ACUTE MORPHINE HYPERALGESIA: A PHARMACOLOGICAL AND PHARMACOGENETIC ANALYSIS

by

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Abstract

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Opioids such as morphine remain the most efficacious and widely used analgesics for moderate to severe pain. However, the clinical use of these opioids is complicated by unwanted side effects, including a paradoxical increase in pain sensitivity (i.e. hyperalgesia). Previous studies have demonstrated that sustained delivery of these opioids causes hyperalgesia independent of opioid receptor activity or analgesia. Unknown, however, is if a single acute morphine bolus dose similarly induces hyperalgesia that is independent of opioid receptor activity or analgesia. The current studies aimed to characterize the pharmacological, physiological, and organismic variables that govern acute morphine hyperalgesia.

Outbred CD-1 male mice, pretreated with the general opioid receptor antagonist, naltrexone (NTX), did not exhibit analgesia, but demonstrated hyperalgesia after systemic administration of morphine. Acute morphine injection caused significant decreases in withdrawal latencies on the tail-withdrawal test and significant increases of licking behavior on the formalin test, both indicative of hyperalgesia, in male CD-1 mice pretreated with NTX and in male mice lacking all three genes encoding the μ, δ, and κ opioid receptors. Additionally, male mice lacking the pronociceptive morphine metabolite, morphine-3β-glucuronide (M3G), also demonstrated acute morphine hyperalgesia. Therefore, acute morphine hyperalgesia was characterized as an active process that is independent of prior or concurrent opioid receptor activity, analgesia, and the

morphine metabolite, M3G.

Additionally, to assess whether spinal and/or supraspinal loci contribute to acute morphine hyperalgesia, mice were pretreated with NTX and then tested for nociception on the tail-flick test before and after receiving an acute morphine via the intrathecal (i.t.) or intracerebroventricular (i.c.v.) route. Acute i.t. and i.c.v. morphine injection both caused significant hyperalgesia in mice subject to concurrent opioid receptor blockade by NTX, indicating that supraspinal and spinal loci significantly contribute to acute morphine hyperalgesia.

Since acute morphine hyperalgesia occurs independently of opioid receptor activity and morphine metabolites, the contribution of *N*-methyl-D-aspartate (NMDA) receptors in acute morphine hyperalgesia was investigated. Systemic administration of the NMDA receptor antagonist MK-801 completely abolished acute morphine-induced hyperalgesia in CD-1 male mice pretreated with NTX. Furthermore, the contribution of NMDA receptors at spinal and supraspinal loci was also studied in NTX-treated mice by assaying nociception before and after i.t. or i.c.v. MK-801 injection in mice treated with an acute systemic morphine. MK-801 injected supraspinally completely blocks hyperalgesia induced by systemic injection of morphine, whereas MK-801 injected into the spinal cord reduces, but does not completely block, hyperalgesia induced by systemic morphine injection.

Lastly, since interindividual differences in magnitude of morphine withdrawal, analgesia, and tolerance have been previously demonstrated to be associated with genotypic variation, we also assessed whether the magnitude of acute morphine-induced hyperalgesia is similarly genotype-dependent. The data revealed marked genetic variation; whereas some strains displayed a 30-50% reduction in withdrawal latency, other strains failed to become hyperalgesic at any time during the 120-minute post-morphine testing interval. Our findings demonstrate that

the magnitude of acute morphine hyperalgesia is genotype-dependent.

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Glossary of Abbreviated Terms

OIH – opioid-induced hyperalgesia
NMDA – *N*-methyl-D-aspartate
TKO – triple knockout
DRG – dorsal root ganglion
PAG – periaqueductal gray
RVM – rostroventral medulla
CCK – cholecystokinin
PKC – protein kinase C
DAMGO – Tyr-D-Ala2-Gly-NMePhe4-Gly-ol5
EAA – excitatory amino acid
s.c. – subcutaneous
i.c.v. – intracerebroventricular
i.t. – intrathecal
M3G – morphine-3β-glucuronide
M6G – morphine-6β-glucuronide
NTX – naltrexone
NK – neurokinin
ORL1 – opioid receptor-like type receptor
GABA – γ-aminobutyric acid
CSF – cerebrospinal fluid
Mrp3 – multidrug resistance protein 3
CGRP – calcitonin gene-related peptide
WIH – withdrawal-induced hyperalgesia

Chapter 1.

I. Introduction

In 1804, German apothecary Friedrich Sertürner isolated the most abundant alkaloid compound found in opium, the dried sap derived from the unripe seedpods of the opium poppy, *Papaver somniferum*. He initially named this substance, “morphium”, after the Greek god of dreams Morpheus, for its tendency to induce sleep. The discovery of the pain relieving effects of morphine stimulated its widespread distribution and use. Today, morphine continues to be recognized as the most efficacious form of treatment for moderate to severe pain (Mao, Sung, Ji, & Lim, 2002) and thus, the most effective analgesic. A term utilized for humans, analgesia is defined as the reversal of the subjective sensation of pain, which involves a decrease in the sensory component of pain as well as affective components such as discomfort or unpleasantness. Similarly, the term antinociception is specifically used to refer to reductions in noxious or painful stimuli without reference to any putative affective changes. Thus, the term antinociception is most commonly and accurately used in studies with animals to avoid the anthropomorphic connotation of the term analgesia.

The long-term clinical use of morphine and other opioids is complicated by unwanted side effects, including an increase in pain sensitivity (i.e., hyperalgesia) (Arner, Rawal, & Gustafsson, 1988; De Conno, et al., 1991). Opioid-induced hyperalgesia (OIH) is defined as the paradoxical response to opioid agonists, whereby instead of an analgesic, or antinociceptive effect, there is an increase in pain perception. In other words, a patient receiving opioids for the treatment of pain could actually become more sensitive to certain painful stimuli. This pain may be described as diffuse, of a different quality, and unassociated with previous tissue damage. These clinical observations are confirmed in laboratory studies, showing that sustained opioid

exposure via continuous infusion or repeated injection reduces sensory nociceptive thresholds in rodents (Angst & Clark, 2006). Further studies have demonstrated that continuous morphine infusion in mice causes hyperalgesia in mice treated concurrently with the general opioid antagonist naltrexone (NTX) or in opioid receptor triple knock-out (TKO) mice, lacking all three genes encoding the μ, δ, and κ opioid receptors (Juni, Klein, & Kest, 2006; Juni, Klein, Pintar, & Kest, 2007). In other words, sustained delivery of these opioids causes hyperalgesia independently of opioid receptor activity or analgesia. Since most of the literature has primarily investigated chronic morphine treatment, a thorough analysis of acute morphine hyperalgesia is necessary to contribute to our understanding of the underlying mechanisms, organismic factors, and neurocircuitry underlying this phenomenon.

The overall goal of the current series of studies is to characterize the pharmacological, physiological, and organismic variables that govern acute morphine hyperalgesia. The first aim of Study 1 was to further assess the opioid nature of acute morphine hyperalgesia in mice treated with the general opioid receptor antagonist naltrexone (NTX) by describing the time- and dose-response characteristics of acute morphine hyperalgesia in a murine model across two pain modalities (Study 1A). The tail-withdrawal test, a commonly used measure of thermal nociception, was used to determine basal nociceptive thresholds and subsequent variations over time. The formalin test is a model of acute inflammatory pain which produces 2 phases of nociceptive behavior (Dubuisson & Dennis, 1977). For both, mice were assayed for nociceptive sensitivity prior to and after an acute morphine dose. Upon establishing reliable protocols, Study 1B repeated this paradigm in TKO mice deficient in μ, δ, and κ opioid receptors (Clarke, et al., 2002; Cox, et al., 2005). To assess the putative role of morphine metabolites in morphine hyperalgesia, Study 1C examined mice lacking multidrug resistance protein 3 (Mrp3) (Zelcer, et

al., 2005), which do not display any measureable levels of the pronociceptive morphine metabolite morphine-3β-glucoronide (M3G). Determination as to whether OIH was present following opioid administration across the neuraxis led to the specific aim of Study 2. In this study, the contribution of spinal and supraspinal loci in acute morphine hyperalgesia was assessed by testing nociception in mice concurrently treated with NTX, after injecting morphine via the intrathecal and intracerebroventricular route, respectively. Other neurochemical mechanisms mediate morphine's analgesic effects and thus, in Study 3, an exploration of the contribution of *N*-methyl-D-aspartate (NMDA) was conducted to identify potential underlying mechanisms of OIH. In order to determine the involvement of NMDA receptors at spinal and supraspinal loci in acute morphine hyperalgesia, nociception was measured before and after intrathecal (i.t.) and intracerebroventricular (i.c.v.) injection of an NMDA-antagonist in NTX-treated mice given acute systemic morphine. Lastly, interindividual variables attributable to genetic background have been shown to be important in morphine analgesia. Therefore, having established an acute morphine paradigm to study OIH in males in Study 1, the specific aim of Study 4 was to investigate acute morphine hyperalgesia in mice of 10 different inbred strains.

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

1. Opioid pharmacology
    a) opioid receptors
    b) opioid receptor modulation of nociception
    c) opioid metabolites
2. Prevalence of opioid-induced hyperalgesia

a) human studies

b) animal studies

3. Mechanisms of opioid-induced hyperalgesia

a) opioid receptor involvement

b) cellular sensitization

c) NMDA receptor involvement

d) pronociceptive opioid metabolites

4. Contribution of genetics to opioid-induced hyperalgesia

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

II. Background

1. Opioid Pharmacology

a) Opioid receptors

In 1973, three different groups simultaneously reported the existence of endogenous receptors for opioids (Pert & Snyder, 1973; Simon, 1973; Terenius, 1973). These findings pointed toward the existence of endogenous pain relieving mechanisms. Technically, the term opiate applies specifically to substances extracted from the exudate of the opium poppy *Papaver somniferum*, and is restricted to two drugs that are naturally found in the exudate: morphine and codeine (Julien, Advokat, & Comaty, 2008). The broader term opioid refers to all substances (endogenous and exogenous) that bind to opioid receptors. Exogenous opioids can be natural (morphine), semisynthetic (heroin), or synthetic (fentanyl). In addition to exogenous opioid

substances, five distinct classes of endogenous opioid peptides have also been discovered: endorphins, enkephalins, dynorphins, endomorphins, and nociceptin (see reviews: Akil, et al., 1984; Fichna, Janecka, Costentin, & Do Rego, 2007; Mustazza & Bastanzio, 2011; Olson, Olson, Kastin, & Coy, 1979). Even though both terms, opiate and opioid, are often used interchangeably within the literature, only the term opioid will be used throughout this dissertation.

With the discovery of an endogenous opioid system, Martin and colleagues (1973) first proposed the existence of multiple subtypes of opioid receptors through numerous pharmacological studies. The study identified and attributed three different behavioral syndromes apparent in spinalized dogs to the interaction of agonists with three distinguishable receptors (mu, kappa, and sigma). Morphine was identified as the prototype agonist for the mu receptor, ketocyclazocine for the kappa receptor, and SKF-10,047 for the sigma receptor. Shortly after, the delta opioid receptor was identified and termed after the observation of differential binding for [Met^{5}]enkephalin and β-endorphin against [^{3}H][Leu^{5}]enkephalin and [^{3}H]naloxone in guinea pig brain (Lord, Waterfield, Hughes, & Kosterlitz, 1977; Waterfield, Leslie, Lord, Ling, & Kosterlitz, 1979; Waterfield, Lord, Hughes, & Kosterlitz, 1978). Advances in cloning and molecular technology led to the clear identification of 3 opioid receptors, mu, kappa, and delta (Y. Chen, Mestek, Liu, Hurley, & Yu, 1993; Evans, Keith, Morrison, Magendzo, & Edwards, 1992; Kieffer, Befort, Gaveriaux-Ruff, & Hirth, 1992; Minami, et al., 1993; Ossipov, et al., 2004). Most recently, a fourth opioid-receptor was identified, the opioid receptor-like type (ORL1) receptor, and has similar structural and functional homology with the other opioid receptors (Mollereau, et al., 1994). The endogenous neuropeptide, termed orphanin FQ or nociceptin (OFQ/N), demonstrates exceedingly high

affinity to the ORL1 receptors, but does not interact with the classical opioid receptors (Meunier, et al., 1995; Reinscheid, et al., 1995).

The distributions and anatomical localizations of the opioid receptor subtypes has been explored via autoradiography, radio-ligand binding, in situ hybridization of mRNA for the receptors, and immunohistochemistry. In terms of spinal sites of action, these receptors are concentrated in the outer laminae of the dorsal horn in the spinal cord. Specifically, the μ-opioid receptor and ORL1 receptor are highly concentrated in the outer laminae of the spinal dorsal horns, δ-opioid receptor is diffusely distributed in the outer laminae, the κ-opioid receptor is concentrated in the outer laminae of the dorsal horns of the lumbosacral cord, receiving nociceptive inputs from the viscera (Besse, Lombard, & Besson, 1991; Besse, Lombard, Zajac, Roques, & Besson, 1990; Quirion, 1984; Quirion, Zajac, Morgat, & Roques, 1983). All subtypes are expressed primarily in nociceptive C- and Aδ fibers of the dorsal root ganglia (DRG) cells as displayed by immunohistochemistry (Arvidsson, et al., 1995; Dado, Law, Loh, & Elde, 1993).

Nociceptive signals entering at the level of the spinal cord are regulated by intrinsic interneurons and by descending inhibitory projections from supraspinal sites that are also activated directly by opioid receptors. Brain regions that express opioid receptors include the frontal cortex, nucleus accumbens, hippocampus, thalamus, and hypothalamus (Quirion, 1984; Quirion, et al., 1983). The periaqueductal gray (PAG) and the rostroventral medulla (RVM), two regions critical in opioid-mediated antinociception, were also identified as expressing opioid receptors. The PAG and RVM are rich in μ-opioid receptors, whereas levels of δ-opioid and κ-opioid receptors are low or undetectable (Mansour, Khachaturian, Lewis, Akil, & Watson, 1987).

b) Opioid receptor modulation of nociception

At the spinal level, opioids are believed to act primarily through opioid receptors residing on the central terminals of these primary afferent C-fibers (Mansour, Fox, Akil, & Watson, 1995) and exert strong antinociceptive effects in animals models and clinical practice. Opioid receptors are presumed to exert their effects by directly inhibiting excitatory transmission and ascending nociceptive inputs, thereby limiting the organism's sensitivity to noxious stimuli (Kohno, Kumamoto, Higashi, Shimoji, & Yoshimura, 1999; Rusin & Moises, 1995; Wu, Chen, & Pan, 2004). Studies employing electrophysiological recordings from isolated nociceptors revealed that spinal opioid receptor activation predictably inhibited C-fiber calcium channels (Cata, Weng, Chen, & Dougherty, 2006; Taddese, Nah, & McCleskey, 1995), which are one of the primary afferent pathways for pain perception. Furthermore, it has been shown that functional blockade of spinal opioid receptors leads to a marked reduction in the analgesic potency of opioids (Hara, et al., 1999; Yaksh, 1981).

Supraspinal (or descending) control of spinal nociception originates from several brain regions, most prominently from the PAG and the RVM and their connections. The PAG is heavily interconnected with the hypothalamus and limbic forebrain structures including the amygdala, anterior cingulate cortex, and medial prefrontal cortex (Heinricher, Tavares, Leith, & Lumb, 2009; Jacquet, 1982; Jacquet & Squires, 1988). The ventrolateral portion of the PAG mediates a strong, naloxone-irreversible antinociceptive effect (Yaksh, Yeung, & Rudy, 1976). The PAG exerts control over different populations of superficial dorsal horn neurons, which then project to deep dorsal horn neurons. Descending inhibitory control from the dorsolateral/lateral and ventrolateral columns of the PAG preferentially targets deep dorsal horn neurons with C-fiber inputs, through direct post-synaptic action in the superficial dorsal horn (Waters & Lumb, 2008). In addition, neuronal activation in the PAG may simultaneously, through indirect

connections, enhance the responses of other neurons with weak or no C-fiber input in the superficial dorsal horn (Heinricher, et al., 2009).

Opioids activate neurons in the PAG and then its descending projections excite neurons in the RVM (Basbaum, Clanton, & Fields, 1978; Basbaum & Fields, 1978, 1984). The RVM is defined as the region of the medulla including the nucleus raphe magnus, the nucleus gigantocellularius pars alpha, and surrounding reticular neurons ventral to the nucleus gigantocellularius and extending between the caudal facial nucleus and the inferior olivary complex (Ossipov, et al., 2004). Mediating bidirectional control of nociception, the RVM projects diffusely to dorsal horn laminae critical in nociceptive processing, including superficial layers and deep dorsal horn (Zhuo & Gebhart, 1997). Specific classes of cells in the RVM contribute in different ways to the descending pathways (Fields & Heinricher, 1985; Fields, Heinricher, & Mason, 1991). The RVM contains “on”, “off”, and “neutral” cells. The on and off-cells provide the neural basis for the bidirectional control from the RVM. On-cells are defined by a burst of activity associated with withdrawal reflexes and have been identified as the source of descending facilitation of nociception. Direct, selective activation of on-cells produces hyperalgesia and reduction in the threshold, at which the on-cell burst is triggered, is associated with a decrease in reflex latency (Heinricher & Neubert, 2004; McGaraughty, et al., 2003; Neubert, Kincaid, & Heinricher, 2004). On the other hand, the off-cells function as the antinociceptive output from the RVM (Heinricher and Tortorici, 1994; Heinricher et al., 1994; Neubert et al., 2004). Lastly, neutral cells show no nocifensor reflex-related changes in firing (Kincaid, Neubert, Xu, Kim, & Heinricher, 2006; Xu, Kim, Neubert, & Heinricher, 2007). In order to determine how these cells contribute to descending control of nociceptive behavior, studies suggest that neutral-cells may be recruited to become on- or off-cells during development

of chronic pain states (Miki, et al., 2002) or their serotonergic characterization may play a role in nociceptive modulation (Heinricher, et al., 2009).

