained in mice with triple opioid receptor knock-out (KO) and B6129F$_1$ controls (n = 6 – 10/strain) immediately before (time 0) and every 24 h during continuous **(A)** morphine and **(B)** oxymorphone infusion at cumulative daily doses of 40.0 and 20.0 mg/kg, respectively. Data are mean (± S.E.M.) latency;

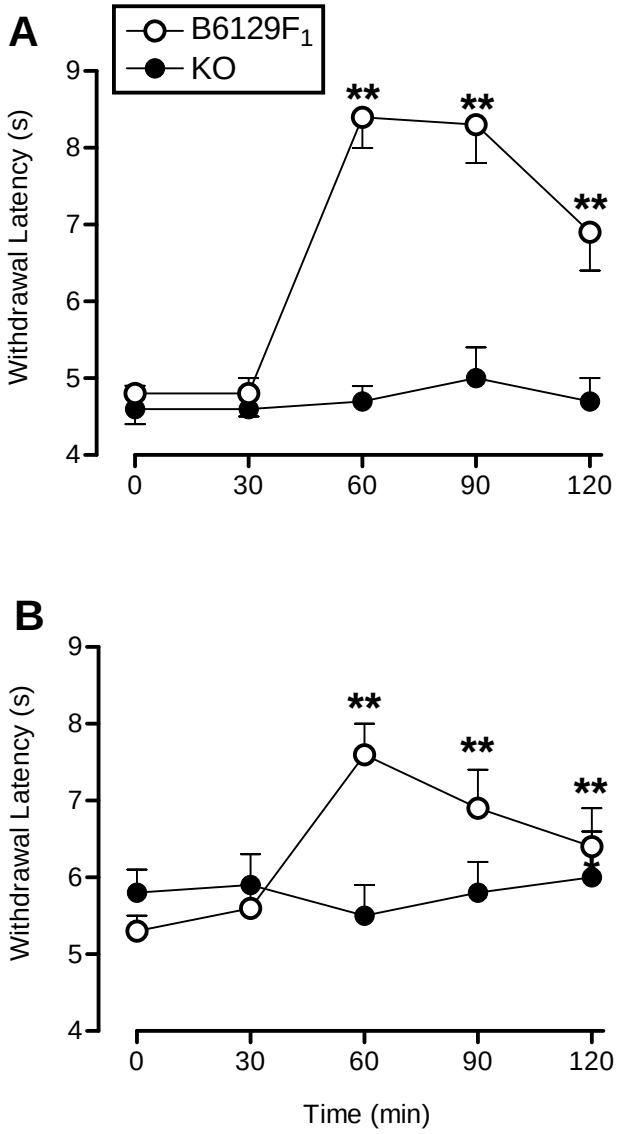
* p <0.05 relative to baseline
** p <0.01 relative to baseline

NMDA receptor blockade does not reverse opioid hyperalgesia in KO mice.

Continuous morphine infusion led to significant hyperalgesia in controls and KO mice by Day 6. Repeated nociceptive assessment following a single MK-801 injection revealed significant differences in tail withdrawal latencies related to strain, time, and their interaction (p <0.01 for all 3 comparisons). *Post-hoc* analysis showed that Day 6 latencies preceding MK-801 exposure (controls: 4.8 $\pm$ 0.4; KO: 4.6 $\pm$ 0.5) were significantly reduced relative to BL values (controls: 8.2 $\pm$ 0.6; KO: 8.4 $\pm$ 0.4; data not shown), indicating comparable hyperalgesia in control and KO mice prior to MK-801 injection. It was also found that MK-801 time-dependently (60-120 min) increased latencies (i.e. reversed hyperalgesia) relative to pre-injection values (time 0) in controls, but was without effect in KO mice (Figure 10A).

Similar results were obtained during 9 days of continuous oxymorphone infusion followed by MK-801 injection. There were significant alterations related to strain, time, and their interaction (p <0.01 for all 3 comparisons). *Post-hoc* analysis revealed comparable latency reductions from BL (controls: 8.9 $\pm$ 0.3; KO: 8.4 $\pm$ 0.6; data not shown) in control and KO mice prior to MK-801 injection on Day 9 (controls, 5.3 $\pm$ 0.4; KO, 5.8 $\pm$ 0.5). MK-801 injection resulted in time-dependent, (60-120 min) significant increases in withdrawal latencies relative to pre-injection values (time 0) in controls, but had no effect in KO mice (Figure 10B). Importantly, MK-801 injection in opioid naïve mice caused no main effect of strain, time, or their interaction (data not shown).

Figure 10. NMDA receptor blockade in triple opioid receptor knock-out mice



Tail-withdrawal latencies were obtained immediately before (time 0) and after a single bolus dose of the non-competitive antagonist MK-801 (0.05 mg/kg) during **(A)** morphine and **(B)** oxymorphone infusion in mice with triple opioid receptor knock-out (KO) and $B6129F_1$ controls. Data are mean (± S.E.M.) tail-withdrawal latencies which were collected on Day 6 (morphine) and 9 (oxymorphone) in hyperalgesic mice.

** Significant analgesia relative to pre-injection values (p <0.01).

DISCUSSION:

The main finding of the present study was that continuous morphine and oxymorphone infusion increased nociception in KO mice lacking all 3 genes encoding for opioid receptors. Despite their hyperalgesic effect, neither morphine nor oxymorphone were able to evoke even minimal analgesia in KO mice during continuous infusion or following an acute bolus dose that produced maximal analgesic responses in controls. The fact that mice devoid of opioid receptors still exhibited opioid-induced enhanced nociception offers direct evidence that the manifest hyperalgesia described in this study could not be a consequence of opioid withdrawal, or an adaptive response following opioid activation. Moreover, these data also demonstrate that morphine and oxymorphone infusion cause hyperalgesia that does not require prior or concurrent opioid receptor activity or analgesia, a finding that is contrary to current conceptualizations of opioid hyperalgesia.

In this context, how did these opioids cause hyperalgesia in KO mice? 1 possibility is active metabolites. As previously described, M3G is the primary metabolic byproduct of morphine, yet functions as a potent neuroexcitant with no detectable opioid receptor affinity. Moreover, M3G accumulation during morphine treatment is proposed to underlie morphine hyperalgesia in rats and humans (Woolf, 1981; Yaksh et al., 1986; De et al., 1991; Sjogren, Thunedborg, Christrup, Hansen, & Franks, 1998). Despite the relatively slow release of opioids by osmotic pumps (1µl/h), several days of unremitting morphine delivery could feasibly allow physiologically relevant M3G levels to

accumulate. Indeed, onset of morphine hyperalgesia was delayed for the first several days in KO mice despite the absence of a competing analgesic response.

However, there is also evidence to suggest that M3G may not have contributed to the hyperalgesia described in this study. For example, in the previous study it was demonstrated that mice receiving 7-14 days of 1.6 mg/kg morphine infusion exhibit hyperalgesic adaptation (i.e. resolution of nociceptive thresholds to pre-morphine baseline values). Acute injection of an effective M3G bolus dose at that time does not increase nociception, suggesting common mechanisms underlying morphine and M3G hyperalgesia. This functional "cross-adaptation", however, was only evident during 1.6 mg/kg morphine infusion and was not observed during the higher dose 40.0 mg/kg morphine infusion utilized in this study.

In addition, the neuro-excitatory effects of M3G are mediated by NMDA receptor activity (Hemstapat et al., 2003), and NMDA receptor antagonists dose-dependently reduce M3G symptomatology, including enhanced nociception (Bartlett et al., 1994a). Here, the non-competitive NMDA antagonist MK-801 did not attenuate morphine hyperalgesia in KO mice although the identical dose was effective in B6129F_1 controls and in CD-1 mice (Specific Aim 1). Gardell et al. (Gardell et al., 2006) similarly conclude that oxymorphone hyperalgesia is unrelated to excitatory metabolites. In their study, only 7 days of sustained (−)-oxymorphone but not (+)-oxymorphone caused hyperalgesia in rats, indicating that the source of hyperalgesia was prior to the conjugation of the opioid parent compound. While the present KO data argue strongly

against an opioid receptor-mediated mechanism for sustained morphine or oxymorphone hyperalgesia, there is no compelling evidence that their neuroexcitatory metabolites underlie this effect. Additional studies are needed to clarify how sustained opioid delivery causes non-opioid receptor mediated hyperalgesia.

As noted, the MK-801 dose effective in reversing opioid hyperalgesia in this study in control mice and in CD-1 mice in Specific Aim 1, was without even minimal effect in hyperalgesic KO mice. The present study thus demonstrates morphine and oxymorphone hyperalgesia that is not reliant on NMDA receptor activity, a finding that is in marked contrast with previously published accounts of NMDA antagonist attenuation of opioid-induced hyperalgesia in various experimental conditions (see reviews:(Simonnet & Rivat, 2003; Xu et al., 2003; Ossipov et al., 2004). The discrepancy may be related to co-localization of NMDA receptors at CNS loci including those regulating nociception (Liu et al., 1994; Liu, Mantyh, & Basbaum, 1997). Thus it is possible that the results obtained here reflect organizational effects of opioid receptor knockout which, in turn, may have led to a related down-regulation of NMDA receptor density or function in the KO mice. However, there is currently no data on the population or functional integrity of NMDA receptor in these mice that can adequately address this possibility.

Mechanistically, there have been some indications that NMDA antagonists may not reverse morphine hyperalgesia directly, but return latencies to pre-drug values by potentiating obfuscated concurrent analgesia during morphine infusion (Kozela et al., 2001; Nemmani et al., 2004). Accordingly, NMDA antagonists would be ineffective in

increasing latencies where KO mice are devoid of opioid receptors and morphine analgesia, as was the case here. The present data do not necessarily dispute that NMDA receptor antagonists modulate morphine hyperalgesia by any of the currently proposed mechanisms (Simonnet & Rivat, 2003; Ossipov et al., 2004), but suggest that the NMDA receptor contributions to opioid hyperalgesia may be restricted to subjects with functional opioid receptors. The supposition of multiple opioid hyperalgesic systems, inclusive and exclusive of NMDA receptors, would be consistent with the diversity of opioid hyperalgesic mechanisms already described above.

It has also been proposed that opioid hyperalgesia competes with concurrent analgesia, consequently diminishing opioid analgesic potency. Accordingly, hyperalgesia has been conceptualized to be a causative factor in opioid analgesic tolerance (Shen & Crain, 2001; Simonnet & Rivat, 2003; Xu et al., 2003; Ossipov et al., 2004). However tolerance is, at minimum, widely understood to be at least initially preceded by opioid receptor activity and/or analgesia, both of which are absent in the KO mice tested here. Tolerance, therefore, is not logically relevant to these mice, yet they demonstrate significant opioid hyperalgesia, suggesting distinct physiological substrates underlying tolerance and hyperalgesia. This conclusion is consistent with the results of Specific Aim 1, which demonstrated their temporal dissociation in CD-1 mice. Collectively, these findings are inconsistent with a causal role for hyperalgesia in opioid analgesic tolerance, and suggest that their mechanistic commonality reflects overlapping but distinct systems.

SPECIFIC AIM THREE

Qualitative sex differences in hyperalgesia during morphine infusion

INTRODUCTION:

Sex differences in hyperalgesia have rarely been directly investigated, and, except for a recent study (Holtman, Jr. & Wala, 2005), all such laboratory investigations of OIH have been conducted using males exclusively. In their study, Holtman et al. reported that acute sub-analgesic morphine exposure yielded hyperalgesia in female rats only. This effect was inversely related to morphine dose and it was only manifested following sub-analgesic morphine exposure. As morphine doses approached analgesic thresholds, males exhibited greater analgesia than females, and neither sex presented with hyperalgesia. Although these findings contrast markedly with the data presented in Specific Aims 1 & 2, where hyperalgesia was demonstrated in males during both sub-analgesic and analgesic morphine infusion, there are several methodological shortcomings that limit the interpretation of their findings including: a) the failure to control for putative sex differences in opioid analgesia may have obfuscated the actual magnitude of the underlying hyperalgesia; b) drug delivery was via acute injection, a paradigm which has been shown to elicit mini-withdrawal episodes (Gutstein, 1996) indicative of WIH; c) floor effects resulting from utilization of a highly noxious stimulus which yields very low baseline values (<4 seconds); d) failure to assess the role of gonadal hormones in the mediation of sex differences in morphine hyperalgesia.

However, extrapolating current findings based on work done in males to females is also inappropriate for the following reasons. First, putative sex differences in basal nociceptive sensitivity are widely reported, and the direction of these differences, and the conditions under which they are observed are unpredictable (Mogil et al., 2000). Second, males and females respond differently to morphine exposure on various behavioral measures and, again, not in a consistent direction (Craft, Stratmann, Bartok, Walpole, & King, 1999; Kest, Adler, & Hopkins, 2000a; Kest et al., 2000b). Finally, NMDA receptor antagonists have been shown to cause uneven sex-related effects on morphine activity, including hyperalgesia (Lipa & Kavaliers, 1990; Nemmani et al., 2004; Bryant, Eitan, Sinchak, Fanselow, & Evans, 2006), (Holtman, Jr. & Wala, 2005).

In this study, the nociceptive responses of male and female mice were compared to gain insight into the physiological mechanisms underlying nociception and pain modulation in general, as well as those mediating sexually dimorphic effects. Tail withdrawal latencies of male and female mice on the warm water tail-withdrawal test were compared during continuous morphine infusion. To abolish any uneven sex-related differences in analgesia concurrent with hyperalgesia, some groups were also implanted with NTX pellets to provide continuous opioid receptor antagonism throughout the period of morphine infusion and nociceptive assessment. Since NMDA antagonists can potentiate morphine analgesia (Bespalov, Zvartau, & Beardsley, 2001; Kozela et al., 2001), their ability to reverse hyperalgesia in both sexes was also tested in mice treated simultaneously with morphine and NTX. Where sex differences were identified, the role of ovarian hormones was evaluated by testing females subject to ovariectomy, with and

without subsequent estrogen replacement treatment, under identical protocols.

METHODS

Subjects: Adult male and female CD-1 mice (Charles Rivers, Kingston, NY) were maintained on a 12:12-h light/dark cycle in a climate-controlled room with free access to food and tap water. Each mouse was used once and for all groups, $n \geq 8$. All surgical procedures were performed while mice were anesthetized using an oxygen/isoflourane inhalant.

Nociceptive Assay: The warm-water tail-withdrawal test (as described above in the General Methods sections) was chosen for its stability in the context of repeated testing and to facilitate comparison to previous studies.