Regulating dorsal horn function, the on and off-cell populations maintain an equilibrium that when shifted, is thought to directly influence nociceptive threshold (Fields & Heinricher, 1985). Nocifensive reflexes such as the tail flick or paw withdrawal to noxious heat are marked by a shift in the balance between the populations such that the on-cells enter an active phase, whereas the off-cells become silent (Heinricher, Barbaro, & Fields, 1989). Specifically, prolonged delivery of a noxious thermal stimulus produced increase on-cell discharge along with a facilitation of nociceptive reflexes, whereas inactivation of RVM activity with lidocaine blocked the facilitated withdrawal response (Morgan & Fields, 1994). In contrast, microinjection of cholecystokinin (CCK_8), an endogenous “anti-opioid” peptide, into the RVM attenuated morphine-induced activation of off-cell activity and elicited enhanced sensitivity to normally innocuous mechanical stimuli (Heinricher, McGaraughty, & Tortorici, 2001).

c) Opioid metabolites

In humans, morphine is metabolized by glucuronidation in the liver. Glucuronidation, catalyzed by a specific set of UDP-glucuronosyl transferases (UGTs), results in the formation of more hydrophilic and less toxic metabolites in order to aid in elimination (Dutton & Storey, 1954). For morphine, UDP-glucuronosyl transferase 2B7 (UGT2B7) converts morphine into morphine-3β-glucuronide (M3G) and morphine-6β-glucuronide (M6G), through the removal of free hydroxyl groups at the third and sixth carbon atom (Bock, Brunner, Hoensch, Huber, & Josting, 1978; Iwamoto & Klaassen, 1977). In humans, approximately 15% of systemically available morphine is converted into M6G while more than 50% is metabolized into M3G (Boerner, 1975). There is a larger proportion of M3G formation because aromatic hydroxyl

groups serve as easier conjugation recipients than do alicyclic hydroxyl groups. For this reason, rodents can not form M6G, only M3G (Aasmundstad, Ripel, Bodd, Bjorneboe, & Morland, 1993; Kuo, Hanioka, Hoshikawa, Oguri, & Yoshimura, 1991).

Membrane transport systems modulate the pharmacokinetics of morphine (Bourasset, Cisternino, Temsamani, & Scherrmann, 2003; M. King, Su, Chang, Zuckerman, & Pasternak, 2001; Schinkel, et al., 1994; Thompson, Koszdin, & Bernards, 2000). MDR1 p-glycoprotein (P-gp), a multidrug transporter belonging to the ATP-bind cassette (ABC) family of membrane transporters and an essential component of the blood brain barrier, limits morphine accumulation in the brain (M. King, et al., 2001; Thompson, et al., 2000). To prevent the intracellular accumulation of the hydrophilic morphine glucuronides after their formation, specific carriers transport them out of the hepatocytes. *In vitro* experiments indicate that Mrp3, multidrug resistant protein 3, transports morphine glucuronides from heptacytes into the bloodstream (Zelcer, et al., 2005). Once in the bloodstream, the glucuronides exert their impact on the organism via an unknown mechanism.

The pharmacological effects of these metabolites differ profoundly. M6G displays affinity at opioid receptors equal to that of morphine and has a 20-40 higher potency than morphine (Christensen & Jorgensen, 1987; Dahan, van Dorp, Smith, & Yassen, 2008; Kilpatrick & Smith, 2005; Pasternak, Bodnar, Clark, & Inturrisi, 1987; Rossi, et al., 1997; Shimomura, Kamata, Ueki, Ida, & Oguri, 1971). Originally identified as a potent opioid analgesic (Pasternak, et al., 1987), some evidence now suggests that acute and chronic M6G infusion can cause hyperalgesia in humans and mice (Kitanaka, Sora, Kinsey, Zeng, & Uhl, 1998; Romberg, et al., 2003; T. W. Smith, Binning, & Dahan, 2009; van Dorp, et al., 2009). In contrast, M3G binds with around a 300 fold lower affinity to opioid receptors and has no analgesic potency (Abazov,

et al., 2001; Bartlett, Cramond, & Smith, 1994; Bartlett & Smith, 1995; Gong, Hedner, Bjorkman, & Hedner, 1992; Loser, et al., 1996; Milne, Nation, & Somogyi, 1996). Intrathecal (Woolf, 1981; Yaksh, Harty, & Onofrio, 1986), intracerebroventricular (Bartlett, Cramond, et al., 1994; Labella, Pinsky, & Havlicek, 1979), and systemic (Juni, et al., 2006; Lipkowski, Carr, Langlade, Osgood, & Szyfelbein, 1994) administration of M3G has been reported to increase nociceptive behaviors and to evoke a range of excitatory behaviors including hyperalgesia, allodynia, myoclonus, and seizures in rats (Christrup, 1997; M. T. Smith, 2000; Wright, Mather, & Smith, 2001; Yaksh & Harty, 1988). Furthermore, these neuroexcitatory effects are not blocked by the general opioid agonist naloxone (Labella, et al., 1979; Woolf, 1981; Yaksh, et al., 1986). Thus, morphine's hyperalgesic effect may be more dependent upon its metabolite activity than its own properties.

2. Prevalence of Opioid-Induced Hyperalgesia

a) Human studies

Opioid-induced hyperalgesia has been demonstrated in the clinical population through observational, cross-sectional, and prospective controlled trials (Angst & Clark, 2006). Most studies have utilized several distinct cohorts and methodologies, including former opioid addicts on methadone maintenance therapy, preoperative exposure to opioids in patients undergoing surgery, healthy human volunteers after acute opioid exposure using human experimental pain testing, and a prospective observational study in opioid-naïve pain patients undergoing initiation of chronic opioid therapy.

Methadone, a long-acting opioid agonist, is administered to former opioid addicts in controlled settings in order to facilitate recovery from heroin addiction. Compton and colleagues

utilized a three-group quasi-experimental design to compare the withdrawal latency for the cold pressor pain test in groups of methadone-maintained, buprenorphine-maintained, and matched-control subjects (P. Compton, Charuvastra, & Ling, 2001). The methadone and buprenorphine-maintained groups had equal and significantly shorter withdrawal latencies than the matched-control subjects. Further investigations compared methadone-maintained patients with matched-controls on electrical stimulation and cold pressor test (Doverty, Somogyi, et al., 2001; Doverty, White, et al., 2001). Methadone-maintained patients are markedly hyperalgesic to pain induced by the cold pressor test. However, hyperalgesia to electrical stimulation was absent, indicating that hyperalgesia may be modality-specific. Pud and colleagues exposed heroin or methadone addicts, who attended a 4-week patient detoxification program, to the cold pressor test at admission, at day 7, and at day 28 subsequent to the cessation of opioid consumption (Pud, Cohen, Lawental, & Eisenberg, 2006). Opioid addicts demonstrated a lower mean latency for pain and decreased pain intensity but their mean time for hand withdrawal (tolerance) was shorter than that of controls. The lower latencies contradict other studies (Doverty, Somogyi, et al., 2001; Doverty, White, et al., 2001; Ho & Dole, 1979), but the decreased tolerance is consistent with other studies (M. A. Compton, 1994; Doverty, White, et al., 2001; Martin & Inglis, 1965). These paradoxical findings may be explained by addicts' pain avoidance behavior and low frustration tolerance and tendency to overreact. Opioid addicts initially deny the feeling of pain and then when the denial becomes impossible, they tend to react to it very quickly. This finding may explain why OIH is much more prominent in the CPT than other assays.

Several clinical studies have investigated OIH in the setting of acute perioperative opioid exposure. Prospective controlled studies with high doses of intraoperative opioids in patients undergoing surgery demonstrated increased postoperative pain despite increased postoperative

opioid use (Chia, Liu, Wang, Kuo, & Ho, 1999; Guignard, et al., 2000). Women undergoing cesarean section and intraoperative fentanyl also showed similar increased postoperative opioid consumption without improved analgesia, compared to women who received placebo intrathecal saline injections (Cooper, et al., 1997). Patients who received acute high-dose intraoperative remifentantil experienced increased peri-incisional wound allodynia and hyperalgesia measured by von Frey hairs compared to low-dose intraoperative remifentanil in patients undergoing major abdominal surgery (Joly, et al., 2005).

Healthy human volunteers after acute opioid exposure also demonstrate hyperalgesia on experimental pain testing. A double-blind, randomized, crossover, and placebo-controlled design measured hyperalgesia in opioid-naïve healthy human volunteers within 30 minutes of stopping a 90-minute infusion with remifentanil, a μ-receptor agonist (Angst, Koppert, Pahl, Clark, & Schmelz, 2003). A skin area with pre-existing mechanical hyperalgesia was significantly enlarged after stopping the infusion but the skin's response to heat was unchanged. This study again demonstrated the differential susceptibility of different pain modalities for the expression of hyperalgesia associated with opioid administration. Koppert and colleagues utilized transcutaneous electrical stimulation to determine the hyperalgesic effects of remifentanil in opioid-naïve male volunteers (Koppert, et al., 2003). Remifentanil reduced pain and areas of punctate hyperalgesia during infusion; however, post-infusion pain and hyperalgesia were significantly higher than controls. An acute opioid physical dependence model was also utilized to test the presence of hyperalgesia to experimental cold pressor pain (P. Compton, Athanasos, & Elashoff, 2003). The study found increased sensitivity to cold pressor pain following precipitated opioid withdrawal after induction of acute physical opioid dependence.

To address some of the limitations in the literature, including cross-sectional designs and

failure to distinguish between tolerance and hyperalgesia, Chu and colleagues conducted a prospective observational study in opioid-naïve pain patients undergoing initiation of chronic opioid therapy (Chu, Clark, & Angst, 2006). Both analgesic tolerance and hyperalgesia through cold pressor and phasic heat experimental pain models were assessed after 1 month of oral morphine therapy. Patients demonstrated significant hyperalgesia and analgesic tolerance in the cold but not heat pain models.

b) Animal studies

Over the past 30 years, there has been an increasingly growing interest in OIH and its underlying mechanisms. Angst and colleagues conducted a comprehensive review 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 (Angst & Clark, 2006). 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 one pain assay may show no hyperalgesic liabilities on other assays, suggesting that OIH is drug and modality specific in animals as well. The literature characterizing OIH will be divided based on the route of administration, including peripheral, systemic, spinal, and supraspinal. With each route of administration, studies that employ acute and chronic models of opioid exposure will be reviewed.

Opioids have been administered peripherally in small volumes to rodent hind paw tissue, due to the expression of μ-opioid receptors on the central and peripheral terminals of primary afferent neurons (Aley, Green, & Levine, 1995; Aley & Levine, 1997a, 1997b; Arts, Holmes, & Fujimoto, 1991). Even though a single injection elicited antinociception, repeated local injection

of the selective μ-opioid agonist Tyr-D-Ala2-Gly-NMePhe4-Gly-ol5 (DAMGO) followed by the local administration of the opioid antagonist naloxone caused mechanical hyperalgesia (Aley, et al., 1995; Aley & Levine, 1997a, 1997b; Arts, et al., 1991).

Systemic administration through an acute dose, repeated injections, and chronic infusion models has also demonstrated hyperalgesia. Most studies demonstrate a consistent, biphasic, and dose-dependent role for morphine and other opioid agonists. The initial response is associated with a marked increase in nociceptive threshold whereas the later response is accompanied by a lowering of the nociceptive threshold below the basal value (Celerier, et al., 2000; Laulin, Larcher, Celerier, Le Moal, & Simonnet, 1998; Waxman, Arout, Caldwell, Dahan, & Kest, 2009). Celerier and colleagues demonstrated that acute injections of fentanyl systemically produced an early response (for 2-5 hours) associated with a marked increase in nociceptive threshold (analgesia) and a later response associated with sustained lowering of the nociceptive threshold below baseline (Celerier, et al., 2000). This significant hyperalgesia persisted for up to 5 days after the fentanyl injections and the hyperalgesic response became more pronounced as the fentanyl dose increased. Furthermore, with opioid receptors blocked by the general opioid antagonist, naltrexone, an acute systemic fentanyl dose (0.25mg/kg) delivered systemically significantly decreased tail-withdrawal latencies to pre-drug values for the next 15-60 minutes (Waxman, et al., 2009). Administered via chronic infusion paradigms or repeated daily injections, morphine produced thermal hyperalgesia and mechanical allodynia for several days after the cessation of morphine administration (Li, Angst, & Clark, 2001b; Trujillo & Akil, 1991, 1994). In differentiating between doses, continuous infusion of 8.0 and 40.0 mg/kg morphine resulted in initial dose-dependent increase of withdrawal latencies for 2 days and then dose-dependent hyperalgesia on Day 4. In contrast, infusion of 1.6 mg/kg created no analgesic effect

and instead decreased latencies were present 6 hours after infusion (Juni, et al., 2006).

The role of the spinal cord in OIH has been demonstrated following direct spinal administration (Yaksh & Harty, 1988). Acute studies first revealed that high morphine doses into the spinal subarachnoid space decreased tail-withdrawal latency, indicative of hyperalgesia (Woolf, 1981). This high spinal dose also elicited a nociceptive behavioral syndrome characterized by biting and licking towards the caudal part of the body without squeaking (Sakurada, et al., 1996). All of these responses failed to be reversed by opioid receptor antagonists and were not subject to tolerance. Conflicting evidence appears for low morphine doses after acute intrathecal administration. In some studies, low morphine doses of the (-) enantiomer produced a significant prolongation of response time for tail immersion whereas the (+) enantiomer, which is inactive to the opioid receptor binding sites, produced hyperalgesia (Woolf, 1981). In contrast, other studies demonstrated that low morphine doses produced brief excitatory effects in the flexor reflex, which is usually marked by the analgesic effect of morphine (Wiesenfeld-Hallin, Xu, Hakanson, Feng, & Folkers, 1991). Furthermore, in the chronic models, rats receiving daily bolus injections of intrathecal morphine for 8 days developed thermal hyperalgesia in association with antinociceptive tolerance (Mao, Price, & Mayer, 1994). In rats, continuous spinal delivery of DAMGO through osmotic minipumps also produced thermal hyperalgesia and tactile allodynia of the hind paws and a decrease in antinociceptive potency and efficacy of spinal DAMGO (Vanderah, et al., 2000).

Lastly, considerable evidence reveals a significant contribution of supraspinal influences to the development and maintenance of hyperalgesia in the rodent model. Systemic opioid-induced mechanical and thermal hypersensitivity can be reversed by lidocaine administration in the rostral ventromedial medulla (RVM) or by lesion placed in the dorsolateral funiculus (DLF),

suggesting that descending pain facilitation from the RVM to the spinal cord is necessary for the manifestation of chronic OIH to noxious and non-noxious stimuli (Vanderah, Suenaga, et al., 2001). Furthermore, an underlying neural basis also contributes to the hyperalgesic action of cholecystokinin (CCK), identified as an anti-opioid peptide. A low dose of CCK microinjected into the RVM blocked the analgesic effect of systemically administered morphine by preventing activation of the off-cells, whereas microinjection of a higher dose of CCK alone activated the on-cells only and also produced hyperalgesia (Heinricher, et al., 2001; Heinricher & Neubert, 2004).

3. Mechanisms of opioid-induced hyperalgesia

a) 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 by alternations in neuronal GM1 ganglioside concentration (Crain & Shen, 2001). In support, cholera toxin-B subunit, which selectively binds to GM1 ganglioside, blocks excitatory opioid receptor function, potentiates morphine hyperalgesia, and blocks morphine tolerance. Furthermore, 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. Therefore, this

evidence suggests 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; Gardell, et al., 2006; Simonnet & Rivat, 2003), 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 receptor-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 is essentially a non-opioid mediated process (Celerier, Laulin, Larcher, Le Moal, & Simonnet, 1999; Dalsgaard, et al., 1982; Devillers, Boisserie, Laulin, Larcher, & Simonnet, 1995; Gardell, et al., 2002; Kest, Palmese, Hopkins, Adler, & Juni, 2001; Labella, et al., 1979; Laulin, et al., 1998; Qian-Ling, Hedner, Bjˆrkman, & Hedner, 1992; G. D. Smith & Smith, 1995; Maree T. Smith, Watt, & Cramond, 1990; X.-J. Wang & Han, 1990; Yaksh, et al., 1986). 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, Laulin, Celerier, Le Moal, & Simonnet, 1998). Despite the ability of low doses of NTX to antagonize excitatory G_s coupled reactions *in vitro* (S. M. Crain & K. Shen, 2000; Crain & Shen, 1995; H. Y. 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 (Cepeda, Africano, Manrique, Fragoso, & Carr, 2002; Devillers, et al., 1995; 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; J. F. Herrero, J. M. A. Laird, & J. A. Lopez-Garcia, 2000).