Drug Doses and Delivery: Osmotic pumps were filled with morphine concentrations providing cumulative daily doses of 1.6 and 40.0 mg/kg, and implanted subcutaneously according to procedures previously described. The latencies of vehicle (saline) infused and sham surgery groups were indistinguishable in pilot and previous studies, so morphine infusion control groups were comprised exclusively of mice subject to sham surgery only. Pellets containing NTX (30 mg) were implanted 24 h prior to the start of morphine infusion to avoid precipitating morphine withdrawal. Acute bolus doses of LY235959 (2.5 mg/kg) and MK-801 (0.05 mg/kg), competitive and non-competitive NMDA receptor antagonists, respectively, were injected s.c. in a volume of 10 ml/kg.

Ovariectomy and estrogen replacement

During surgery, female mice were subject to either ovariectomy or sham surgery protocols. All mice were allowed to recover for 20 days before the start of estrogen replacement. Estrogen replacement consisted of subcutaneous implantation of silastic tubules filled with EB (50 μg suspended in 0.25 ml SO) during surgery as previously described (Cohen & Milligan, 1993). Only OVX mice received EB (OVX+EB). Control groups were comprised of OVX (OVX+SO) and SHAM mice (SHAM+SO) implanted with SO filled capsules. A 10 day recovery period was allowed to elapse between the start of estrogen treatment and testing.

Data analysis

Withdrawal latencies were analyzed using a 2-way ANOVA followed by Fisher's LSD (protected *t*-tests) for post hoc comparisons. Since sex differences in BL were occasionally detected, latencies are presented as difference scores (post-infusion latency – BL latency) to facilitate comparison. Thus, for all figures, the 0 value on the *y* axis represents BL at any given infusion interval. For all comparisons, $\alpha = 0.05$.

RESULTS:

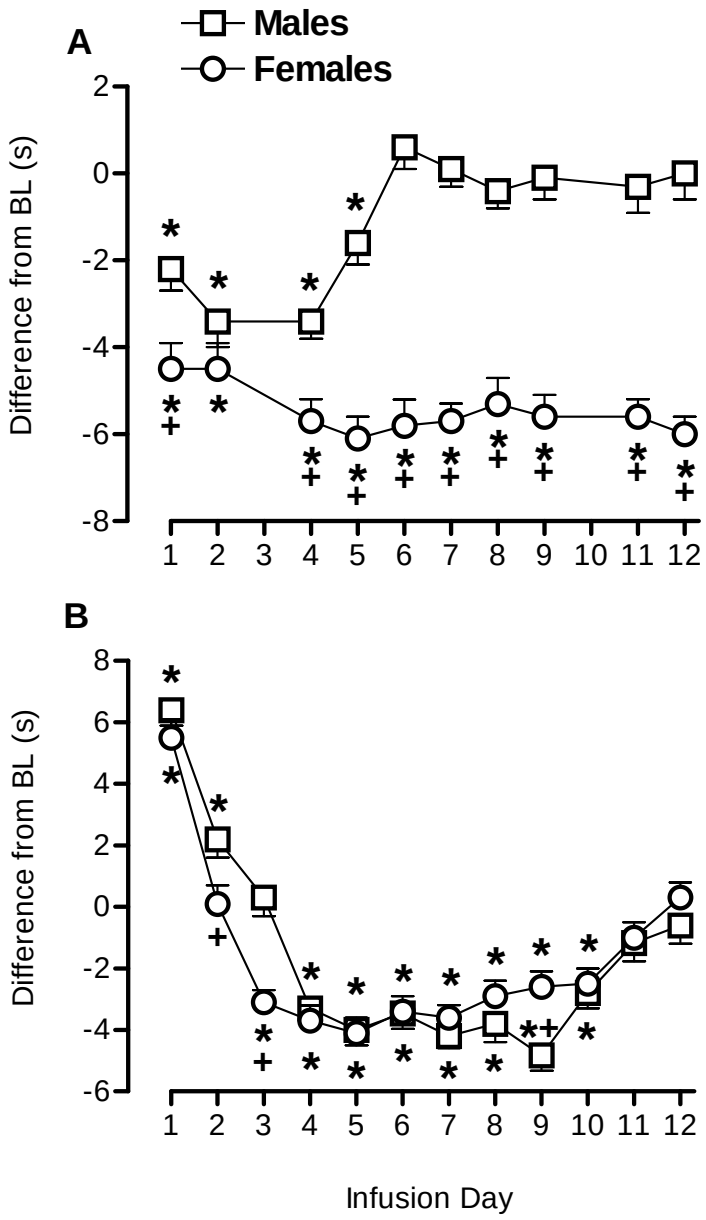
Sex Differences in morphine hyperalgesia time course

Infusing 1.6 mg/kg morphine caused latency reductions from pre-morphine BL values, indicative of hyperalgesia, in placebo pelleted mice of both sexes on Day 1 (Figure 11 A). But almost without exception, females displayed greater hyperalgesic magnitude

relative to males throughout all 12 infusion days. This sex difference was particularly salient starting on Day 6, when hyperalgesia resolved in males (i.e. returned to BL latencies values) but not in females.

In contrast, withdrawal latencies in males and females were equally increased relative to BL values 24 h after the start of continuous 40.0 mg/kg morphine infusion (Day 1; Figure 11B). This analgesic effect of morphine was still evident on Day 2 in males only. Significant hyperalgesia was subsequently observed in females and males starting on Days 3 and 4, respectively, demonstrating a sex difference in hyperalgesic onset. The magnitude of hyperalgesia was quite similar between sexes on all subsequent days and no sex differences were noted other than on Day 9. Hyperalgesia resolved in both sexes by Day 11. There were no significant latency fluctuations in placebo-pelleted control mice of either sex during daily testing for 12 days (data not shown).

Figure 11. Sex Differences in hyperalgesia during morphine infusion.



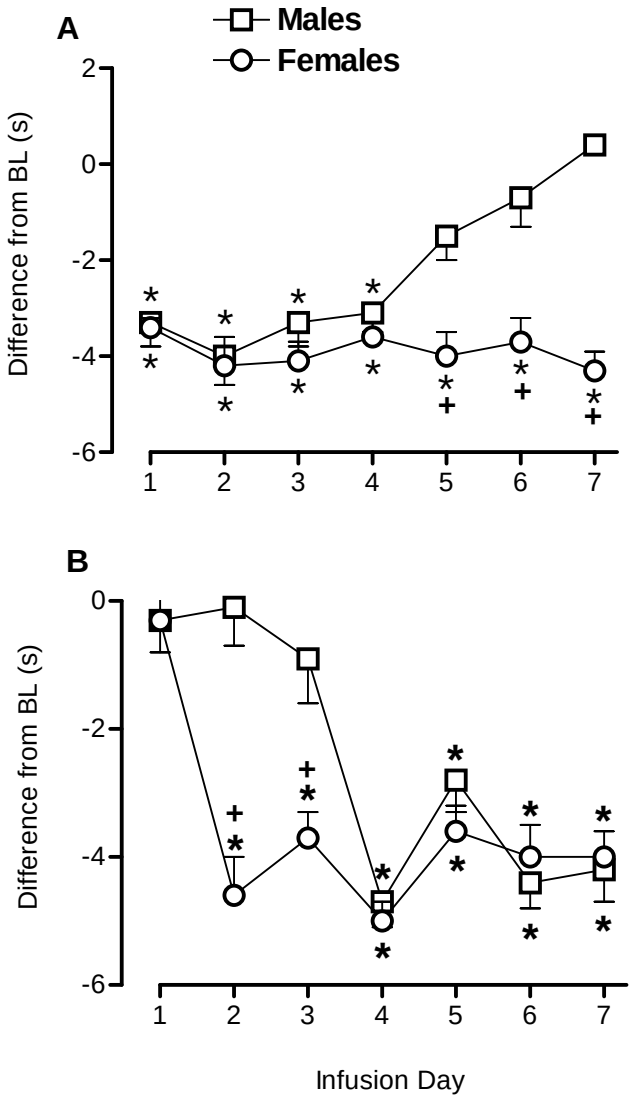
Daily measures of nociception in mice of both sexes infused with cumulative daily morphine doses of 1.6 (A) or 40.0 mg/kg (B). Data are mean ± S.E.M. latency differences from BL (pre-morphine values). The 0 value on the *y* axis in this and all subsequent figures represents BL at any given infusion interval (see text).

* Significant difference relative to same sex BL latency ($p < 0.01$);
+ Significant difference relative to corresponding male values ($p < 0.05$).

Sex Differences in morphine hyperalgesia during analgesic blockade

During 1.6 mg/kg morphine infusion, NTX pelleted mice of both sexes displayed similar latency reductions from BL on Days 1-4 (Figure 12A). By Day 5, significant sex differences were noted as hyperalgesia resolved in males but not females. During 40.0 mg/kg morphine infusion, NTX pellets completely abolished the analgesic response typically observed on Days 1 and 2 in placebo pelleted mice (Figure 12B). But whereas withdrawal latencies in males remained similar to BL values on Days 1-3, significant latency reductions were already observed by Day 2 in females, indicating again the earlier onset of hyperalgesia in this sex. By Day 4, both sexes displayed significant hyperalgesia of similar magnitude that endured for the duration of testing. NTX treatment without morphine infusion did not alter latencies from BL values over 7 days of repeated daily testing (data not shown).

Figure 12. Effect of NTX on hyperalgesia during morphine infusion.



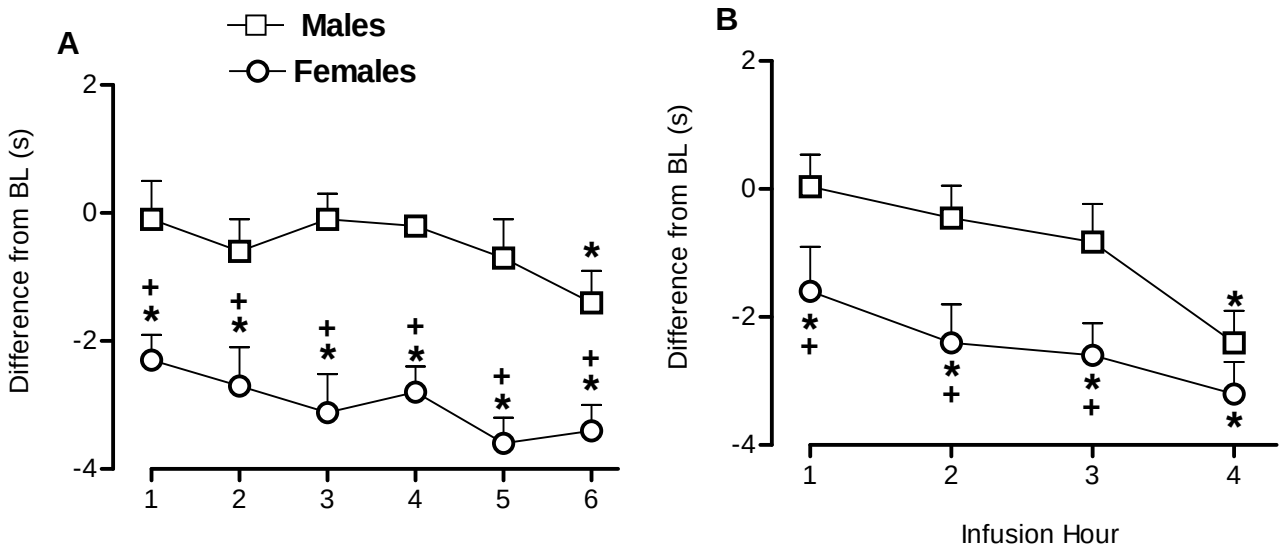
Tail withdrawal latencies were obtained in mice of both sexes implanted with NTX pellets 24 h prior to starting the infusion of morphine at cumulative daily doses of 1.6 **(A)** or 40.0 mg/kg **(B)**. Data are mean ± S. E. M. latency differences from baseline values.

* Significant difference relative to same sex BL latency ($p < 0.01$);
+ Significant difference relative to corresponding male values ($p<0.05$).

Sex differences in latency to hyperalgesic onset

In the above studies, both sexes were already hyperalgesic 24 h after starting 1.6 mg/kg morphine infusion. Thus, we tested placebo and NTX pelleted mice at hourly intervals after the start of infusion until both sexes were hyperalgesic. In placebo pelleted female and male mice, latencies reductions relative to BL were noted after 1 and 6 h, respectively (Figure 13A). In NTX pelleted female and male mice, hyperalgesia was evident 1 and 4 h later, respectively (Figure 13B).

Figure 13. Sex differences in hyperalgesic onset during morphine infusion.



Placebo (A) or NTX (B) pelleted mice were implanted with osmotic pumps infusing a cumulative daily dose of 1.6 mg/kg of morphine. Nociception was assayed at hourly intervals immediately after starting infusion until both groups were hyperalgesic. Data are mean ± S. E. M. latency differences from BL (i.e. pre-morphine values).

* Significant difference from same sex BL latency ($p < 0.01$);
+ Significant difference relative to corresponding male values ($p < 0.05$).