Recent studies have specifically targeted the non-opioid receptor involvement of OIH and report that the μ-opioids, morphine, M6G, fentanyl, and oxymorphone can cause hyperalgesia in mice treated concurrently with the general opioid receptor antagonist naltrexone (Juni, et al., 2006; Juni, et al., 2007; van Dorp, et al., 2009; Waxman, et al., 2009). Thus, they cause hyperalgesia independently of opioid receptor activity or analgesia. Juni and colleagues first showed that continuous opioid receptor antagonism using NTX pellets abolished analgesia during continuous morphine administration, transiently potentiated hyperalgesia, and revealed difference in hyperalgesia onset between morphine infusion doses (Juni, et al., 2006). For mice receiving the 40 mg/kg infusion dose of morphine, hyperalgesia was not manifest until Day 4 of the infusion; however, for mice receiving a 1.6 mg/kg infusion dose, tail-withdrawal latencies significantly decreased below baseline on Day 1. However, for both doses, hyperalgesia continued through Day 6, the final day of testing. Furthermore, studies further showed that continuous morphine and oxymorphone infusion increased nociception in opioid receptor TKO mice lacking all three genes encoding the μ, δ, and κ opioid receptors (Juni, et al., 2007). Even though the absence of the classical opioid genes leads to a selective up-regulation of the ORL1 receptor in primarily noncortical areas in TKO mice (Clarke, et al., 2002), mice lacking the ORL1 receptor demonstrated the antinociceptive effect induced by morphine (Nishi, et al., 1997). This evidence confirms that morphine and oxymorphone cause hyperalgesia that does not require prior or concurrent opioid analgesia and/or classical opioid receptor activity and indicate

the involvement of other mechanisms in OIH.

b) Cellular sensitization

Studies indicated that prolonged opioid treatment not only results in a loss of opioid antinociceptive efficacy, a negative sign of system adaptation (desensitization), but also leads to activation of a pronociceptive system manifested as a reduction of nociceptive thresholds, a positive sign of system adaptation (sensitization) (Mao, 2002). Central sensitization refers to the enhancement of firing rates in the central nervous system in response to noxious input. OIH may result from direct sensitization of primary afferent neurons, from the enhanced production and release of excitatory amino acid neurotransmitters and the suppressed reuptake of neurotransmitters, from neuroplastic changes in the peripheral and central nervous system that lead to sensitization of pronociceptive pathways, and/or from a dampening of pain inhibitory states.

A mechanism underlying OIH may be the sensitization of primary afferent neurons and evidence demonstrates that the "wind-up" response of wide-dynamic neurons as a possible indication of this peripheral sensitization (Cata, et al., 2006; Staud, Vierck, Robinson, & Price, 2005). 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 substance P (Ikeda, Heinke, Ruscheweyh, & Sandkuhler, 2003). Windup responses have been proposed to contribute to the hyper-responsiveness to sensory stimuli seen in OIH and other forms of hyperalgesia (J. F. Herrero, J. M. Laird, & J. A. Lopez-Garcia, 2000; Melzack, Coderre, Katz, & Vaccarino, 2001; Schulte, Sollevi, & Segerdahl, 2004).

The enhanced production and release of excitatory amino acid neurotransmitters and the

suppressed reuptake of neurotransmitters may also contribute to OIH. Most studies have focused on the role of glutamate, substance P, and protein kinase C and guanosine triphosphate proteins in the development and maintenance of OIH. Glutamate, a major excitatory neurotransmitter mediating synaptic transmission between primary afferent fibers and dorsal horn neurons, activates NMDA receptors. In mice treated for several days to induce OIH, the intrathecal injection of glutamate leads to greatly enhanced nociceptive behaviors when compared with saline treated mice (Li & Clark, 2002). Chronic intrathecal morphine administration also downregulates both neuronal and glial glutamate transporters in the spinal cord, resulting in an increased availability of extracellular spinal glutamate (Mao, et al., 2002). This augmented extracellular glutamate level led to an exaggerated pain response, including an increased magnitude and prolonged time course.

Substance P (SP), a neuropeptide that activates neurokinin-1 (NK-1) receptors, is synthesized in small-diameter primary afferent fibers (McCarthy & Lawson, 1989). This neuropeptide is released into the spinal cord after noxious stimulation (Duggan, Morton, Zhao, & Hendry, 1987), activates NK-1 receptors, which then excites nociceptive dorsal horn neurons (Khasabov, et al., 2002; Moochhala & Sawynok, 1984). Studies have shown that SP in mice chronically exposed to morphine, evoked hyperalgesia (Li & Clark, 2002). Selective ablation of spinal neurons possessing SP receptors using the selective cytotoxin conjugate SP-saporin (SP-SAP) decreases hyperalgesia and central sensitization (Khasabov, Ghilardi, Mantyh, & Simone, 2005). Furthermore, SP conjugated to saporin was used as an intrathecal neurotoxin to ablate NK-1 receptor expressing cells in the spinal cord (Vera-Portocarrero, et al., 2007) and this treatment prevented the normally observed morphine-induced sensitization in rats. Further investigation demonstrated that the serotonin 5-HT3 receptor, which participates in a spinal-

supraspinal-spinal loop to maintain nociceptive sensitization, needed to be active for the manifestation of OIH (Vera-Portocarrero, et al., 2007).

Additionally, OIH may result from neuroplastic changes in the peripheral and central nervous system that lead to sensitization of pronociceptive pathways. The RVM has been identified as a source of spinopetal inhibitory and facilitatory modulation of nociceptive inputs and is critical to the integration of nociceptive processing and descending modulation (Ossipov, Lai, Malan, & Porreca, 2000; Vanderah, Ossipov, Lai, Malan, & Porreca, 2001). The activities of the on and off-cells, subsets of neurons within the RVM, may contribute to the mechanisms of descending facilitation that influence spinal nociceptive processing. Additionally, on-cell activity within the RVM increases in association with the behavioral manifestation of naloxone-precipitated hyperalgesia (Bederson, Fields, & Barbaro, 1990). Furthermore, bilateral lesioning of the dorsolateral funiculus, an anatomical pathway connecting the brainstem and spinal cord, blocks the increase in spinal excitatory neuropetides in opioid-treated animals (Gardell, et al., 2002), suggesting that descending facilitation may function in part through the modulation of spinal neuropeptide contents.

This descending facilitation induces an increase in spinal dynorphin content (Gardell, et al., 2002; Z. Wang, et al., 2001). Dynorphin, an endogenous kappa-opioid receptor agonist that was originally thought to be antinociceptive, was shown to exert non-opioid pronociceptive actions by potentiating NMDA receptors, facilitating release of excitatory transmitters and increasing intracellular calcium levels (Lai, Ossipov, Vanderah, Malan, & Porreca, 2001). Spinal administration of dynorphin has been shown to induce pain (Laughlin, et al., 1997; Vanderah, et al., 1996). Chronic intrathecal administration of μ-opioid receptor agonist DAMGO enhanced spinal expression of dynorphin (Vanderah, et al., 2000). Spinal MK-801 or

dynorphin antiserum blocks morphine-induced abnormal pain and antinociceptive tolerance (Vanderah, et al., 2000). Increased levels of dynorphin lead to the release of spinal excitatory neuropeptides such as calcitonin gene-related peptide from primary afferents (Gardell, et al., 2002).

Upregulation of NK-1 receptors in the spinal cord may also signify another neuroplastic change contributing to OIH. Lamina I cells of the dorsal horn expressing the NK-1 receptors project to the supraspinal areas that mediate nociceptive processing (Nichols, et al., 1999). Repeated electrical stimulation of the dorsal root afferents has been shown to increase the synaptic strength between primary afferent C-fibers and these projections neurons, but not with other neurons in lamina I (Ikeda, et al., 2003). Sustained morphine treatment induces NK-1 receptor dependent hyperalgesia, increases spinal SP content, enhances evoked SP release within the spinal cord, increases spinal NK-1 receptor expression, and elicits stimulus-induced NK-1 receptor internalization in both superficial and deep spinal dorsal horn neurons (T. King, et al., 2005).

Lastly, a reduction of the inhibitory effects of the neurotransmitter γ-aminobutyric acid (GABA) and its effects on descending facilitation may underlie OIH. Supraspinal GABA's inhibition of ascending dorsal horn nociceptive inputs has been repeatedly identified as one 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 thus, 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). Spinal (Hara et al., 1999) and supraspinal (Ammon-Treiber & Hollt, 2005; Dunbar et al., 2006) GABA-opioid interactions also 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). Pharmacological blockade of spinal GABAergic neurons also leads to severe hyperalgesia and allodynia (Dickenson et al., 1997). 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 modulate nociceptive processes, but cannot be linked definitively to OIH.

c) NMDA receptor involvement

Evidence demonstrates that NMDA receptors play a pivotal role in the development and maintenance of hyperactive states underlying the behavioral manifestations of pain facilitation such as hyperalgesia, allodynia, and spontaneous pain. NMDA receptors expression exists in the cell bodies, as well as peripheral and central processes of primary sensory neurons in rodents (Carlton, Hargett, & Coggeshall, 1995; Lovinger & Weight, 1988) and humans (Kinkelin, Brocker, Koltzenburg, & Carlton, 2000). Evidence also confirms that NMDA receptors are located on nociceptive C and Aδ fibers (Du, Zhou, Coggeshall, & Carlton, 2003). Coadministration of competitive (AP-5) or non-competitive (MK-801) NMDA receptor antagonists with systemic or intrathecal morphine reduces thermal hyperalgesia to radiant heat in animal models of neuropathic pain (Davar, Hama, Deykin, Vos, & Maciewicz, 1991; Mao, Price, Hayes, Lu, & Mayer, 1992a; Mao, et al., 1993; Tal & Bennett, 1993; T. Yamamoto & Yaksh, 1992), carrageenan-induced acute peripheral pain (Ren, Williams, Hylden, Ruda, & Dubner,

1992), heat injury (Coderre & Melzack, 1991; Juni, et al., 2006), and formalin-induced nociception (Coderre & Melzack, 1992a, 1992b). Mechanistically, it has been suggested that hyperalgesia may result from increased activity at pre-synaptic NMDA receptors localized to central primary afferent fibers, where they may be anatomically linked with opioid receptors, causing spinal sensitization and increased nociceptive input (Ossipov, et al., 2004). However, because NMDA antagonists also potentiate opioid analgesia, they might attenuate hyperalgesia indirectly, by increasing the latent opioid analgesia obfuscated by the concurrent increased nociception. This possibility is not supported by the demonstration that MK-801 reverses morphine hyperalgesia in NTX-pelleted mice (Juni, et al., 2006).

Although it is clear that NMDA receptors play an important role in OIH, the locations of these opioid-NMDA interactions have yet to be clearly identified. Evidence reveals that activation of spinal cord NMDA receptors contributes to the development and maintenance of hyperalgesia that occurs following peripheral nerve injury or inflammation (Urban & Gebhart, 1998). Intrathecal coadministration of MK-801 and morphine effectively prevented the elicitation of OIH (Mao, et al., 1994). A conditioned deletion of the NR1 subunit of the NMDA receptor, a subunit expressed in all lumbar dorsal root ganglia, has demonstrated the essential contribution of the NMDA receptor on lumbar spinal cord neurons to central sensitization and injury-induced pain (South, et al., 2003). Known to reduce the specific binding ligands for the NMDAR1 receptor in the spinal dorsal horn, NMDAR1 antisense treatment for 5 days blocks the thermal hyperalgesic effects of i.t. NMDA-induced pain behaviors in the rat (Shimoyama, Shimoyama, Davis, Monaghan, & Inturrisi, 2005). In addition, i.t. administration of NMDAR1 antisense treatment for 3 days results in a reduction in formalin-induced nociceptive behaviors during phase 2, the phase associated with central sensitization (Shimoyama, et al., 2005). This

evidence provides additional support for the critical role of the spinal NMDA receptor in the development of hyperalgesia.

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). Anatomical studies involving bilateral lesion or lidocaine injections into the RVM resulted in attenuation of OIH and restored previously masked morphine analgesia (Vanderah, Ossipov, et al., 2001; Vanderah, Suenaga, et al., 2001). Furthermore, in a model of thermal hyperalgesia involving topical application of mustard oil, evidence shows that blocking on-cell activation by local infusion of the competitive NMDA-receptor antagonist AP5 into the RVM prevented hyperalgesia (Xu, et al., 2007). The decrease in off-cell firing following mustard oil may also make some contribution but is not by itself sufficient to induce measurable behavioral hyperalgesia. Therefore, NMDA receptors have two opposing roles in the RVM, producing analgesia when recruited to activate off-cells by opioid administration, and hyperalgesia when contributing to activation of on-cells following an acute inflammatory stimulus. These studies provide evidence that a novel, NMDA receptor-mediated activation of on-cells is required for secondary thermal hyperalgesia in acute inflammation. Thus, although evidence suggests important roles for both spinal and supraspinal NMDA receptors in the modulation of OIH, the precise pathway by which opioid receptor agonists and their derivatives interact with NMDA receptors still remains to be discovered.

Activation of NMDA receptors initiates a variety of intracellular processes that may underlie this heightened nociceptive sensitivity. Studies have shown that mu-receptor agonists increase the NMDA-receptor mediated glutamate *in vitro* response via protein kinase C (PKC)-facilitated removal of the magnesium blockade of the NMDA receptor channel (L. Chen &

Huang, 1992). This magnesium removal enhances the reactivity of the receptor, leading to subsequent increases in intracellular Ca^{2+} concentration, which further stimulates calcium-sensitive protein kinase, especially the gamma isoform (PKCγ), activity. PKCγ catalyzes NMDA receptor phosphorylation, leading to enhancement of the NMDA mediated glutamate responses and to long-term potentiation of synaptic transmission (L. Chen & Huang, 1992). GM1 ganglioside and H-7, intracellular inhibitors of PKC translocation/activation, effectively attenuate hyperalgesia *in vitro* and *in vivo* (Bederson, et al., 1990; Magal, Louis, Aguilera, & Yavin, 1990), induced by peripheral nerve injury (Hayes et al., 1992; Mao et al., 1992c-e) or formalin injection into the rat's hind paw (Coderre, 1992). Studies involving mice lacking the PKCγ gene (Abeliovich, et al., 1993) provided further confirmation of the importance of PKC in the development of hyperalgesia. There was no difference in second phase formalin-induced nociceptive behaviors between morphine- and placebo-pelleted PKCγ mutant mice (Zeitz, Malmberg, Gilbert, & Basbaum, 2001). Furthermore, in these mice, an enhancement of the early fentanyl antinociceptive effects and a complete prevention of the fentanyl delayed hyperalgesic effects were observed (Celerier, Simonnet, & Maldonado, 2004). Thus, PKC may represent a key element which links the opioid receptor activation and the recruitment of the glutamatergic/NMDA systems implicated in the promotion of pain.

d) Pronociceptive opioid metabolites

Researchers have also proposed that OIH may be mediated by pronociceptive non-opioid glucuronide metabolites of the parent opioid compound. Specifically, morphine undergoes hepatic glucuronidation to form hydrophilic metabolites, M3G and M6G, to facilitate renal elimination (Kuo, et al., 1991). There is a larger proportion of M3G formation because aromatic hydroxyl groups serve as easier conjugation recipients than do alicyclic hydroxyl groups. For

this reason, rodents can not form M6G, only M3G. Studies demonstrate that injecting M3G in rodents via the i.c.v. (Bartlett, Cramond, et al., 1994; Labella, et al., 1979), i.t. (Woolf, 1981; Yaksh, et al., 1986), or systemic (Juni, et al., 2006; Lipkowski, et al., 1994) route increases nociception and evokes potent neuroexcitatory effects on behavior (Christrup, 1997; M. T. Smith, 2000). Systemic administration of M3G in rodents has proven to be at least 10-fold more potent as a neuro-excitant (Bartlett, Dodd, & Smith, 1994) in its ability to elicit a wide range of negative sequelae including myoclonus, allodynia, seizures and hyperalgesia (Bartlett, Cramond, et al., 1994; Wright, et al., 2001). Moreover, systemic morphine administration (oral or subcutaneous) has been to shown to produce M3G:morphine ratios in the human cerebrospinal fluid (CSF) ranging from 3:1 to 10:1, which due to M3G's ability to easily cross the blood brain barrier, has been linked to a host of neuroexcitatory behaviors in cancer patients (M. T. Smith, Wright, Williams, Stuart, & Cramond, 1999; Wolff, Samuelsson, & Hedner, 1996). On the contrary, following i.c.v. administration, morphine's concentration in the CSF increases 50-fold, accompanied by a noticeable drop in ventricular M3G concentration and no demonstrable neuroexcitatory effects (Wolff, et al., 1996). Importantly, M3G has no appreciable affinity at any opioid receptor subtype (Bartlett, Dodd, et al., 1994; Z. R. Chen, Irvine, Somogyi, & Bochner, 1991; Labella, et al., 1979) and its neuroexcitatory effects are not blocked by the general opioid antagonist naloxone (Labella, et al., 1979; Woolf, 1981; Yaksh, et al., 1986). Thus, morphine's primary metabolite seems to be 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 preliminary evidence suggesting that it may act through the NMDA receptor (Bartlett, Cramond, et al., 1994). 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; Hemstapat, Monteith, Smith, & Smith, 2003; Moran & Smith, 2002), 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 and colleagues further demonstrated 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).

M6G, the other metabolite formed by the hepatic glucuronidation of morphine in humans only, displays affinity at μ-opioid receptors equal to that of morphine and is a potent opioid analgesia in humans and rodents. However, evidence continues to accumulate suggesting that acute M6G doses can cause hyperalgesia as well. A single acute injection of M6G reduced tail-withdrawal latencies by up to 40% in mice lacking exons 1 and/or 2 of the μ-opioid receptor (Kitanaka, et al., 1998; Romberg, et al., 2003) as well as increased pain ratings in healthy volunteers subject to a cutaneous heat pain assay (van Dorp, Romberg, Sarton, Bovill, & Dahan, 2006). To understand whether M6G causes hyperalgesia independently of opioid receptor activity and whether NMDA contribute to this effect, van Dorp and colleagues (2009) demonstrated that acute and chronic delivery of M6G caused hyperalgesia in CD-1 mice treated concurrently with NTX and in TKO mice. MK-801 blocked and reversed hyperalgesia after the acute injection and continuous infusion of M6G, respectively. In humans, they demonstrated that M6G increased heat pain sensitivity for at least 6 hours independently of simultaneous

naloxone infusion (van Dorp, et al., 2009). Thus, these studies indicate that M6G causes hyperalgesia independent of previous opioid receptor activity or analgesia in mice and humans.

Although a role for the bioactive neuroexcitatory glucuronide metabolites have logically been suggested (Juni, et al., 2006; van Dorp, et al., 2009), several studies demonstrate inconsistencies. For example, NMDA receptor antagonists do not reverse morphine hyperalgesia in female CD-1 mice (Juni, Klein, Kowalczyk, Ragnauth, & Kest, 2008) or morphine or oxymorphone hyperalgesia in TKO mice (Juni, et al., 2007) despite evidence that NMDA receptor antagonists are also functional M3G antagonists (Bartlett, Cramond, et al., 1994). Furthermore, although both the R- and S-enantiomers of oxymorphone are transformed into oxymorphone-3-glucuronide, only sustained (−)-oxymorphone, but not (+)-oxymorphone, caused hyperalgesia in rats, suggesting that hyperalgesia was initiated prior to the conjugation of the opioid parent compound. We report that fentanyl, too, causes hyperalgesia independently of opioid receptor activity, despite the fact that it is not subject to glucuronidation (Waxman, et al., 2009). Collectively, these data provide a challenge to the supposition that glucuronide metabolites are broadly involved in OIH. Like with the other μ-opioids, morphine, M6G, and oxymorphone, it still remains to be determined how exactly fentanyl causes hyperalgesia independently of opioid receptor activity.

4. Contribution of genetics to opioid-induced hyperalgesia

In order to explain the interindividual differences in nociception, the influence of genetics in clinical and animal populations has gained attention in the literature. Animal studies specifically have demonstrated the contribution of genetics to OIH (Hoffmann, Plesan, & Wiesenfeld-Hallin, 1998). The CXB series of mice demonstrated the first genetic differences in

an opioid phenotype. Eleven strains of the CXB series were divided into three statistically significant groups with respect to whole-brain [^{3}H]naloxone binding: CXBH > all others > CXBK (Baran, Shuster, Eleftheriou, & Bailey, 1975). CXBK displayed the lowest analgesic response to morphine as measured by the tail-flick assay. This strain demonstrated supraspinal μ_1 opioid receptor binding deficiencies (Moskowitz & Goodman, 1985). They also displayed deficient to absent morphine analgesia, morphine hypolocomotion, opioid footshock stress-induced analgesia, opioid swim stress-induced analgesia, and ethanol-induced analgesia and hypothermia and exhibited naloxone-insensitive, non-opioid analgesia.

Liang and colleagues also performed a series of studies that investigated the genetic contribution to OIH (Liang, Guo, et al., 2006). Their first study showed that genetic background and chronic inflammatory nociception function together to influence baseline nociceptive thresholds, opioid analgesic sensitivity, opioid tolerance, and physical dependence. In their genetic analysis of OIH in mice, they used *in silico* mapping (which relies on the availability of high resolution single nucleotide polymorphism (SNP) databases) to identify haplotypic blocks associated with OIH (Liang, Liao, Wang, et al., 2006). *In silico* genetic mapping identified the β2-AR gene as a candidate gene involved in modulating mechanical OIH in mice. They also found increased β2-AR density in the central nervous system after chronic morphine treatment in rats as well as upregulation of guanosine triphosphate binding proteins, molecules utilized by β2-AR to activate ion channels and second messenger systems. Furthermore, the β2-AR gene is linked to the coding of the P-glycoprotein drug transporter (Liang, Liao, Lighthall, Peltz, & Clark, 2006). This nonselective drug transporter is able to control brain levels of opioids by mediating the efflux of the drug across the blood brain barrier. Inhibition of P-glycoprotein eliminated OIH as did genetic deletion of the abcb1a/b gene coding for P-glycoprotein

transporters in mice.

5. Rationale for the Present Dissertation

Given the prominence of morphine and other opioids in the clinical treatment of pain, identifying the physiological process underlying OIH and other unintended sequelae of acute 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, morphine metabolites, NMDA receptors, and genotype. 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.

Rationale for the use of morphine. 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 all experiments described in this thesis. Obtaining an understanding of the hyperalgesic liabilities following acute morphine exposure can thus provide a substantial and important scientific contribution to the field. Furthermore, the proposal that hyperalgesia may be mediated by pronociceptive non-opioid glucuronide metabolites of the parent opioid compound can be investigated with the use of morphine. Through the use of morphine, we hope that these results can be generalized to multiple opioid analgesics, particularly those with mu binding properties.

Rationale for the use of mice. Hyperalgesia 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 availability of inbred mouse strains.

Rationale for investigating different routes of drug administration. The studies described in this dissertation feature different routes of drug administration. Systemic administration results in widespread distribution along the neuraxis and periphery, leading to activation at spinal, supraspinal and peripheral levels. 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. More focal administration paradigms, including i.c.v. and i.t. administration, were also utilized in order to assess and isolate whether spinal and/or supraspinal loci contribute to acute morphine-induced hyperalgesia.

Rationale for selected experimental assays of nociception. Multiple experimental 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 investigate distinct modalities such as thermal pain, mechanical pressure, noxious chemicals or nerve injury. Mogil and colleagues (1999) used multivariate analysis of the responses of 11 inbred mouse

strains on 12 common measures of nociception to identify three clusters of pain tests which seem to share common genetic substrates and presumably underlying physiology (Mogil, et al., 1999a, 1999b). The analysis revealed three major clusters of nociception: “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, and peripheral nerve injury). 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. For the proposed sets of studies described within this dissertation, two experimental assays of nociception, the tail-withdrawal test and the formalin test, are utilized to characterize acute morphine hyperalgesia (Table 1).

Dimension	Tail-Withdrawal Test	Formalin Test
Type of Noxious Stimulus	Thermal	Chemical
Stimulus Duration	Acute	Biphasic
Stimulus Location	Cutaneous	Subcutaneous
Stimulus Intensity	High	Moderate
Intensity-time Relationship	Consistent	Increasing
Presence/Absence of Tissue Damage and/or Inflammation	Absence	Presence
Stimulus Escapability	Escapable	Inescapable
Response Characteristics	Reflexive	Spontaneously emitted
Level of Nociceptive Processing	Spinal	Spinal and supraspinal

Table 1. Comparison of Tail-Withdrawal Test and Formalin Test on Different Dimensions (Mogil, Kest, Sadowski, & Belknap, 1996).

The tail-withdrawal assay. The tail-withdrawal assay is a well-known measure of nociceptive sensitivity based on reflexive limb withdrawal from a phasic noxious stimulus of high intensity. 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 this classic test was selected for this dissertation (Janssen, Niemegeers, & Dony, 1963), based on its minimally invasive procedure and its stability in the context of repeated testing (Wilson & Mogil, 2001). The assay involves immersing the distal portion of the animal's tail into a hot-water bath and measuring the latency between water immersion and reflexive withdrawal of the tail. For the experiments discussed in this dissertation, latency of tail-withdrawal from the hot-water bath was used as the dependent measure to indicate nociceptive sensitivity.

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 dorsal root ganglion synapse in the dorsal horn of the spinal cord synapse onto 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 (Y. P. Chen, Chen, & Pan, 2005). Regulation of the tail-withdrawal can therefore occur via modulation of either peripheral or central mechanisms (McCormack, Prather, & Chapleo, 1998).

Formalin test. The formalin test assesses the way an animal responds to moderate, continuous pain generated by injured tissue. Evidence suggests that the formalin test provides a more valid model of clinical pain than the tests with phasic mechanical or thermal stimuli. The formalin test is biphasic, consisting of an acute "early" phase and a tonic "late" phase, each with clearly dissociable properties (Dubuisson & Dennis, 1977; Hunskaar, Fasmer, & Hole, 1985). The "early" phase starts immediately following injection of formalin, lasts for approximately 5-10 minutes, and is due to direct chemical stimulation of C fibers, not Aδ afferents (Tjolsen, Berge, Hunskaar, Rosland, & Hole, 1992). Then, there is a quiescent period of 10-15 minutes when the animals display very little behavior suggestive of nociception. The "late" phase starts approximately 15-20 minutes after formalin injection and lasts for 20-40 minutes. Studies demonstrate that this tonic phase is dependent on peripheral inflammatory processes and central plasticity (Tjolsen, et al., 1992). For this dissertation, the time spent licking/biting the affected hindpaw in each phase was used as the dependent measure to indicate nociceptive duration. This measure is the mostly commonly used when using the formalin test in mice.

Opioid-induced hyperalgesia versus opioid tolerance, allodynia, and withdrawal-associated hyperalgesia. Since these syndromes can manifest similar symptoms, OIH needs to be distinguished from opioid tolerance, allodynia, and withdrawal-induced hyperalgesia (WIH). Tolerance to opioids is classically defined as the progressive reduction of their analgesic effect, explaining the need for increasing amounts of opioids to achieve the same pharmacological effect. Allodynia is the experience of pain from an innocuous stimulus. WIH is the experience of nociceptive hypersensitivity in opioid-dependent organisms following cessation of opioid treatment. However, these syndromes have been clinically differentiated through their different effective interventions.

Tolerance occurs when there is a progressive lack of response to a drug, thus requiring increased dosing. OIH can not be overcome by increased dosage of the drug. Thus, OIH causes a downward shift of the opioid dose–response curve whereas tolerance leads to a rightward shift of the curve (Chu, Angst, & Clark, 2008). Furthermore, Juni and colleagues (2006) showed that morphine hyperalgesia is unrelated to tolerance, by demonstrating tolerance in the absence of hyperalgesia and vice versa (Juni, et al., 2006). Since presence and magnitude of tolerance are related to increasing morphine treatment duration and/or dose, the acute morphine paradigms utilized in this dissertation will not induce tolerance.

Allodynia is the experience of pain due to stimulus that does not usually provoke pain. For example, individuals suffering from temporomandibular joint disorder will report excruciating pain from light touch or cold foods. Allodynia is initially treated by opioids, but if pain persists, effective treatment strategies may include antiepilectics, antidepressants, or surgery (Tompkins & Campbell). This dissertation measures sensitivity to painful thermal or chemical stimuli, thus, distinguishing between hyperalgesia and allodynia.

This dissertation will consider the influence of WIH since dependence and withdrawal can occur after an acute injection of morphine. In order to prevent dependence and withdrawal, pretreatment with NTX will be used in this dissertation. WIH is interpreted as an indication of opioid dependence severity (Angst, et al., 2003; Bederson, et al., 1990; P. Compton, et al., 2003; Gutstein, 1996; Kayan, Woods, & Mitchell, 1971; Tilson, et al., 1973; VonVoigtlander & Lewis, 1983). 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 (X. H. Chen, Geller, DeRiel, Liu-Chen, & Adler, 1996; Houshyar, Cooper, & Woods, 2001; Pinelli, Trivulzio, & Spezia, 1998), and appetitive changes (Kanof,

Aronson, & Ness, 1993; Pinkofsky, et al., 2005; Schoenbaum, Martin, & Roane, 1989). 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 (Dunbar, Karamian, & Zhang, 2007; Harris, Hanes, & Gewirtz, 2004). For example, many of the investigations into WIH have employed naloxone, a wide spectrum opioid antagonist that shows a high affinity for all 3 receptor subtypes and can induce immediate withdrawal behaviors. Following treatment with opioids such as morphine or alfentanil, naloxone administration produces significant increases in nociceptive-related slow ventral root potentials above pre-opioid control values (Feng & Kendig, 1996), and elevates the firing rates of the 'hyperalgesic' on-cells in the RVM relative to pre-opioid 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 Bars, Guilbaud, Jurna, & Besson, 1976). In addition, naloxone administration in non-opioid treated animals produces no discernable nociceptive effects (Kest, Palmese, et al., 2002), thus further suggesting that WIH is strictly an adaptive response to opioid removal. There is 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.

6. Rationale for Specific Aims of the Present Dissertation

As evidenced in the literature reviewed above, OIH is a growing clinical problem that continues to be poorly understood. There have been relatively few studies documenting the

pharmacological and pharmacogenetic contribution of acute OIH. 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 define the opioid nature of acute morphine hyperalgesia across two pain modalities. Since previous studies have shown that chronic administration of morphine causes hyperalgesia independently of opioid receptor activity, we first aimed to determine whether acute morphine causes hyperalgesia in naïve, outbred CD-1 mice treated concurrently with NTX. We assessed nociception via two pain modalities in mice, the tail-withdrawal test and the formalin test, before and after receiving an acute bolus dose of morphine. The tail-withdrawal test is a model designed to investigate phasic pain, mainly peripheral and spinal mediated responses (Lutfy, Cai, Woodward, & Weber, 1997), whereas the formalin test produces two phases of nociceptive behavior (Dubuisson & Dennis, 1977) and involves both peripheral and central sensitization (South, et al., 2003). We also conducted studies assessing the time course of this hyperalgesic effect in order to determine the peak hyperalgesic effect of morphine on the tail-withdrawal test. We hypothesized that acute morphine hyperalgesia will be independent of opioid receptor activity.

The second aspect of Specific Aim 1 involved assessing whether acute morphine causes hyperalgesia in opioid receptor triple knockout (TKO) mice. These mice were obtained by cross-breeding mice singly deficient in the genes coding for μ, δ, and κ receptors using homologous recombination techniques (Clarke, et al., 2002; Cox, et al., 2005). The knockout animals were subject to the same bolus dose of morphine as the naïve CD-1 mice, to firmly establish the role of opioid receptors in the mediation of hyperalgesic states. We hypothesized that TKO mice will demonstrate hyperalgesia after a systemic acute dose of morphine.

The final aspect of Specific Aim 1 involved determining whether morphine causes hyperalgesia by opioid receptors in mice lacking multidrug resistance protein 3 ($Mrp3^{(-/-)}$) pretreated with NTX. Mrp3 is the major transporter able to excrete M3G from hepatocytes into the bloodstream in order to prevent accumulation of the hydrophilic metabolite in the liver. $Mrp3^{(-/-)}$ mice display 50-fold decreased plasma M3G levels and brain M3G levels are below the limit of detection following morphine treatment (Zelcer, et al., 2005). If indeed M3G contributes to morphine hyperalgesia, then the reduction of systemic levels of M3G in these mice should result in the absence or marked reduction in the magnitude of morphine hyperalgesia in these mice relative to their FVB wild-type control mice. In accordance with the existing literature, we hypothesized that $Mrp3^{(-/-)}$ mice pretreated with NTX, will not demonstrate any reduction in withdrawal latencies after a systemic dose of morphine.

The goal of Specific Aim Two was to determine the contribution of route of administration to acute morphine hyperalgesia. To localize whether spinal and/or supraspinal CNS loci contribute to systemic morphine hyperalgesia, mice were injected with NTX and then tested for nociception on the tail-withdrawal test before and after receiving an acute morphine via the i.t. or i.c.v. route. This study aimed to determine whether morphine injected into spinal and supraspinal loci can increase nociception, and that this ensuing hyperalgesia is not mediated by opioid receptors. We hypothesized that spinal and supraspinal loci will contribute to OIH, independent of opioid receptors.

The goal of Specific Aim Three was to determine the contribution of *N*-methyl-D-asparate (NMDA) receptors to acute morphine hyperalgesia. The activation of excitatory amino acid receptors such as NMDA receptors has been implicated in the mechanisms of OIH. NMDA antagonists, such as MK-801, attenuate chronic morphine-induced hyperalgesia in NTX-

treated mice, demonstrating a role for this receptor in morphine hyperalgesia unrelated to its effect upon morphine analgesia (Juni, et al., 2006).

Mechanistically, antagonists might block NMDA receptors localized to central primary afferent terminals that cause spinal sensitization and increased nociceptive input (Ossipov, et al., 2004). Since the localization and expression patterns of NMDA receptors have been mapped in the periaqueductal gray, rostral ventromedial medulla, and the cell bodies of the dorsal root ganglion primary afferent neurons, NMDA receptors at these loci may also contribute to OIH. Therefore, the study determined the contribution of NMDA receptors at spinal and supraspinal loci on acute morphine hyperalgesia by assaying nociception before and after i.t. and i.c.v. MK-801 injection in NTX-treated mice treated with acute systemic morphine. We hypothesized that blockade of NMDA receptors at the systemic, spinal, and supraspinal levels will abolish acute morphine-induced hyperalgesia.