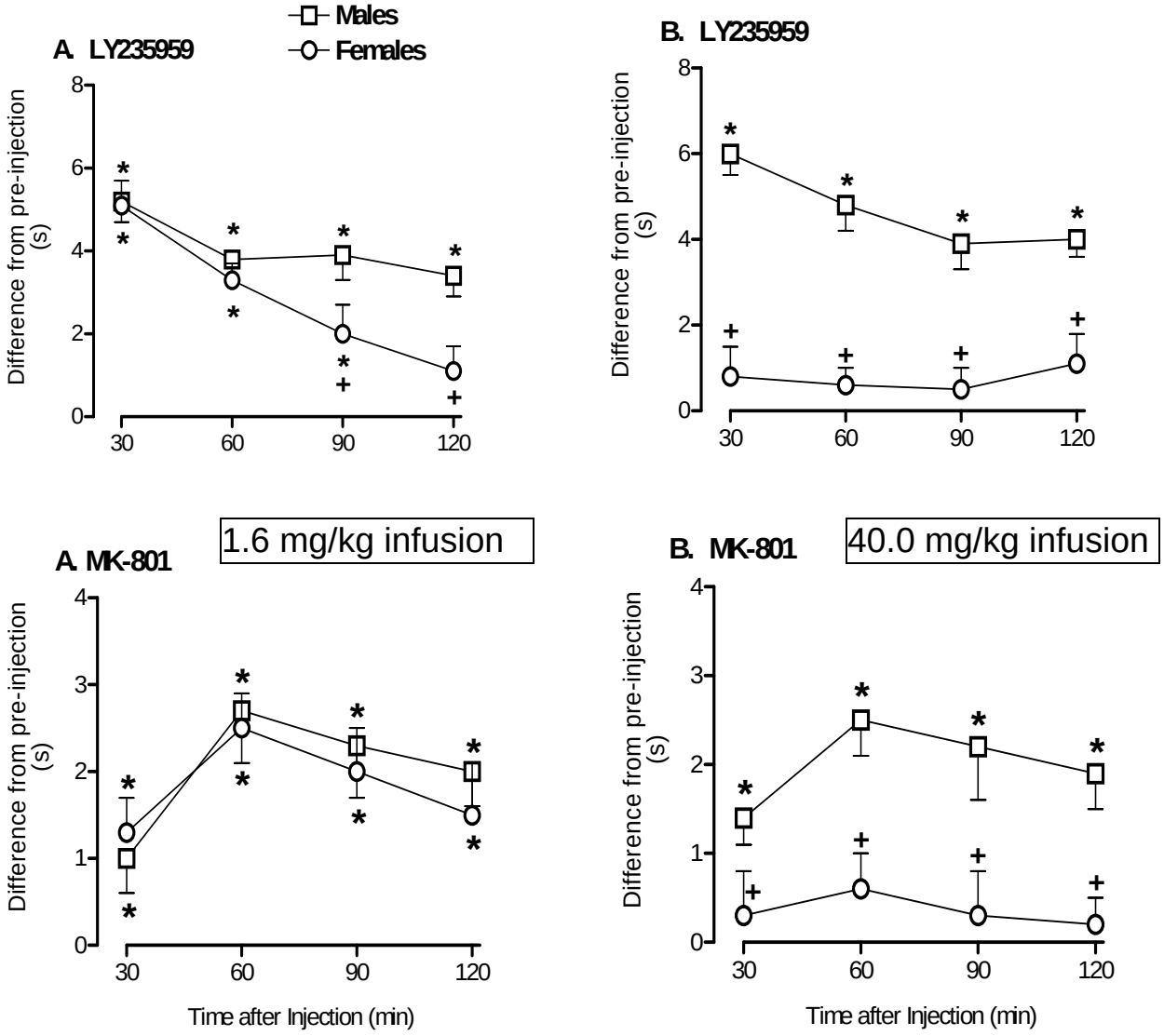
Sex differences in NMDA receptor blockade of morphine hyperalgesia.

Groups of NTX pelleted male and female mice were infused with either 1.6 or 40.0 mg/kg morphine. As in the above studies, both sexes displayed similar latency reductions on Days 2 and 6, respectively, and were subsequently injected with an NMDA receptor antagonist.

In males and females undergoing 1.6 mg/kg morphine infusion (Figures 14A), both LY235959 (top) and MK-801 (bottom) increased latencies (i.e. reversed hyperalgesia) relative to pre-injection values starting at 30 min, and generally lasting throughout the 120 min of testing. LY235959 was significantly more effective in males than females 90-120 min after injection (Figure 14A, top).

LY235959 and MK-801 also reversed hyperalgesia during 40.0 mg/kg morphine infusion in males, increasing their latencies relative to pre-injection values over the same time 120 minute time course, but had no effect on female latencies at any time (Figures 14B, top and bottom, respectively). For both males and females, withdrawal latencies were not altered by saline injection in morphine treated mice, or by NMDA receptor antagonist injection in morphine naïve mice (data not shown).

Figure 14. Sex differences in NMDA antagonism of morphine hyperalgesia



Mice of both sexes were subjected to morphine infusion with cumulative daily doses of 1.6 mg/kg **(A Figures)** or 40.0 mg/kg **(B Figures)**. On infusion Days 2 and 6, respectively, they were assayed for nociception (pre-injection latency) and subcutaneously injected with either LY235959 (2.5 mg/kg) or MK-801 (0.05 mg/kg). Nociception was reassessed every 30 minutes for the next 120 minutes. Data are mean ± S. E. M. post-injection latency differences from pre-injection values (0 value on the *y*-axis).

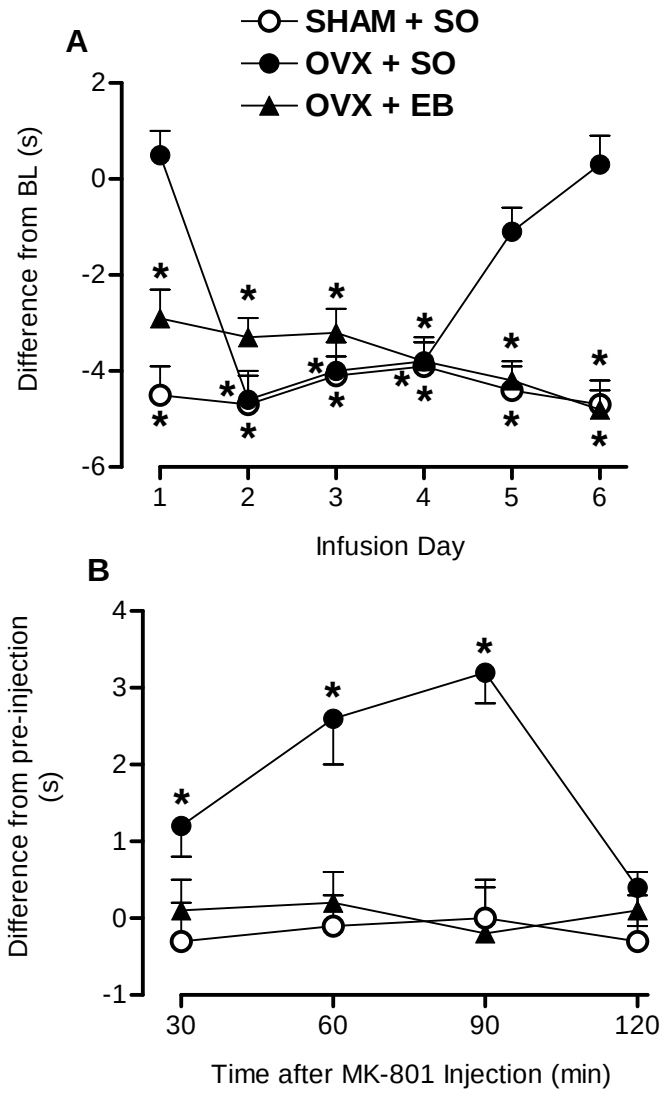
* Significant difference relative to same sex pre-injection value ($p < 0.01$);
+ Significant difference relative to corresponding male values ($p < 0.05$).

Effect of ovariectomy and estrogen replacement on morphine hyperalgesia

In the above studies, infusing 1.6 mg/kg morphine caused hyperalgesia in both sexes. But whereas hyperalgesia resolved in males by Day 6, it persisted in females for at least 14 days. To assess the contribution of ovarian sex steroids in sustaining morphine hyperalgesia, we subjected OVX mice, with and without subsequent EB treatment, and SHAM controls to continuous infusion of cumulative daily does of 1.6 mg/kg morphine. Figure 15A illustrates that 1.6 mg/kg morphine infusion caused significant latency reductions relative to BL starting on Day 2 in all 3 groups. However, whereas hyperalgesia in OVX + SO mice resolved by Day 5, withdrawal latencies in the OVX + EB and SHAM + SO mice remained significantly reduced through Day 6.