The goal of Specific Aim Four was to determine the contribution of genotype to acute morphine hyperalgesia. To investigate the contribution of genetics on acute OIH, nociception before and after acute morphine injection was tested in 10 inbred mouse strains pretreated with NTX. We also determined if these inter-strain differences exist across a wide range of cumulative morphine doses. We hypothesized that inter-strain variability of OIH will exist and thus, will demonstrate the contribution of genotype to acute morphine hyperalgesia.

Chapter 2.

General Methods

1. Approval

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

2. Subjects

All of the experiments described in this dissertation were performed using mice. All mice were maintained on a 12:12-hour light/dark cycle in a climate-controlled room with free access to food (Purina chow) and tap water. Each subject was used once and for all groups, $n \geq 8$.

CD-1, B6, and FVB mice. Adult CD-1, B6, and FVB mice were obtained commercially (Charles Rivers, Kingston, NY).

TKO mice. Opioid receptor triple knockout (TKO) mice (Gift of John Pintar, University of Medicine and Dentistry, NJ) were obtained by cross-breeding mice singly deficient in the genes coding for μ, δ, and κ receptors using homologous recombination techniques (Clarke, et al., 2002; Cox, et al., 2005). These combinatorial mice lack spinal cord [^{3}H] naloxone receptor labeling, opioid analgesia (Juni, et al., 2007), or gross behavioral or anatomical alterations (Clarke, et al., 2002; Cox, et al., 2005).

Mrp3$^{(-/-)}$ mice. Mice lacking the multidrug resistance protein 3 (Mrp3$^{(-/-)}$) (Gift of Piet Borst, The Netherlands Cancer Institute, The Netherlands) are unable to excrete M3G from the liver into the bloodstream, the major hepatic elimination route for morphine. They shift their excretion of M3G from primarily urinary to primary fecal. These mice display 50-fold decreased

plasma M3G levels and brain M3G levels are below the limit of detection following acute morphine treatment (Zelcer, et al., 2005).

Inbred strains. An inbred strain is created by repeated sibling matings for at least 20 generations (Mogil, Sternberg, et al., 1996). Once fully inbred, the offspring of a given strain are genetically identical to each other. The specific allele that becomes fixed at each genetic locus is randomly determined. Therefore, individuals from different inbred strains will be different at many genetic loci. Male mice of the following inbred strains were obtained from The Jackson Laboratory (Bar Harbor, ME). Strains included 129P3/J, A/J, AKR/J, BALB/c/J, C3H/He/J, C57BL/6/J, CBA/J, DBA/2/J, SJL/J, and SWR/J.

3. Drugs

Morphine. Morphine was gifted by the National Institute of Drug Abuse (Rockville, MD) and was delivered in a 0.9% physiological saline vehicle.

Naltrexone. Naltrexone powder, gifted by the National Institute of Drug Abuse (Rockville, MD), was delivered in a 0.9% physiological saline vehicle. Pellets containing 30 mg of drug were also obtained from the National Institute of Drug Abuse (Rockville, MD) and surgically implanted subcutaneously (see below).

MK-801. A non-competitive NMDA antagonist, MK-801 was obtained from Sigma-Aldrich (St. Louis, MO) and delivered in a 0.9% physiological saline vehicle.

4. Drug Delivery Mechanisms.

Acute injection paradigms. Throughout this dissertation, drug injection paradigms involved subcutaneous (s.c.), intrathecal (i.t.), or intracerebroventricular (i.c.v.) 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 formula of 10ml drug solution per kilogram of mouse weight. I.c.v. injections were made into the lateral ventricles using the method of Haley and McCormick (T. J. Haley & McCormick, 1957). Specifically, a small midline incision was made in the scalp of mice under oxygen/isoflurane inhalant anesthesia, and lambda located. Injections (5 µl volume) were made directly through the skull at a point 2mm rostral and lateral to lambda at a depth of 3mm using a 10-µl Hamilton micro-syringe fitted with a 27-gauge needle. A stainless steel wound clip was used to close the incision after each injection. I.t. injections were made under light oxygen/isoflurane inhalant anesthesia using a 10-µl Hamilton micro-syringe fitted with a 27-gauge needle and administered by lumbar puncture.

Pellet implantation. For the dose response studies, NTX pellets were wrapped in a sterile nylon mesh and subcutaneously implanted into the nape of the neck under oxygen/isoflurane inhalant anesthesia, through a small incision and closed with stainless steel surgical staples. Pellets were always implanted at least 24 hours prior to the onset of each experiment and have been shown not to influence baseline withdrawal latencies (Juni, et al., 2006; Yoburn, Cohen, & Inturrisi, 1986).

Dose–response measures. A dose-response approach, typically utilized for assessing analgesic potency, was adapted to measure hyperalgesic potency (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 1 mg/kg, given a series of acute morphine subcutaneous injections of increasing (~0.25 log units) magnitude. Latencies are reassessed 30 minutes after each injection. This sequence of injection and testing continued for the following

doses: 2, 3.6, 6.5, 11.7, and 21 mg/kg. All mice (even those who were already hyperalgesic) 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.

5. 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 8 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 & Hirst, 1983).

Warm water tail-withdrawal. The modified version of the tail-withdrawal test of D'amour and Smith (1941) was chosen for its stability in the context of repeated testing (Elliott, Kest, Man, Kao, & Inturrisi, 1995; Kest, Hopkins, Palmese, Adler, & Mogil, 2002; Nemmani & Mogil, 2003). Studies described in this dissertation were performed with a water temperature of 47.3°C ± 0.2°, since in pilot studies baseline latencies of 9–10 seconds were consistently obtained, thus minimizing the possibility of floor effects during hyperalgesia. Nociception was tested near mid-photophase to reduce possible circadian effects on nociception (Kavaliers & Hirst, 1983). 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 the 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 two measures were then averaged. A 30 second cutoff latency was employed to prevent possible tissue damage. Ambient room temperature for all assessments trials was held stable at 22-23°C, as it has been documented that changes in tail skin temperature can affect tail-withdrawal latencies (Tjolsen & Hole, 1993).

Formalin test. A modified version of the formalin test, described by Hunskaar and colleagues for use in mice (Hunskaar, et al., 1985) and originally described by Dubuisson and Dennis in rats and cats, was also utilized (Dubuisson & Dennis, 1977). Formalin is the aqueous solution of 37% (weight/weight) formaldehyde. In this assay, mice were placed on an aluminum table within Plexiglas cylinders (30 centimeters high, 30 centimeters diameter) and allowed to habituate to this novel environment for 30 minutes. They were then weighed, injected with morphine subcutaneously, and returned to the cylinders. Ten minutes later, mice were lightly restrained, and 20ul of 1% formalin was injected just under the skin of the plantar surface of the right hindpaw by use of a microsyringe with a 30-gauge needle. Mice were returned to the

cylinders and immediately observed for licking of the affected paw. The total time spent licking the right hindpaw over the next 40 minutes was measured with a stopwatch and recorded to the nearest second in 5-minute blocks. Based on previous literature, the “early phase” (F_{early}) was defined as 0 to 10 minutes post-injection of formalin, interphase as 10 to 20 minutes post-injection, and the “late phase” (F_{late}) as 20 to 40 minutes post-injection (Tjolsen, et al., 1992).

6. Data analysis

Acute morphine-induced hyperalgesia was expressed as raw withdrawal latencies. Withdrawal latencies were analyzed using one, two or three-way analysis of variance, where appropriate, followed by a Fisher’s LSD (protected t-test) for post-hoc comparisons. *P*-values < 0.05 were considered significant.

For the dose-response study with the different inbred strains, latencies obtained after each test dose were used to calculate the latency values at which each strain achieved 50% of their maximum hyperalgesic response (i.e., HD_{50}). Data were fitted with a one-phase exponential decay function, which showed better goodness-of-fit than any other non-linear regression function available (Prism v5.04). The maximum hyperalgesia response was defined as the plateau of this function for each strain, and the HD_{50} as the half-life.

Chapter 3.

I. Study 1: To define the opioid nature of acute morphine hyperalgesia across two pain modalities.

1. Introduction

Opioids such as morphine remain the most efficacious and widely used analgesics for moderate to severe pain. However, the clinical use of these opioids is complicated by unwanted side effects, including a paradoxical increase in pain sensitivity (i.e., hyperalgesia). Previous studies have demonstrated continuous infusion of opioids can paradoxically increase nociception in humans and rodents (Angst & Clark, 2006; Chu, et al., 2008). Furthermore, our studies show that chronic morphine, oxymorphone, and fentanyl cause hyperalgesia that is not blocked in mice concurrently treated with NTX nor is it diminished in mice completely devoid of the three classic opioid receptors (Juni, et al., 2006; Juni, et al., 2007; Waxman, et al., 2009). These findings indicate that the chronic administration of these opioids causes hyperalgesia independent of opioid receptor activity.

In order to explore and define the opioid nature of acute morphine hyperalgesia, we first aimed to determine whether acute morphine causes hyperalgesia in naïve CD-1 mice treated concurrently with NTX. We assessed nociception via two pain modalities in mice, the tail-withdrawal test and the formalin test, before and after receiving an acute bolus dose of morphine. The tail-withdrawal test is a model designed to investigate phasic pain, mainly peripheral and spinal mediated responses (Lutfy, et al., 1997), whereas the formalin test produces two phases of nociceptive behavior (Dubuisson & Dennis, 1977) and involves both peripheral and central sensitization (South, et al., 2003). We also conducted studies assessing the time course of this

hyperalgesic effect in order to determine the peak hyperalgesic effect of morphine on the tail-withdrawal test.

The second aspect of Study 1 directly tested the assumption that prior analgesia and/or opioid receptor activation are critical to opioid hyperalgesia using opioid receptor knock-out mice lacking all 3 opioid receptor genes (Clarke, et al., 2002; Cox, et al., 2005). These mice were obtained by cross-breeding mice singly deficient in the genes coding for μ, δ, and κ receptors using homologous recombination techniques. This approach 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 approaches also prevent 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 (A. L. Vaccarino, Plamondon, & Melzack, 1992). Although this study was limited to the BALB/c strain and the formalin test, opioid blockade via gene knockout obviously represents a much more comprehensive form of opioid blockade. In addition, the TKO 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.

Lastly, in defining the opioid nature of acute morphine hyperalgesia, the active glucuronide metabolites of morphine, morphine-3β-glucuronide (M3G) and morphine-6β-glucuronide (M6G), may mediate acute morphine hyperalgesia. M3G is the primary product of

morphine biotransformation in rodents and has no appreciable affinity at any opioid receptor subtype. Injecting M3G in rodents via the i.c.v. (Bartlett, Cramond, et al., 1994; Labella, et al., 1979), i.t. (Woolf, 1981; Yaksh, et al., 1986), or systemic (Juni, et al., 2006; Lipkowski, Carr, Langlade, Osgood, & Szyfelbein, 1994) route enhances nociception. Furthermore, the neuroexcitatory effects of M3G are not blocked by the general opioid antagonist naloxone (Labella, et al., 1979; Woolf, 1981; Yaksh, et al., 1986). Therefore, the final aspect of Study 1 involved determining whether morphine causes hyperalgesia by opioid receptors in mice lacking multidrug resistance protein 3 ($Mrp3^{(-/-)}$) pretreated with NTX. Mrp3 is the major transporter able to excrete M3G from hepatocytes into the bloodstream in order to prevent accumulation of the hydrophilic metabolite in the liver. $Mrp3^{(-/-)}$ mice display 50-fold decreased plasma M3G levels and brain M3G levels are below the limit of detection following morphine treatment (Zelcer, et al., 2005). If indeed M3G contributes to acute morphine hyperalgesia, then the reduction of systemic levels of M3G in these mice should result in the absence or marked reduction in the magnitude of morphine hyperalgesia in these mice relative to their FVB wild-type control mice.

2. Materials and Methods

Subjects. Adult male CD-1 mice 6 – 8 weeks of age, were obtained from Charles River Laboratories (Kingston, NY). Opioid-receptor triple knock-out (TKO) 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). 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 opioid receptor subtypes,

and lack gross behavioral or anatomical alterations (Clarke et al., 2002; Cox et al., 2005). Accordingly, B6x129 F_1 mice were bred and served as controls. Lastly, mice lacking the multidrug resistance protein 3 (Mrp3$^{(-/-)}$) (Gift of Piet Borst, The Netherlands Cancer Institute, The Netherlands) are unable to excrete M3G from the liver into the bloodstream, the major hepatic elimination route for morphine. They shift their excretion of M3G from primarily urinary to primary fecal. Since Mrp3$^{(-/-)}$ mice are on a 99% FVB background, FVB mice served as controls.

All mice were housed separately, housed 4 to a cage, and maintained on a 12:12-hour light/dark cycle in a climate-controlled room (22 °C ± 2°C) with free access to food and tap water. To eliminate any interaction of sex with nociceptive sensitivity and morphine hyperalgesia (Kest et al., 2000b), only males were tested. Each subject was used once and for all groups, $n \geq 8$.

Behavioral assays. The warm-water tail-withdrawal test, as described above in the General 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). The formalin test, also described above in the General Methods section, assesses the way an animal responds to moderate, continuous pain generated by injured tissue. Evidence suggests that the formalin test is biphasic, consisting of an acute "early" phase and a tonic "late" phase, and provides a more valid model of clinical pain than the tests with phasic mechanical or thermal stimuli (Dubuisson & Dennis, 1977; Hunskaar, et al., 1985).

Drugs and drug delivery. Morphine and naltrexone were dissolved in a saline vehicle and delivered using a 10ml/kg injection volume. They were injected subcutaneously and obtained from the NIDA Drug Supply Program.

Morphine hyperalgesia time–response studies. All mice were first weighed and assayed for baseline withdrawal latencies. Then, they received either a subcutaneous injection of NTX (15mg/kg) or saline. Thirty minutes later, mice were injected with an acute morphine dose (2mg/kg or 15mg/kg) or saline subcutaneously and retested for nociception over the next 30–120 minutes.

Only CD-1 mice were subjected to the formalin test. They first received a subcutaneous injection of either saline or NTX (15mg/kg). Thirty minutes later, mice were injected with either an acute morphine dose (15mg/kg) or vehicle subcutaneously. Ten minutes later, mice were lightly restrained, and 20 uL of 1% formalin solution was injected just under the skin of the plantar surface of the right hindpaw by use of a microsyringe with a 30-gauge needle. These parameters are common for use in mice (see Tjolsen et al*.,* 1992). Mice were returned to the cylinders and immediately observed for licking of the affected hindpaw. The total time spent licking the right hindpaw over the next 40 minutes was measured with a stopwatch and recorded to the nearest second in 5-minute blocks.

Data analysis. Withdrawal latencies and time spent licking were analyzed using a two-way (treatment, time) analysis of variance followed by a Fisher's LSD (protected t-test) for post-hoc comparisons. *P*-values < 0.05 were considered significant. Values reported are mean ± SEM.

3. Results

Study 1A: Morphine hyperalgesia time-response studies. Two behavioral assays, the tail-withdrawal test and the formalin test, were utilized to assess the hyperalgesic magnitude and duration of acute systemic morphine doses. On the tail-withdrawal test, mice received an acute

subcutaneous injection of either 2 mg/kg or 15 mg/kg morphine, after pretreatment with NTX (Figure 1). Two groups of animals served as controls: those who received an acute saline injection and were pretreated with saline and those who received an acute saline injection and were pretreated with NTX. Latencies of control animals (saline + saline) did not differ from NTX + saline-treated animals, indicating that NTX has no effect on nociception. Acute injection of 2 mg/kg and 15 mg/kg morphine in NTX-pelleted mice caused significant latency reductions from baseline, indicative of hyperalgesia, 30-60 minutes post-injection. Additionally, there is a significant difference between the latencies obtained from the 2 mg/kg morphine dose and those obtained from the 15 mg/kg dose. Therefore, this finding provides evidence that a dose-dependent hyperalgesia also exists.

On the formalin test, mice were injected with either saline or NTX 30 minutes prior to receiving either an acute bolus dose of saline or 15 mg/kg morphine. Ten minutes later, formalin was injected into the plantar surface of the right hindpaw. A clear, biphasic pattern of licking behavior was observed in all groups (Figure 2). Licking behavior of control animals (saline + saline) did not differ from licking behavior of NTX + saline-treated animals in any phase, indicating that NTX has no effect on nociception on the formalin test. However, mice receiving acute morphine spent significantly more time licking in F_{early} and the interphase than mice receiving saline. For the late phase, no significant differences were detected between groups. Therefore, an acute morphine bolus dose only causes significant hyperalgesia in the early phase and interphase phase of the formalin test.