Earlier, (see Figures 14B), 40.0 mg/kg morphine infusion caused hyperalgesia that was refractory to reversal by NMDA receptor antagonists in females only. Here, the contribution of ovarian sex steroids to this sex difference was assessed by testing OVX mice, with and without EB treatment, and SHAM controls under identical conditions. All 3 groups were tested on Day 6 of 40.0 mg/kg morphine infusion, confirming hyperalgesia (data not shown), and then injected with MK-801. Within 30 min, the withdrawal latencies of OVX + SO were significantly increased relative to pre-injection values, and remained elevated for 90 min (Figure 15B). MK-801 injection did not alter the responses of OVX + EB or SHAM + SO mice. Injecting saline instead of MK-801 did not alter latencies in any of the 3 groups (data not shown).

Figure 15. Effect of ovariectomy on morphine induced hyperalgesia.



Female mice previously subjected to either ovariectomy (OVX) or SHAM surgery and then treated with estradiol benzoate (EB) or the sesame oil (SO) vehicle were infused with morphine.

Figure A: Tail withdrawal latencies at daily intervals during infusion of 1.6 mg/kg morphine. Data are mean ± S. E. M. latency differences from BL (i.e. pre-morphine values). * Denotes a significant difference from BL ($p < 0.01$).

Figure B: Mice were infused with 40.0 mg/kg morphine. On infusion Day 6, nociception was assayed before (pre-injection latency) and for 120 min after (post-injection latency) subcutaneously injection of MK-801 (0.05 mg/kg). Data are mean ± S.E.M. post-injection latency differences from pre-injection values.
* Denotes a significant difference from pre-injection values ($p < 0.01$).

DISCUSSION:

Hyperalgesia has been previously demonstrated in male mice undergoing continuous morphine infusion and treated simultaneously with NTX (Specific Aim 1). These findings are replicated here and extended to include female mice, support previous conclusions that morphine can activate mechanisms facilitating pain independently of those inhibiting pain or opioid receptor activity (Woolf, 1981; Crain & Shen, 2001; Holtman, Jr. & Wala, 2005). Although non-opioid morphine metabolites such as M3G have previously been implicated in this effect (Woolf, 1981; Sjogren et al., 1998), there are discrepancies in the present data that preclude drawing definitive conclusions regarding its role here. These include the rapid onset of hyperalgesia (within 1 hour) in females undergoing infusion of the low systemic morphine dose, and the inability of NMDA antagonists, which are also functional M3G antagonists (Bartlett et al., 1994a), to reverse hyperalgesia in females infused with the higher morphine dose. Identifying the basis of non-opioid hyperalgesia under the present morphine delivery protocols will require further work.

The data also reveal a persistent pattern of sex differences in hyperalgesic onset such that females always preceded males regardless of morphine infusion dose. Specifically, hyperalgesia in females was already evident just 1 h after starting 1.6 mg/kg morphine infusion, irrespective of concurrent treatment with NTX or placebo. In contrast, male latencies were not reduced until 4 and 6 h later, respectively. While it is not possible to accurately determine the cumulative morphine dose delivered by osmotic pumps filled

with a low morphine concentration during such a short infusion interval, it will assuredly be very low. This finding is consistent with the rapid hyperalgesic onset (5 min post-injection) in female, but not male rats, after low (0.002-0.02 mg/kg) acute systemic morphine doses (Holtman, Jr. & Wala, 2005), and suggests that morphine directly activates pain facilitating mechanisms rather than recruiting adaptive processes. Sex differences in onset were not limited to low morphine doses, as 40 mg/kg morphine also caused hyperalgesia in females that preceded onset in NTX and placebo pelleted males by 1 and 2 days, respectively. Whether this earlier hyperalgesic onset is related to reports of greater M3G accumulation in females following morphine exposure (Baker & Ratka, 2002) is an interesting possibility.

Infusing 1.6 mg/kg morphine in placebo or NTX treated mice caused hyperalgesia in males that resolved by Day 6, consistent with the findings of Specific Aim 1. In contrast, hyperalgesia persisted throughout the entire assessment period in identically treated females. OVX caused hyperalgesia in females to resolve at about the same time as males (Day 5), but EB treatment subsequent to OVX prevented this resolution. These findings are not easily attributable to OVX effects on nociception per se, since BL latencies were similar between OVX and non-OVX groups, and basal withdrawal latencies obtained during pilot studies conducted with OVX+SO mice were indistinguishable from naïve controls. Furthermore any OVX-induced changes in general nociceptive sensitivity would in any case be irrelevant, since latencies obtained during morphine infusion were always compared to within-group BL values. Likewise, changes in estrous cycle secondary to OVX are unlikely to be responsible for the changes noted here, as thermal

nociception in CD1 mice has previously been shown to relatively independent of alterations in gonadal hormone cyclicity (Mogil et al., 2000).

Interestingly, hyperalgesia in females resolved simultaneously with males after increasing the daily morphine infusion dose to 40.0 mg/kg, a 25-fold increase, suggesting that females utilized a different hyperalgesic mechanism for the 2 morphine doses. Nevertheless, there was still a qualitative sex difference in the efficacy of NMDA receptor antagonists' reversal of 40mg/kg hyperalgesia. Although both sexes displayed equal hyperalgesic magnitude on morphine infusion Day 6, NMDA antagonists returned latencies to, or near, BL values in males, but were entirely ineffective in females. Larger antagonist doses were not tested due to their adverse side-effects in our pilot studies and elsewhere (Mogil, Sternberg, Kest, Marek, & Liebeskind, 1993; Brosnan-Watters, Wozniak, Nardi, & Olney, 1996). Furthermore, the lack of efficacy is unlikely to be a dose related effect since identical antagonist doses were sufficiently large to reverse hyperalgesia in males and OVX females. As EB treatment reinstated MK-801 insensitivity in OVX mice, the data instead suggest that ovarian steroids inhibited the NMDA receptor contribution to hyperalgesia in intact females. How estrogen regulates MK-801 sensitivity is unknown, but it seems unlikely to be related to the regulation of NMDA receptor binding. In general, whereas OVX decreases specific NMDA receptor binding relative to intact subjects, estradiol after OVX increases binding (Cyr et al., 2001). Accordingly, NMDA receptor ligands should be less effective in OVX subjects and more effective when OVX is followed by EB. Here, the converse pattern is observed.

These data also indicate important qualitative sex differences in hyperalgesia for both morphine infusion doses. The ability of OVX to abolish these differences, and for EB treatment after OVX to reinstate them, suggests that intact females innately possess male-typical mechanisms but are diverted from their use by ovarian sex steroids. However, given that each morphine dose elicited distinct qualitative sex differences, the data suggest that morphine dose-dependently recruits distinct hyperalgesic systems in females. These morphine doses have already been shown in males to be mechanistically dissociable in Specific Aims 1 and 2. Similarly, other studies have reported variations in hyperalgesic susceptibility to naloxone blockade as a function of intrathecal morphine dosing regimen (Woolf, 1981; Crain & Shen, 2001). Whether the distinct systems described in this study are similar to any of the hyperalgesic mechanisms already described (Crain & Shen, 2000; Simonnet & Rivat, 2003; Xu et al., 2003; Ossipov et al., 2004) remains to be determined.

GENERAL DISCUSSION

The data reported in this dissertation illustrate several novel findings about opioid induced hyperalgesia. 1) Manifestation of OIH independent of prior or concurrent analgesia or opioid receptor activity. 2) Temporal independence of analgesic tolerance and hyperalgesia. 3) NMDA receptor regulation of OIH that is not related to attenuation or potentiation of morphine analgesia. 4) M3G contributes to the development of hyperalgesia during low-dose morphine infusion in males. 5) Demonstration of OIH in animals that are devoid of all 3 opioid receptors. 6) Unremitting hyperalgesia in females that persists long after cessation of opioid administration. 7) Sexually dimorphic mechanisms underlying identical hyperalgesic expression. 8) The simultaneous existence of both 'male' and 'female' mechanisms of OIH in females, with utilization dependent upon current gonadal hormone status. Given the different temporal pattern of hyperalgesia by the 2 morphine infusion doses used, and the different qualitative sex differences discovered for each, these data collectively indicate the existence of multiple mechanistically distinct morphine dose-dependent hyperalgesia systems in both males and females.

There are several limitations to the studies detailed within this dissertation. All assessments were conduced using either morphine or oxymorphone, substances that preferentially bind to the μ opioid receptor, a characteristic common to virtually all opioids reported to cause hyperalgesia in humans and rodents (Xu et al., 2003; Ossipov et al., 2004). Since we do not yet know how these opioids cause hyperalgesia

independently of opioid receptor activity, we can not predict whether the present findings can be extrapolated to include delta and kappa receptor opioids, or even other μ-preferring opioids. Further studies that assess the hyperalgesic tendencies of different opioids are required before such comparisons can be made. Likewise, it should be noted that findings of sex differences (when they occur) are frequently limited to the species, strain, and experimental methods described in a particular study (Riley, III et al., 1998; Mogil et al., 2000; Myers et al., 2001; Wang, Traub, & Murphy, 2006). Furthermore, since the dependent nociceptive measure in all the studies described in this dissertation, the tail withdrawal test, is a measure of thermal pain, it is also possible that different results would be obtained on other nociceptive measures such as mechanical pain or inflammation (see review by Mogil et al. 1999a). Applicability beyond the narrow conditions described above should thus not be assumed.

One possible explanation for the non-opioid receptor mediated hyperalgesia described in this dissertation involves secondary behavioral effects of morphine exposure. In addition to analgesia, morphine administration is also associated with a host of side effects including respiratory depression, euphoria, sedation, nausea, constipation, alterations of the endocrine and autonomic nervous system, temperature dysregulation, pruritus, and flushing of the skin (Andersen et al., 2003). In theory, alterations in tail withdrawal latencies could have been related to changes in core body temperature or motor excitation, which may have been mediated by non-opioid receptors. However, this supposition is unlikely for a number of reasons. First, these side effects have long been established to be dose-response effects, yet hyperalgesia was manifest immediately

during low-dose infusion, but was delayed during high-dose infusion, even under conditions of opioid receptor blockade. Second, many opiate induced side effects including motor excitation are blocked by naloxone (Snead, III, 1986; Shohami, Evron, Weinstock, Soffer, & Carmon, 1986), while hyperalgesia in our studies was unaffected by opioid receptor blockade. Finally, reports of morphine induced hyperthermia being blocked by opioid antagonists (Baker & Meert, 2002; Rawls, Amin, & Zisk, 2007), suggests that it is mediated by opioid receptors, and thus cannot account for the patterns of hyperalgesic expression described in the current set of experiments.

A more plausible explanation involves M3G, morphine's non-opioid primary metabolite. Although there is still much to be learned about its physiological properties and pharmacodynamics, its pronociceptive nature, negligible opioid receptor affinity, and cross-adaptation with low-dose hyperalgesia are certainly suggestive of it playing a primary role during 1.6 mg/kg morphine infusion in males. Although the relationship is not as clear, sex differences in M3G may also be at least partially responsible for the altered hyperalgesic expression (characterized by lack of hyperalgesic adaptation and earlier onset), observed during low-dose infusion in females. For example, a recent study comparing morphine analgesia with metabolic accumulation (Baker & Ratka, 2002) reported that relative to males, female rats demonstrate significantly lower levels of morphine analgesia, and mean plasma M3G:morphine ratios nearly 3 times greater than males. In addition, we have observed that whereas continuous M3G infusion (in drug naïve animals) reduces withdrawal latencies for 12 days in males before returning to baseline values, hyperalgesia persists in females (unpublished observation). These

findings suggest that gender differences in M3G may be related to the earlier onset and prolonged duration of hyperalgesia observed during low-dose infusion in females. Interestingly, OVX markedly reduces plasma M3G levels in females to values obtained in males (Baker & Ratka, 2002), and in Specific Aim 3, the hyperalgesia time course during low-dose infusion in OVX females is shortened, becoming identical to that of males. This quality is unlikely to be related to estrogen's effect on analgesia (Mogil et al., 2000; Barrett et al., 2002; Stoffel et al., 2003), as the results in our study were obtained with sub-analgesic doses of morphine (1.6 mg/kg) as well as during periods of opioid receptor blockade. Moreover, NMDA antagonists, which can also act as functional M3G antagonists (Bartlett et al., 1994a), reversed hyperalgesia only at the lower, but not the higher, morphine infusion dose in females. This dovetails nicely with the findings presented here, and provides ancillary support for the notion that only low-dose hyperalgesia is mediated by M3G, whereas high-dose hyperalgesia is mediated by another unidentified mechanism.

Clearly, the relationship between M3G and the current data is speculative, as there are number of outstanding issues that remain unaddressed at this time. If M3G accumulation is indeed responsible for low-dose hyperalgesia, it should certainly have a demonstrable effect following the 25-fold larger 40.0 mg/kg infusion dose. Yet, there was no cross-adaptation between M3G and morphine hyperalgesia in specific aim 1. Furthermore, assuming that morphine hyperalgesia in CD-1 and C57BL/6 mice are mediated by identical mechanisms, why is hyperalgesia in the latter not reversed by antagonists for the NMDA receptor, a putative site of M3G activity? Future studies utilizing clofibrate, a

drug that can prevent morphine glucoronidation (Faura, Olaso, Garcia, & Horga, 1996), might begin to elucidate the contribution of M3G to morphine hyperalgesia.

It is interesting to consider whether unremitting hyperalgesia may exist in human females, analogous to the findings manifested in female mice during low-dose infusion. Although numerous surveys of opioid addicts (Sosa-Zapata, Colon, Robles, & Cabassa, 2007; Larson et al., 2007) have not demonstrated this sex difference, this may be due to dosage effects. Unremitting hyperalgesia was only demonstrated in our study following low-dose infusion which had no analgesic or presumably reinforcing properties, and achieved its effects through non-opioid receptor mediated mechanisms. Opioid addicts, who are ostensibly receiving some form of mu-receptor mediated euphoric effect, are thus more akin to the high-dose morphine groups which manifested adapting hyperalgesia equally in both sexes. Patients receiving long-term opioid agonist therapies (OAT) for addiction, such as methadone or buprenorphine maintenance treatment, may therefore provide for better comparisons to the low-dose paradigm due to 1) controlled administration paradigms, 2) use of low doses of opioid agonists, and 3) the relative lack of analgesia and euphoria associated with OAT treatment. Indeed, there are indications that patients receiving OAT report higher levels of acute pain (Alford, Compton, & Samet, 2006; Dursteler-MacFarland et al., 2006), but no mention was made of sex differences. Experimental paradigms using the cold pressor test revealed that opioid addicts manifested persistent hyperalgesia even after cessation of opioid use (Pud et al., 2005), but also did not report sex differences.