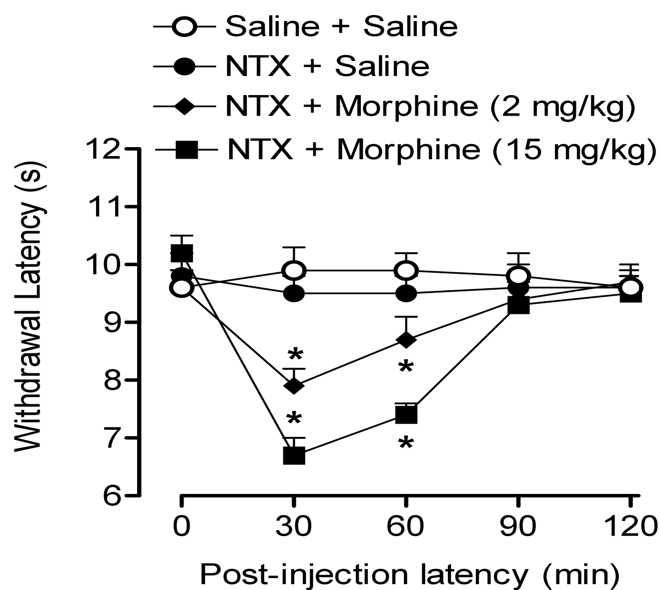

Figure 1. Time course of acute morphine hyperalgesia with CD-1 male mice pretreated with NTX on tail-withdrawal test. Mice were injected with either saline or NTX (15 mg/kg) 30 minutes before receiving an acute bolus dose of saline or morphine (2 mg/kg or 15 mg/kg) and nociception was assayed for the next 120 minutes. Values represent mean tail-withdrawal latency ± S.E.M.; significant decreases (*) in withdrawal latencies relative to values obtained prior to morphine treatment (time 0) are indicated.

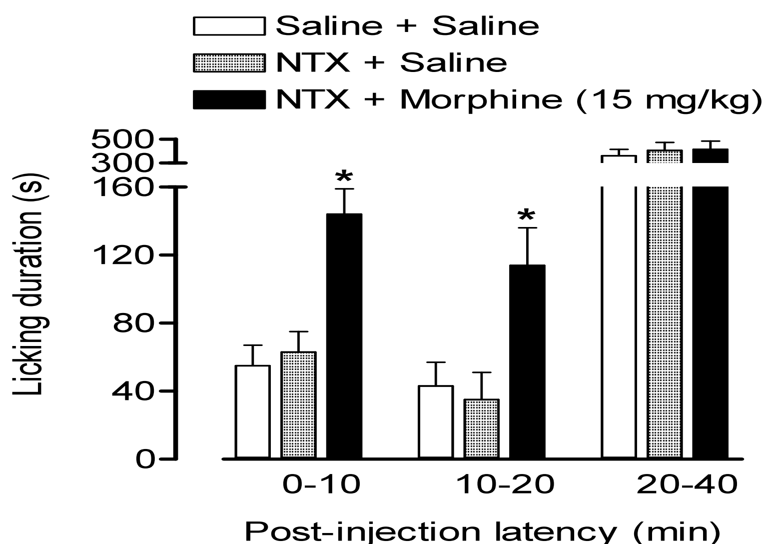

Figure 2. Acute morphine hyperalgesia with CD-1 male mice pretreated with NTX on the formalin test. Mice were injected with either saline or NTX (15 mg/kg) 30 minutes before receiving an acute bolus dose of saline or morphine (15 mg/kg). Bars represent the mean total time spent licking the affected paw S.E.M. in F_{early} (0-10 minutes), interphase (10-20 minutes), and F_{late} (20-40 minutes). Significant increases (*) in licking behavior relative to values obtained from other groups are indicated.

Study 1B: Acute morphine hyperalgesia in mice lacking μ, δ, and κ opioid receptors. Whereas saline injection had no subsequent effect on withdrawal latencies relative to baseline (time 0) values, an acute morphine injection caused significant hyperalgesia during the subsequent 30-90 minutes for TKO male mice (Figure 3). Serving as controls, all B6x129 F_1 male mice were pretreated with NTX. Therefore, those animals that received an acute dose of morphine demonstrated significant decreases in withdrawal latencies during 30-120 minutes compared to those receiving saline.

Study 1C: The role of morphine metabolites in acute morphine hyperalgesia. An acute systemic morphine injection caused significant reduction of withdrawal latencies from 30-120 minutes post-injection in $Mrp3^{(-/-)}$ and FVB male mice pretreated with NTX (Figure 4). $Mrp3^{(-/-)}$ and FVB mice pretreated with NTX that received saline did not show any reductions from baseline and therefore, served as controls.

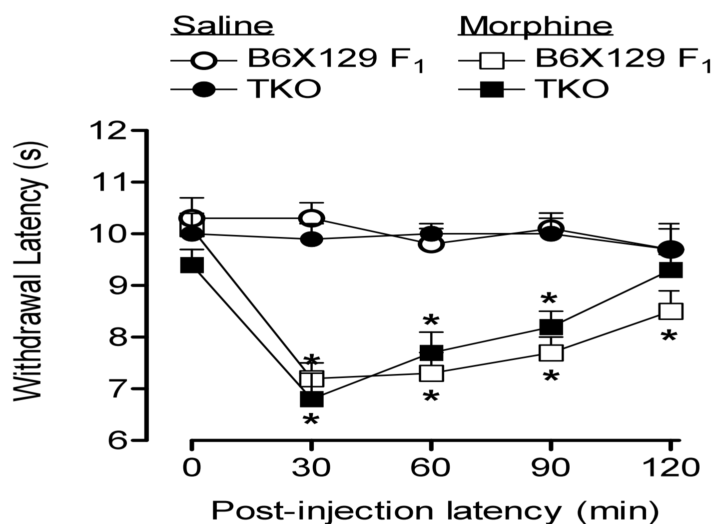

Figure 3. Time course of acute morphine hyperalgesia with TKO and B6x129 F_1 male mice. All mice received an acute bolus dose of saline or morphine (15 mg/kg) and nociception was assayed via the tail-withdrawal test for the next 120 minutes. B6129F_1 male mice were pretreated with NTX in order to serve as controls. Values represent mean tail-withdrawal latency ± S.E.M.; significant decreases (*) in withdrawal latencies relative to values obtained prior to morphine treatment (time 0) are indicated.

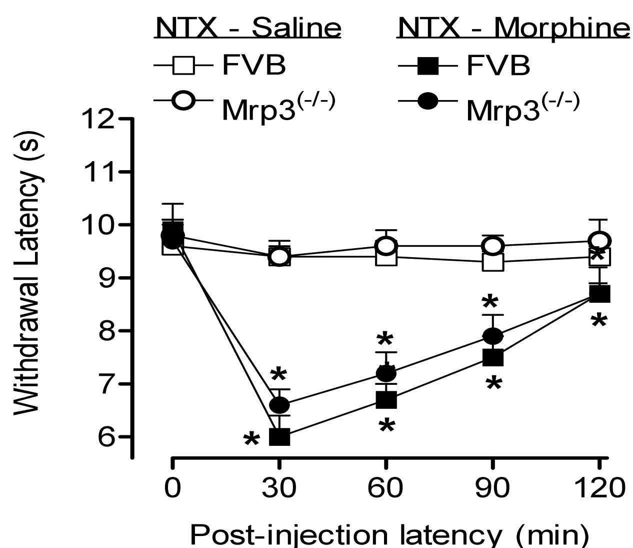

Figure 4. Time course of acute morphine hyperalgesia with Mrp3$^{(-/-)}$ and FVB male mice. All mice were pretreated with NTX (15 mg/kg) 30 minutes before receiving an acute bolus dose of saline or morphine (15 mg/kg) and nociception was assayed on the tail-withdrawal test for the next 120 minutes. Values represent mean tail-withdrawal latency ± S.E.M.; significant decreases (*) in withdrawal latencies relative to values obtained prior to morphine treatment (time 0) are indicated.

4. Discussion

The major findings of Study 1 were as follows: 1) Pre-treatment with NTX providing continuous opioid receptor blockade blocked analgesic expression, but did not prevent hyperalgesia; 2) Acute bolus morphine injection causes significant decreases in withdrawal latencies and significant increases of licking behavior, both indicative of hyperalgesia, in male CD-1 mice pretreated with NTX; 3) Acute bolus morphine injection causes hyperalgesia in male mice lacking opioid receptors; 4) Mice lacking the multidrug resistance protein 3 ($Mrp3^{(-/-)}$) also demonstrate acute morphine hyperalgesia that is independent of opioid receptors. These findings are discussed in greater detail below.

Non-opioid mediation of hyperalgesia. In order to begin to define acute morphine hyperalgesia, injection of NTX was used to ensure opioid receptor blockade during acute morphine injection. Since NTX was injected 30 minutes prior to morphine injection, it is logical to assume that functional opioid receptor blockade was in effect throughout different time courses. The NTX dose utilized in these studies was significantly larger in magnitude than those necessary to selectively block excitatory G_s-coupled opioid receptors that yield hyperalgesia (S. M. Crain & K. F. Shen, 2000; Crain & Shen, 2001) and, as presently demonstrated, inhibitory $G_{i/o}$-coupled opioid receptors that yield analgesia. Therefore, these findings demonstrate acute morphine hyperalgesia that is independent of concurrent or prior opioid receptor activation.

Two experimental assays of nociception, the tail-withdrawal test and the formalin test, were utilized to characterize acute morphine hyperalgesia in CD-1 male mice. The tail-withdrawal test measures transient pain, in which a brief noxious thermal stimulus is applied to the distal portion of the tail. The reduction in withdrawal latencies from 30-60 minutes post-injection of acute systemic morphine indicates that acute morphine-induced hyperalgesia occurs

over this time course. The formalin test is a model of inflammatory pain that produces two phases of nociceptive behavior (Dubuisson & Dennis, 1977). The early or acute phase occurs immediately after injection of formalin and the late or tonic phase occurs 20-40 minutes after the formalin injection. Acute morphine hyperalgesia only occurs during the "early" phase and the interphase, not the "late" phase.

These findings for the tail-withdrawal test are supported by previous research utilizing a chronic morphine infusion model with NTX-pelleted CD-1 male mice. Juni and colleagues (2006) demonstrated that continuous infusion of both 8.0 and 40.0 mg/kg morphine resulted an initial dose-dependent increase of withdrawal latencies relative to their respective baseline latencies, illustrating an initial analgesic effect of about 2 days of duration followed by dose-dependent hyperalgesia starting on Day 4. In contrast, 1.6 mg/kg morphine infusion produced no detectable initial analgesic effect. Instead, significantly decreased tail-withdrawal latencies (i.e. hyperalgesia) were already evident 6 hours after the start of morphine infusion, and continued unabated until Day 6. Likewise, the initial analgesic phase elicited by the 40.0 mg/kg morphine dose was already visible 3 hours after the start of infusion. 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), demonstrating hyperalgesic adaptation. Furthermore, acute and chronic administration of fentanyl and M6G cause hyperalgesia, as indicated by the tail-withdrawal test, that is not blocked in mice concurrently treated with NTX (van Dorp, et al., 2009; Waxman, et al., 2009).

To confirm the findings from CD-1 male mice pretreated with NTX, acute morphine injection increased nociception in knock-out mice lacking all 3 genes encoding for μ, δ, and κ opioid receptors. The fact that mice devoid of opioid receptors still exhibited OIH 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 confirm that an acute morphine injection causes hyperalgesia that does not require prior or concurrent opioid receptor activity or analgesia. The non-opioid nature of acute morphine hyperalgesia in TKO mice is supported by identical findings from a chronic model of morphine-induced hyperalgesia (Juni, et al., 2007) as well as acute and chronic studies of fentanyl-induced hyperalgesia in TKO mice (Waxman, et al., 2009). With the lack of involvement of the opioid receptors, the mechanisms underlying acute morphine hyperalgesia are still unknown and further investigations to understand these underlying mechanisms need to be conducted.

For the findings from the formalin test, we expected acute morphine hyperalgesia to occur during the "early" phase and the "late" phase, not the interphase. The nociceptive response to subcutaneous formalin is matched by a corresponding biphasic increase in the activity of dorsal horn neurons after formalin injection. Behavioral and electrophysiological studies demonstrate that central neural changes that occur during the early phase of the formalin test are essential for the development of the later tonic phase. Intrathecal administration of DAMGO, a potent and selective μ-opiate agonist, significantly inhibits the prolonged increase in dorsal horn activity produced by subcutaneous formalin (Dickenson & Sullivan, 1987). However, this inhibition occurs only if the drug is given before the formalin injection, implying that the dorsal horn activity associated with the late phase of the formalin test depends upon spinal activation during the early phase immediately after formalin injection. Thus, with opioid receptor blockade, morphine failed to inhibit the increase in dorsal horn neuron responses in the early phase and the interphase in our study. Since excitatory amino acids (EAAs) acting at the NMDA receptor contribute to the injury-induced sensitization of the dorsal horn (Coderre &

Melzack, 1992a), morphine may also contribute to the increase in EAAs, leading to greater sensitization and manifestation of hyperalgesia in the early phase and interphase. However, the development of plasticity or sensitization by the activation of NMDA receptors occurs in the early phase, as indicated by the selective effectiveness of NMDA receptor antagonists in the early phase (Coderre & Melzack, 1992a). Therefore, since the NMDA receptors do not contribute to the ongoing pain responses after sensitization has occurred (late phase), morphine hyperalgesia may not be demonstrated in the late phase.

Furthermore, evidence also shows that activity in supraspinal structures, including the cingulum bundle and the fornix, during the early phase response to formalin is critical to the development of the late phase response. The cingulum bundle and fornix are part of a neural loop that projects from the anterior thalamic nuclei to the cingulate cortex, hippocampus, and mammillary bodies, and returns to the anterior thalamic nuclei (Coderre, Vaccarino, & Melzack, 1990; Anthony L. Vaccarino & Melzack, 1992). Morphine may lead to increased activation of this "closed" circuit during the early phase of the formalin response, which induces enhancement in sensitization and thus, a hyperalgesic response.

However, the present study also demonstrates significant hyperalgesia that is independent of opioid receptors, in the "interphase" of the formalin test. Even though the interphase has been largely ignored by the scientific community, evidence demonstrates that an active inhibitory process, instead of a lack of excitation, occurs during the interphase (Henry, Yashpal, Pitcher, & Coderre, 1999). In few studies to date, the findings show that this inhibition is not under regulation of a GABAergic or the opioid receptors mechanism (Henry, et al., 1999). However, this active inhibitory mechanism underlying interphase still requires further investigation and may be disabled by the administration of morphine.

Non-metabolite mediation of hyperalgesia. Non-opioid metabolites of morphine have been proposed to serve as a possible mechanism underlying morphine-induced hyperalgesia (Woolf, 1981; Yaksh et al., 1986; De et al., 1991; Sjogren, Thunedborg, Christrup, Hansen, & Franks, 1998). M3G is the primary metabolic byproduct of morphine biotransformation in rodents (Wahlstrom, Hammar, Lundin, & Rane, 1986; Kuo, Hanioka, Hoshikawa, Oguri, & Yoshimura, 1991), yet has no detectable affinity at any opioid receptor subtype (LaBella et al., 1979; Bartlett et al., 1994a; Bartlett et al., 1994b). M3G is also devoid of an 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), i.t. (Woolf, 1981; Yaksh et al., 1986), and systemic (Ekblom, Gardmark, & Hammarlund-Udenaes, 1993; Lipkowski et al., 1994) administration, that is naloxone insensitive (LaBella et al., 1979; Yaksh et al., 1986). In addition, the neuroexcitatory 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).

However, in the present study, M3G accumulation from acute systemic morphine administration did not underlie OIH. Mrp3$^{(-/-)}$ mice, pretreated with NTX, demonstrated hyperalgesia for 30-120 minutes following an acute morphine bolus dose. Therefore, acute morphine injection causes hyperalgesia that does not require the actions of morphine metabolite, M3G. Multidrug resistance protein (Mrp3), a protein present in the basolateral membrane of polarized cells, is the major transporter of M3G and M6G *in vitro* (Zelcer, et al., 2005). Furthermore, Mrp3 transports these metabolites from hepatocytes, where glucuronidation occurs, into the bloodstream in order to prevent accumulation of the hydrophilic metabolites in the liver.

Mrp3$^{(-/-)}$ mice, lacking the Mrp3 transporter, display 50-fold decreased plasma M3G levels and brain M3G levels are below the limit of detection following morphine treatment (Zelcer, et al., 2005). Furthermore, since these mice are unable to excrete M3G from the liver into the bloodstream, the main disposition route for morphine and M3G shifts from the urine to the feces (Zelcer, et al., 2005). Since M3G is the only morphine metabolite formed in mice and these mice lack circulating M3G and primarily eliminate it through their feces, our findings demonstrate that M3G does not contribute to the hyperalgesic state of Mrp3$^{(-/-)}$ mice.

The literature provides limited evidence to support that acute morphine hyperalgesia is not mediated by morphine metabolites. In a previously reported mouse study, systemic injection of M3G doses between 10 and 100 mg/kg failed to produce overt behavioral responses or hyperalgesia (Bian & Bhargava, 1996). Acute and chronic systemic administration of fentanyl, a synthetic opioid, causes hyperalgesia independently of prior or concurrent opioid receptor activity or analgesia (Waxman, et al., 2009). Since the biotransformation of fentanyl does not yield any known pronociceptive metabolites, these findings challenge the supposition that glucuronide metabolites are broadly involved in OIH. Additionally, Gardell and colleagues concluded that oxymorphone hyperalgesia is unrelated to excitatory metabolites (Gardell, et al., 2006). 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. Therefore, with this evidence of non-metabolite mediated OIH, additional studies are needed to clarify how acute morphine administration causes non-opioid receptor mediated hyperalgesia.