For the larger morphine infusion dose, it was surprising to find that despite identical hyperalgesic expression in males and females, NMDA receptor antagonism only reversed hyperalgesia in males but was without effect in females. Although gonadal hormones were responsible for this insensitivity (not apparent in OVX mice but was reinstated in OVX+EB mice), this effect is clearly unrelated to widely reported sex differences in morphine analgesia (Cicero et al., 1997; Kest et al., 2000a; Barrett et al., 2002; Wang et al., 2006; Bernal et al., 2007), as the studies with NTX implantation and KO mice proved that prior analgesia and opioid receptor activity are not relevant to subsequent hyperalgesic expression. It is also interesting to note that this effect was only limited to 40 mg/kg infusion, whereas low-dose infusion, despite the lack of hyperalgesic adaptation in females, was equally responsive to NMDA antagonism in both sexes. The method by which estrogen mediates these changes will hopefully be addressed in future studies.

While a comprehensive understanding of the mechanisms underlying high-dose hyperalgesia still remains at large, the current series of studies have highlighted a number of important factors. As suggested by its susceptibility to NMDA receptor antagonism, high-dose hyperalgesia in males (but not intact females) is under the influence of NMDA receptors. It thus comes as no surprise that others have reported (Lipa & Kavaliers, 1990) that greater increases in morphine analgesia are found in male mice following MK-801 administration relative to females. The fact that MK-801 does not alter morphine pharmacokinetics (Maeda et al., 2002), indicates that sex differences observed following MK-801 administration are unlikely to be related to alterations in M3G or other

metabolite formation. This supports our assertion derived from the cross adaptation findings that high-dose hyperalgesia is not mediated by M3G. Moreover, as M3G has been shown to initiate its effects through indirect activation of NMDA receptors (Hemstapat et al., 2003), the failure of MK-801 to reverse hyperalgesia in females provides additional support favoring a non-contributory role for M3G in this process, and the existence of sex-specific hyperalgesic mechanisms.

It is intriguing to consider the current findings in relation to previous studies reporting analogous qualitative sex differences in opioid analgesia. Specifically, NMDA receptor antagonists have been shown to effectively reduce kappa opioid (Kavaliers & Choleris, 1997) and non-opioid swim stress-induced analgesia (Mogil et al., 1993) in male but not female mice. The latter study also reported that MK-801 is an effective antagonist when testing analgesia only in OVX females, while OVX followed by estrogen injection reinstates the MK-801 insensitivity characteristic of intact females. Based on these findings, the authors concluded that estrogen diverts pain modulation in females towards a system that is independent of the NMDA receptor contribution which is evident in males. Here, the data suggest identical conclusions with respect to morphine hyperalgesia, as estrogen replacement reinstated NMDA receptor antagonist insensitivity during high-dose infusion and terminal hyperalgesia during low-dose infusion. Despite these very intriguing similarities, the data can not address whether they represent a single phenomenon, nor whether the presence of both male- and female- typical systems in intact female subjects affords some adaptive benefit.

The current findings also have interesting implications within the greater context of gender-specific neural mechanisms of nociception. For example, there are a number of clinical disorders such as irritable bowel syndrome, fibromyalgia, temporomandibular disorder, and chronic fatigue syndrome that exhibit much higher prevalency rates in females relative to males (Staud et al., 2003; Payne, 2004; Ouyang & Wrzos, 2006). Surprisingly, despite much research, investigators have been unable to find adequate explanations for these pathological pain states (Lucas, Brauch, Settas, & Theoharides, 2006; Spiller, 2007), leading them to focus on identifying gender differences in neuronal circuitry and nociception (Bespalov et al., 2001; Wood, 2006). Thus, further elucidation of the mechanisms underlying the unrelenting hyperalgesia in females described above may provide important insights into these pathological pain states as well. For instance, i.t. morphine administration was shown to elicit analgesia that in males was solely dependent upon μ receptor activation, while analgesia in females also required the additional activation of spinal κ receptors and the production of spinal dynorphin (Liu, von, & Gintzler, 2007). Likewise, numerous studies have reported that κ activating opioids utilize different physiological circuitry in males and females (Sternberg, Ritchie, & Mogil, 2004; Holtman, Jr. & Wala, 2006; Lomas, Barrett, Terner, Lysle, & Picker, 2007), leading to them being significantly more effective analgesic agents in both rodent and human females (Miller & Ernst, 2004; Mogil et al., 2005). A striking example of this phenomenon was demonstrated by (Mogil et al., 2003) in a study involving inbred mutant mice strains and healthy red-headed female volunteers, groups which are identically deficient in melanocortin-1 receptors. The authors reported significantly greater nociceptive thresholds and M6G induced analgesia in both experimental female groups

relative to male controls, indicating the presence of cross-species gender specific neuronal mechanisms of nociception. Future studies have been planned to assess the role of this *MC1R* gene in morphine hyperalgesia, specifically in the mediation of the sex differences described above.

Based on the current findings, opioid-induced hyperalgesia appears to be an active process leading to pain sensitization that is initiated by the first opioid exposure as suggested from the early time course studies, and the demonstration of hyperalgesia following premature removal of infusion pumps. Although its mechanisms are not fully understood, it is clear that OIH is not mediated by opioid receptors, is independent of prior analgesia, and pharmacologically and physiologically distinct from withdrawal induced hyperalgesia and analgesic tolerance. However, because it is initiated and maintained by circulating levels of opioids, it frequently opposes the intended analgesic effect that was the basis for the opioid therapy in the first place, necessitating dose escalation and fostering the illusion of drug tolerance. Thus while the findings of this dissertation certainly do not advocate limiting the clinical use of opioids for pain management, they will hopefully encourage further investigation into the mechanisms underlying OIH in an attempt to enhance analgesic potencies and reduce debilitating side effects. Understanding the causes of this heightened sensitivity is the first step in providing safer and more effective pain management techniques. Furthermore, pharmacological agents that can reduce or prevent the functioning of some of the pronociceptive mechanisms described in this paper (NMDA receptor activity, M3G accumulation, gonadal hormones), hold great promise as possible adjuvants to opioid

therapy by reducing the likelihood of pain escalation and sensitization, thereby increasing the quality of life for those suffering from chronic pain and other medical conditions.

APPENDIX

Abbreviations

AD_{50} Analgesic Dose 50

BL baseline

CI confidence interval

^{o}C degrees Celsius

CNS central nervous system

DLF dorsal lateral funiculus

DRG dorsal root ganglion

EB estradiol benzoate

ED_{50} Effective Dose 50

h hour

i.c.v. intracerebroventricular

i.t. intrathecal

kg kilogram

LH leutenizing hormone

LTP long term potentiation

M3G morphine-3-β-glucoronide

mg milligram

ml milliliter

MPE maximum possible effect

NMDA *N*-methyl-*D*-aspartate

NTX naltrexone

OAT opioid agonist therapy

OIH opioid induced hyperalgesia

OVX ovariectomy

PAG periaqueductal gray

PKC protein kinase C

PNS peripheral nervous system

RVM rostral ventromedial medulla

s seconds

SHAM sham surgery

SO sesame oil

TMD temporomandibular disorders

TW tail withdrawal

WIH withdrawal induced hyperalgesia

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