II. Study 2: To determine the contribution of route of administration to acute morphine hyperalgesia.

1. Introduction

The spinal cord is believed to be a critical site for the mediation of the antinociceptive effects of morphine (Akil, et al., 1984; Basbaum & Fields, 1984; Yaksh, 1981). Previous studies show that administration of opioids via the spinal route produces a hyperalgesic response in rodents and humans (Sakurada, Komatsu, & Sakurada, 2005). Acute studies first revealed that high morphine doses into the spinal subarachnoid space decreased tail-withdrawal latency, indicative of hyperalgesia (Woolf, 1981). In rats with chronically implanted i.t. catheters, high concentrations of morphine yielded a syndrome of pain behavior that involved biting and scratching at the dermatomes innervated by levels of the spinal cord proximal to the catheter tip (Yaksh & Harty, 1988; Yaksh, et al., 1986). Furthermore, pretreatment with NTX did not reduce the biting, licking, and scratching behaviors elicited by an i.t. injection of morphine (Sakurada, et al., 1996). In response to the Yaksh and colleagues (1986) study, a case report of a patient with terminal cancer receiving high concentrations of spinal morphine demonstrated that she showed a hyperalgesic response in her lower extremities (Ali, 1986). Research then accumulated about hyperalgesic states in humans following administration of high-dose subarachnoid morphine (Arner, et al., 1988; De Conno, et al., 1991; Krames, et al., 1985; Penn & Paice, 1987; Sjogren, Jonsson, Jensen, Drenck, & Jensen, 1993; Werz & MacDonald, 1982).

Conflicting evidence appears for low morphine doses via i.t. administration. In some studies, low acute morphine doses of the (-) enantiomer produced a significant prolongation of response time for tail immersion whereas the (+) enantiomer, which is inactive to the opioid receptor binding sites, produced hyperalgesia (Woolf, 1981). In contrast, other studies

demonstrated that low acute morphine doses produced brief excitatory effects in the flexor reflex, which is usually marked by the analgesic effect of morphine (Wiesenfeld-Hallin, et al., 1991). Furthermore, in the chronic models, rats receiving daily bolus injections of i.t. morphine for 8 days developed thermal hyperalgesia in association with antinociceptive tolerance (Mao, et al., 1994). In rats, continuous spinal delivery of DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin), a synthetic opioid peptide with high opioid receptor specificity, through osmotic minipumps also produced thermal hyperalgesia and tactile allodynia of the hind paws and a decrease in antinociceptive potency and efficacy of spinal DAMGO (Vanderah, et al., 2000).

Descending projections from supraspinal sites may also play a role in acute OIH. Considerable evidence reveals a significant contribution of supraspinal influences to the development and maintenance of hyperalgesia in the rodent model. Opioid-induced mechanical and thermal hypersensitivity can be reversed by lidocaine administration into the rostral ventromedial medulla (RVM) or by lesion of the dorsolateral funiculus (DLF), suggesting that descending pain facilitation from the RVM to the spinal cord is necessary for the manifestation of chronic OIH to noxious and non-noxious stimuli (Vanderah, Suenaga, et al., 2001). Furthermore, an underlying neural basis also contributes to the hyperalgesic action of cholecystokinin (CCK), identified as an anti-opioid peptide. A low dose of CCK microinjected into the RVM blocked the analgesic effect of systemically administered morphine by preventing activation of the off-cells whereas microinjection of a higher dose of CCK alone activated the on-cells only and also produced hyperalgesia (Heinricher, et al., 2001; Heinricher & Neubert, 2004).

Previous studies demonstrate that OIH via systemic administration of morphine is not mediated by opioid receptors. To localize whether spinal and/or supraspinal central nervous

system loci contribute to systemic acute morphine hyperalgesia, mice were injected with NTX and then tested for nociception on the tail-withdrawal test before and after receiving acute morphine via the i.t. or i.c.v. route. This study aimed to determine whether morphine injected into spinal and supraspinal loci can increase nociception, and that this ensuing hyperalgesia is not mediated by opioid receptors.

2. Materials and Methods

Subjects. Naïve, adult male CD-1 mice were obtained commercially from Charles River Laboratories (Kingston, NY). All mice were maintained on a 12:12-hour light/dark cycle in a climate-controlled room with free access to food and tap water. Each subject was used once and for all groups, $n \geq 8$.

Nociceptive assay. Nociception was assessed using a modified version of the tail-withdrawal test as described previously.

Drugs and drug delivery. Naltrexone and morphine were dissolved in a saline vehicle. Naltrexone was injected via the systemic route, and morphine was injected via the systemic, i.t., or i.c.v. route. i.c.v. injections were made into the lateral ventricles using the method of Haley and McCormick. Specifically, a small midline incision was made in the scalp of mice under oxygen/isoflurane inhalant anesthesia, and lambda located. Injections (5 µl volume) were made directly through the skull at a point 2mm rostral and lateral to lambda at a depth of 3mm using a 10-µl Hamilton micro-syringe fitted with a 27-gauge needle. A stainless steel wound clip was used to close the incision after each injection. i.t. injections were made under light oxygen/isoflurane inhalant anesthesia using a 10-µl Hamilton micro-syringe fitted with a 27-gauge needle and administered by lumbar puncture.

Procedure. Groups of mice were injected subcutaneously with either an acute NTX (15 mg/kg) dose or saline and assayed for nociception on the tail-withdrawal test (time 0). Thirty minutes later, an acute morphine injection was given via the i.t. (1 mg/kg) or i.c.v. route (1 mg/kg) and nociception was assayed for the next 60 and 90 minutes, respectively.

Data analysis. Withdrawal latencies were analyzed using a two-way (treatment, time) analysis of variance followed by a Fisher's LSD (protected t-test) for post-hoc comparisons. *P*-values < 0.05 were considered significant. Values reported are mean ± SEM.

3. Results

Whereas saline injection had no subsequent effect on withdrawal latencies relative to baseline (time 0) values, an acute i.t. morphine injection caused significant hyperalgesia during the subsequent 15-45 minutes for NTX-pretreated CD-1 male mice (Figure 5). Withdrawal latencies returned to baseline at 60 minutes post-injection of morphine. CD-1 male mice pretreated with NTX and saline and injected i.t. with saline, served as control groups. Therefore, those animals that received an acute dose of morphine via the spinal route demonstrated significant decreases in withdrawal latencies during 15-45 minutes compared to those receiving saline.

An acute i.c.v. injection of morphine in NTX-pretreated CD-1 male mice caused significant withdrawal latency reductions from baseline values, indicative of hyperalgesia, which persisted for the duration of the 90-minute testing time course (Figure 6). Latencies of CD-1 mice pretreated with saline or NTX and injected i.c.v. with saline did not show any reductions in withdrawal latencies from their respective baseline values at any post-injection testing interval over the time course.

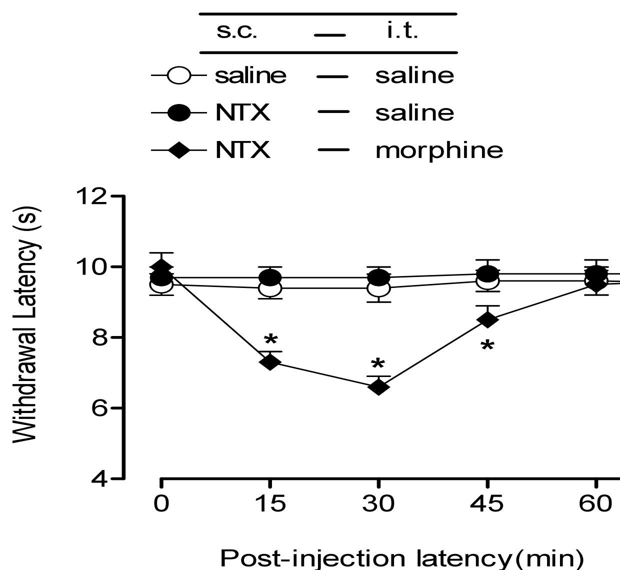

Figure 5. Time course of acute morphine hyperalgesia after intrathecal injection of morphine in NTX-treated CD-1 male mice. Mice were subcutaneously injected with either saline or NTX (15 mg/kg) and assayed for nociception on the tail-withdrawal test (time 0). Thirty minutes later, mice received an acute i.t. injection of saline or morphine (1 mg/kg) and nociception was assayed for the next 60 minutes. Data are mean ± S.E.M. latencies obtained prior to NTX and morphine injection (0) and at 15-minute intervals post-morphine injection. Significant treatment, time, and time x treatment effects were observed (all $P < 0.001$). Post-hoc comparisons: * $P < 0.01$ versus BL (pre-morphine baseline).

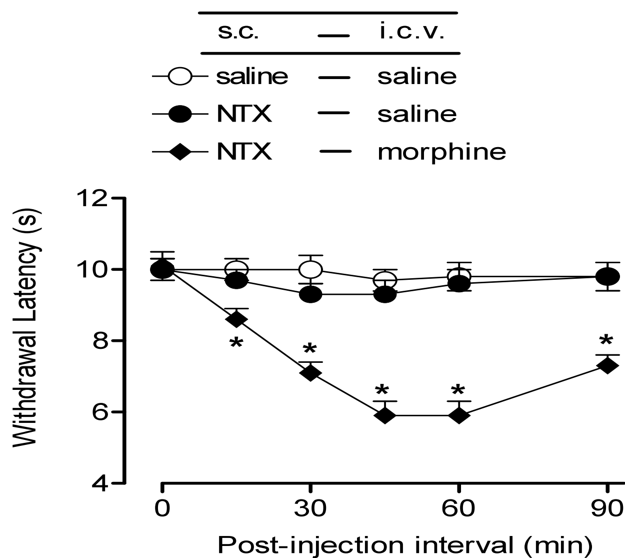

Figure 6. Time course of acute morphine hyperalgesia after intracerebroventricular injection of morphine in NTX-treated CD-1 mice. Mice were subcutaneously injected with either saline or NTX (15 mg/kg) and assayed for nociception on the tail-withdrawal test (time 0). Thirty minutes later, mice received an acute i.c.v. injection of saline or morphine (1 mg/kg) and nociception was assayed for the next 90 minutes. Data are mean ± S.E.M. latencies obtained prior to NTX and morphine injection (0) and at 15-minute intervals post-morphine injection. Significant treatment, time, and time x treatment effects were observed (all $P < 0.001$). Post-hoc comparisons: * $P < 0.01$ versus BL (pre-morphine baseline).

4. Discussion

The major findings of Study 2 were as follows: 1) Acute morphine injection via spinal administration causes hyperalgesia in male CD-1 mice pretreated with NTX; 2) Acute morphine injection via supraspinal administration causes hyperalgesia in male CD-1 mice pretreated with NTX; 3) The time course of hyperalgesia was longer for the supraspinal administration of morphine compared to the spinal administration of morphine. These findings are discussed in greater detail below.

Spinal contribution to acute morphine hyperalgesia. The findings from this study demonstrate that acute morphine injected directly into the spinal cord increases nociception, but that the ensuing hyperalgesia is not mediated by opioid receptors. Therefore, this study confirms the importance of these spinal loci in acute OIH and perpetuates the search to identify the underlying mechanisms of OIH. In previous literature, a critical role has been attributed to an endogenous pain facilitatory system involving NMDA and NK-1 receptors and their respective neurotransmitters, glutamate and substance P (SP), and the actions of dynorphin, an endogenous pronociceptive ligand, in the spinal cord.

The role of glutamate and SP, both excitatory amino acid (EAA) neurotransmitters, in the spinal cord has been implicated as an underlying mechanism in OIH. SP and glutamate induces a series of scratching, biting, and licking behaviors indicative of nociceptive behavior when administered intrathecally in mice and rats (Hylden & Wilcox, 1981; Seybold, Hylden, & Wilcox, 1982; Takahasi, et al., 1987). Spinal injection of glutamate or substance P also evoked an exaggerated pain response in mice chronically exposed to morphine (Li, Angst, & Clark, 2001a). An increased content of EAA neurotransmitter in dorsal root ganglion neurons in the spinal cord has been confirmed through studies investigating chronic i.t. morphine administration

(Belanger, Ma, Chabot, & Quirion, 2002; Ibuki, Marsala, Masuyama, & Yaksh, 2003). This increased availability of spinal glutamate may be caused partly by a decreased activity of spinal glutamate transport systems associated with i.t. opioid administration (Mao, et al., 2002). Therefore, OIH may be connected to increased availability of glutamate and SP in spinal cord tissue after i.t. morphine administration.

Enhancement of NMDA receptor function in dorsal horn neurons may also underlie OIH. The NMDA receptors are located postsynaptically and extrasynaptically in the membrane of the dorsal horn neurons and are activated by the presynaptic release of glutamate from nerve terminals of the dorsal root ganglia cells depolarized by pain-provoking thermal, mechanical, or chemical stimuli. Activation of spinal NMDA receptors causes the dorsal horn neurons to depolarize and fire action potentials, resulting in further release of glutamate and propagation of pain signal through the spinal cord and to the brain. Intrathecal administration of NMDA elicits scratching, biting, and licking behaviors indicative of nociceptive response in mice (Aanonsen & Wilcox, 1987; Sakurada, Manome, Tan-No, Sakurada, & Kisara, 1990) or thermal hyperalgesia (Malmberg & Yaksh, 1993). Furthermore, the competitive NMDA receptor antagonists, D-APV and CPP, and the non-competitive NMDA receptor antagonist, MK-801, reduce the behavioral response of intrathecally administered high-dose morphine (Dougherty, Palecek, Paleckova, Sorkin, & Willis, 1992; Sakurada, et al., 2002; Watanabe, Sakurada, et al., 2003). Specifically, i.t. administration of selective NMDA receptor antagonists produce antinociceptive effects in both phasic and tonic behavioral nociceptive tests in rats (Cahusac, Evans, Hill, Rodriquez, & Smith, 1984) as well as reduce hyperalgesia associated with inflammatory (Ren, et al., 1992) and neuropathic injury (Mao, Price, Mayer, Lu, & Hayes, 1992; T. Yamamoto & Yaksh, 1992) in

rats. Therefore, the increased activation of NMDA receptors may contribute to the underlying mechanism of OIH.

NMDA receptors and their intracellular cascade may play an important role in hyperalgesia evoked by i.t. administration of morphine. The effect of NMDA receptor activation and the associated Ca^{2+} influx result in production of nitric oxide (NO), an endogenous short-living free radical gas (Meller & Gebhart, 1993). Inhibitors of NO synthase (NOS), including L-N^G-nitro arginine methyl ester (L-NAME) and L-N^G-monomethyl arginine, were utilized in behavioral studies to determine the functional significant of NO (Moncada, Palmer, & Higgs, 1991). Pretreatment with L-NAME causes a significant inhibition of nociceptive behavioral response to i.t. high-dose morphine (Watanabe, Sakurada, et al., 2003). Therefore, i.t. morphine activates NMDA receptors in the spinal cord, which in turn leads to increased NO production. Increase in intracellular protein kinase C (PKC) activity that occurs in response to NMDA receptor activation has also been implicated in intracellular mechanisms of OIH. GM1 ganglioside and H-7, intracellular inhibitors of PKC translocation and activation (F. Vaccarino, Guidotti, & Costa, 1987), effectively attenuate hyperalgesia induced by peripheral nerve injury (Hayes, et al., 1992; Mao, Hayes, et al., 1992; Mao, Price, Hayes, Lu, & Mayer, 1992b) and reduce formalin-induced hyperalgesic responses (Coderre & Melzack, 1992b). This evidence demonstrates a critical role for central NMDA receptor activation and its subsequent intracellular changes in the mechanisms of OIH.

Tachykinin neurokinin 1 (NK-1) receptors in the spinal cord have also been investigated and shown to contribute to OIH (T. King, et al., 2005). NK-1 antagonists block scratching, biting and licking responses to i.t. administration of SP. Intrathecal co-administration of NK_1 receptor antagonists, sendide and CP-96,345, also inhibit spinally-mediated behavioral responses

evoked by i.t. high dose morphine (Sakurada, et al., 1996). Furthermore, the ablation of spinal dorsal horn cells expressing the NK-1 receptor, with an intrathecal injection of SP-saporin (SP-SAP) prevented chronic morphine-induced thermal and mechanical hyperalgesia, increased touch-evoked spinal FOS expression, and prevented upregulation of spinal dynorphin content (Vera-Portocarrero, et al., 2007). In contrast, fentanyl-induced hyperalgesia was not completely blocked by the ablation of NK-1 expressing neurons, suggesting active participation of other mechanisms in OIH (Rivat, et al., 2009). Additionally, long-term spinal infusion with either morphine or naloxone did not produce any changes in NK-1 receptor densities in the dorsal horns of the spinal cord, suggesting that changes in SP availability is implicated in OIH (Gouarderes, et al., 1993).

Evidence also implicates the non-opioid actions of spinal dynorphin as a possible underlying mechanism of OIH. Chronic spinal DAMGO in rats caused tactile allodynia and thermal hyperalgesia of the hindpaws as well as elicited an increase in lumbar dynorphin content (Vanderah, et al., 2000). Furthermore, i.t. administration of dynorphin A antiserum blocked tactile allodynia and reversed thermal hyperalgesia to above baseline levels. Vanderah and colleagues (2000) suggested the involvement of opioid receptors in the expression and regulation of spinal dynorphin, due to the finding that spinal dynorphin content was elevated after DAMGO infusion. However, in this experiment with the blockade of μ, δ, and κ opioid receptors, the involvement of the opioid receptors is not a possibility. Instead, this overexpression of dynorphin likely affects the release of excitatory neurotransmitters. Studies demonstrated that dynorphin A (1-17) can increase the release of capsaicin-stimulated SP (Arcaya, Cano, Gomez, Maixner, & Suarez-Roca, 1999) and that non-opioid fragments of dynorphin can increase capsaicin-stimulated calcitonin gene-related peptide (CGRP) release (Gardell, et al., 2002).

Furthermore, the increase in evoked CGRP release from spinal tissues taken from morphine-exposed rats is blocked by antiserum to dynorphin, but not by a control serum (Gardell, et al., 2002). Therefore, the increased expression and release of dynorphin and CGRP provide a physiological basis for increased stimulus-induced excitation of the spinal cord that may underlie OIH.

Supraspinal contribution to acute morphine hyperalgesia. In this study, the findings demonstrate that acute morphine injected directly into the brain increases nociception, but that the ensuing hyperalgesia is not mediated by opioid receptors. Additionally, an acute injection of morphine in the brain causes significant hyperalgesia for thirty minutes longer than an acute injection of morphine in the spinal cord. Therefore, these findings confirm the role of supraspinal loci in acute OIH and suggest that increased activation of neurons in the brain, through the release of excitatory neurotransmitters, including cholecystokinin (CCK), glutamate, and SP, and their respective receptors, may contribute to the underlying mechanisms of OIH.

This evidence supports that neuroplastic changes in supraspinal loci may also contribute to the underlying mechanisms of OIH. Supraspinal sites, including the midbrain periaqueductal gray (PAG) and the rostral ventral medulla (RVM), have been identified as modulating spinal nociceptive input (Basbaum & Fields, 1984; Fields, et al., 1991). The majority of efferent projections from the PAG terminate in the RVM (Abols & Basbaum, 1981), which sends descending projections that terminate in laminae I, II, and V of the trigeminal n. caudalis and laminae I-III and V-VII of the spinal cord dorsal horn (Basbaum, et al., 1978). Serving as the source of spinopetal inhibitory and facilitatory modulation of nociceptive inputs, the RVM is composed of specialized groups of neurons, on, off, and neutral cells, and has descending projections that once activated, release serotonin and CCK at the spinal cord (Heinricher &

Neubert, 2004). Studies demonstrate that local application of CCK into RVM activates on-cells, which in turn produce behavioral allodynia and hyperalgesia (Heinricher & Neubert, 2004; Kovelowski, et al., 2000; Xie, et al., 2005). Furthermore, increased activity of CCK in the RVM activates spinal pathways that upregulate spinal dynorphin and consequently enhance nociceptive inputs at the spinal level (Vanderah, Suenaga, et al., 2001). To confirm the role of the RVM in OIH, microinjection of lidocaine into the RVM or dorsolateral funiculus (DLF) lesions abolishes OIH (Rivat, et al., 2009; Vanderah, Suenaga, et al., 2001). Furthermore, microinjection of lidocaine into the RVM abolished hyperalgesia on the first day after acute fentanyl administration, demonstrating that acute OIH requires activation of descending pain facilitatory mechanisms in the RVM (Rivat, et al., 2009).

Since the localization and expression patterns of NMDA receptors have been mapped in the PAG and RVM, NMDA receptors at these supraspinal loci may also contribute to OIH. Previous studies have indicated that opioids activate NMDA pain facilitatory processes in the RVM. Blockade of medullary NMDA receptors or of NO generation attenuates somatic and visceral hyperalgesia (Coutinho, Urban, & Gebhart, 2001; Urban & Gebhart, 1999). NMDA-receptor mediated activation of on-cells in the RVM is also required for secondary thermal hyperalgesia in acute inflammation (Xu, et al., 2007). Furthermore, an abundance of literature demonstrates that NMDA receptor antagonists administered at the systemic level block the development of OIH in rodents. The studies focusing on the direct contribution of supraspinal NMDA receptors on OIH are limited and thus, further investigation should be conducted to elucidate the role of supraspinal NMDA receptors in OIH.

Lastly, SP and its NK-1 receptor in the RVM also have been detected to participate in mechanisms of descending facilitation and behavioral hyperalgesia (LaGraize, et al., 2010). NK-

1 receptors are localized in the RVM (Maeno, Kiyama, & Tohyama, 1993; Saffroy, Torrens, Glowinski, & Beaujouan, 2003) and spinomedullary neurons, that express NK-1 receptors and project to the DLF, modulate descending circuitry (Khasabov, et al., 2005). SP microinjected into the RVM produces hyperalgesia in non-inflamed animals and NK-1 receptor antagonists in the RVM attenuate inflammation-induced hyperalgesia (Budai, Khasabov, Mantyh, & Simone, 2007; LaGraize, et al., 2010; Pacharinsak, Khasabov, Beitz, & Simone, 2008). Therefore, further investigations must be performed in order to explore the role of supraspinal NK-1 receptors and SP in acute OIH.

III. Study 3: To determine the contribution of *N*-methyl-D-asparate (NMDA) receptors to acute morphine hyperalgesia.

1. Introduction

The activation of excitatory amino acid receptors such as NMDA receptors has been implicated in the mechanisms of OIH. For example, co-administration of competitive (AP-5) or non-competitive (MK-801) NMDA receptor antagonists with systemic or i.t. morphine reduces or abolishes thermal hyperalgesia to radiant heat in animal models of neuropathic pain (Davar, et al., 1991; Mao, Price, Hayes, et al., 1992a; Mao, et al., 1993; Tal & Bennett, 1993; T. Yamamoto & Yaksh, 1992), carrageenan-induced acute peripheral pain (Ren, et al., 1992), heat injury (Coderre & Melzack, 1991; Juni, et al., 2006), and formalin-induced nociception (Coderre & Melzack, 1992a, 1992b). Systemic administration of MK-801 also attenuates chronic morphine-induced hyperalgesia in NTX-treated mice, demonstrating a role for this receptor in morphine hyperalgesia unrelated to its effect upon morphine analgesia (Juni, et al., 2006). Therefore, evidence supports the involvement of NMDA receptors, independent of opioid receptor activity, as a possible mechanism for chronic OIH and needs to be investigated after an acute morphine injection.

Although it is clear that NMDA receptors play an important role in OIH, the locations of these opioid-NMDA interactions have yet to be clearly identified. Evidence suggests that activation of spinal cord NMDA receptors contributes to the development and maintenance of OIH (Mao, et al., 1994; Shimoyama, et al., 2005; South, et al., 2003; Urban & Gebhart, 1998) whereas other findings propose a critical role for supraspinal NMDA activity in OIH (Urban & Gebhart, 1999; Vanderah, Ossipov, et al., 2001; Vanderah, Suenaga, et al., 2001; Xu, et al., 2007). Since the localization and expression of NMDA receptors have been mapped in the

central primary afferent fibers as well as in the periaqueductal gray (PAG) and rostral ventromedial medulla (RVM), NMDA receptors at spinal and supraspinal loci may contribute to OIH. Therefore, this study determined the contribution of NMDA receptors at spinal and supraspinal loci on acute morphine hyperalgesia by assaying nociception before and after i.t. and i.c.v. MK-801 injection in NTX-treated mice treated with acute systemic morphine.

2. Materials and Methods

Subjects. Naïve, adult, outbred CD-1 male mice were obtained commercially from Charles River Laboratories (Kingston, NY). They were maintained on a 12:12-hour light/dark cycle in a climate-controlled room with free access to food and tap water. Each mouse was used only once, and for all groups, $n \geq 8$.

Nociceptive assay. Nociception was assessed using a modified version of the tail-withdrawal test as described previously.

Drugs and drug delivery. Naltrexone and morphine were dissolved in a saline vehicle. Naltrexone was injected via the systemic route, morphine was injected via the systemic route, and MK-801 was injected via the systemic, i.t., or i.c.v. route.

Contribution of NMDA receptors in systemic acute morphine-induced hyperalgesia. To assess the contribution of NMDA receptors, CD-1 male mice were injected with a single subcutaneous injection of MK-801 (0.05 mg/kg) 30 minutes prior to acute systemic morphine (15 mg/kg) or saline injection. All mice also received a subcutaneous injection of NTX (15 mg/kg) or saline 30 minutes prior to MK-801 injection. Nociception was assayed at baseline and after acute systemic morphine/saline bolus dose at 30-minute intervals for 120 minutes. The MK-801 dose chosen for study does not increase tail-withdrawal latencies in naïve or saline infused mice.

Contribution of NMDA receptors at spinal and supraspinal loci. To assess the role of NMDA receptors at spinal and supraspinal loci, separate groups of CD-1 mice were injected with a single i.c.v. or i.t. bolus dose of MK-801 (0.05 mg/kg) 30 minutes prior to acute systemic morphine (15 mg/kg) or saline injection. All mice received a subcutaneously injection of NTX (15 mg/kg) 30 minutes prior to MK-801 injection. Nociception was assayed at baseline and after acute systemic morphine/saline bolus dose at 15-minute intervals for 60 minutes. The MK-801 dose chosen for study does not increase tail-withdrawal latencies in naïve or saline infused mice. Control mice were injected with saline vehicle instead of MK-801.

Data analysis. Withdrawal latencies were analyzed using a two-way (treatment, time) analysis of variance followed by a Fisher's LSD (protected t-test) for post-hoc comparisons. *P*-values < 0.05 were considered significant. Values reported are mean ± SEM.

3. Results

NMDA receptor blockade in NTX-treated male CD-1 mice. Withdrawal latencies obtained for NTX-pretreated mice with acute morphine and MK-801 injections from 30 to 120 minutes post-injection did not significantly differ from the baseline values (Figure 7). Therefore, the NMDA receptor antagonist blocks morphine hyperalgesia in NTX-pretreated mice. In mice pretreated with saline, MK-801 injection yielded no discernable behavioral or nociceptive effects and did not significantly alter latencies relative to baseline.

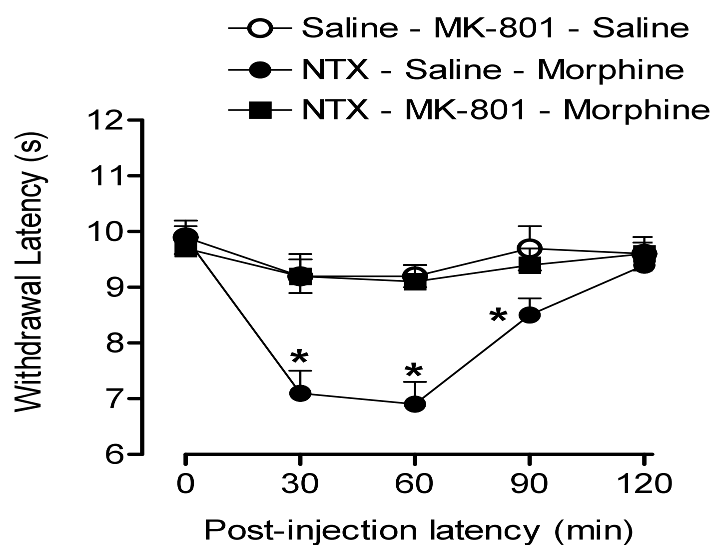

Figure 7. NMDA receptor blockade with MK-801 in NTX-treated male CD-1 mice. All mice received an acute subcutaneous injection of NTX or saline and baseline latencies on the tail-withdrawal test were assayed. Subjects were then injected with a single subcutaneous bolus dose of MK-801 (0.05 mg/kg) or saline 30 minutes prior to an acute morphine injection dose (15 mg/kg) or saline. Withdrawal latencies were reassessed at 15-minute intervals post-morphine injection for 60 minutes. Data are mean withdrawal latencies ± SEM; * indicates significant reductions in withdrawal latencies relative to baseline values obtained prior to morphine/saline injections.

NMDA receptor blockade at spinal and supraspinal loci. In order to determine the contribution of NMDA receptors at spinal loci on acute morphine hyperalgesia, nociception was assayed before and after i.t. MK-801 injection in NTX-treated mice treated with acute systemic morphine. A single i.t. MK-801 injection reduced the hyperalgesic effect that was evident in mice injected with saline prior to morphine (Figure 9). However, NMDA antagonism in the spinal cord did not completely abolish morphine-induced hyperalgesia since the withdrawal latencies of these mice were significantly reduced compared to the control group. In mice pretreated with saline and NTX, MK-801 injection yielded no discernable behavioral or nociceptive effects and did not significantly alter latencies relative to baseline.

In order to determine the contribution of NMDA receptors at supraspinal loci on acute morphine hyperalgesia, nociception was assayed before and after i.c.v. MK-801 injection (0.05 mg/kg) in NTX-treated mice treated with acute systemic morphine. A single i.c.v. MK-801 injection prevented the subsequent development of hyperalgesia that was evident in mice injected with saline prior to morphine (Figure 8). In mice pretreated with saline and NTX, MK-801 injection yielded no discernable behavioral or nociceptive effects and did not significantly alter latencies relative to baseline.

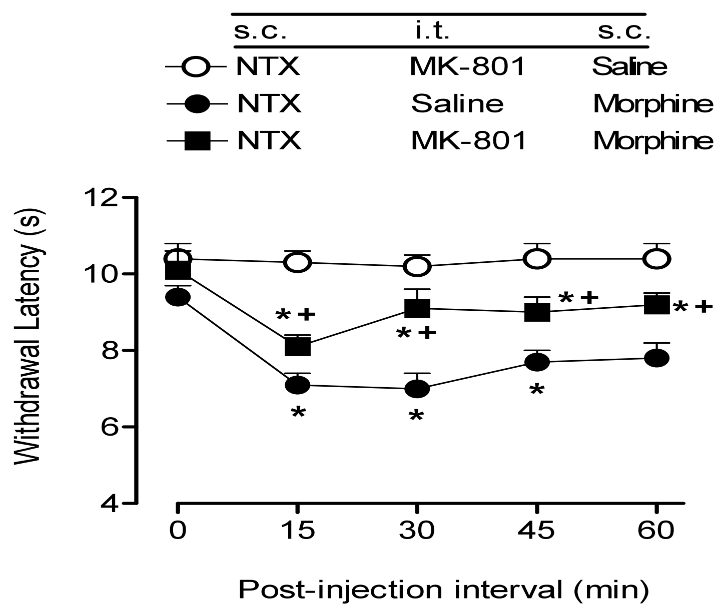

Figure 8. NMDA receptor blockade with MK-801 at spinal loci of hyperalgesia induced by systemic injection of morphine in NTX-treated mice. All mice received an acute subcutaneous injection of NTX and baseline latencies on the tail-withdrawal test were assayed. Subjects were then injected with a single i.c.v bolus dose of MK-801 (0.05 mg/kg) or saline 30 minutes prior to an acute morphine injection dose (15 mg/kg) or saline. Withdrawal latencies were reassessed at 15-minute intervals post-morphine injection for 60 minutes. Data are mean withdrawal latencies ± SEM; * indicates significant reductions in withdrawal latencies relative to baseline values obtained prior to morphine/saline injections. + indicates significant reversal of withdrawal latencies compared to morphine-saline group.

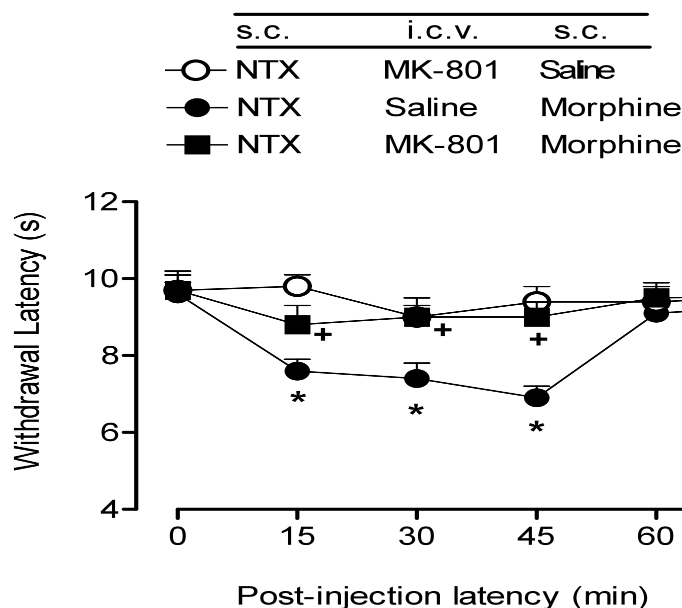

Figure 9. NMDA receptor blockade with MK-801 at supraspinal loci of hyperalgesia induced by systemic injection of morphine in NTX-treated mice. All mice received an acute subcutaneous injection of NTX and baseline latencies on the tail-withdrawal test were assayed. Subjects were then injected with a single i.c.v bolus dose of MK-801 (0.05 mg/kg) or saline 30 minutes prior to an acute morphine injection dose (15 mg/kg) or saline. Withdrawal latencies were reassessed at 15-minute intervals post-morphine injection for 60 minutes. Data are mean withdrawal latencies ± SEM; * indicates significant reductions in withdrawal latencies relative to baseline values obtained prior to morphine/saline injections. + indicates significant reversal of withdrawal latencies compared to morphine-saline group.

4. Discussion

The major findings of Study 3 were as follows: 1) systemic administration of the NMDA receptor antagonist MK-801 completely abolished acute morphine-induced hyperalgesia; 2) MK-801 injected into the spinal cord reduces, but does not completely block, hyperalgesia induced by systemic morphine injection; and 3) MK-801 injected supraspinally via the lateral ventricles blocks hyperalgesia induced by systemic injection of morphine. These findings are discussed in greater detail below.

Mediating excitatory neurotransmission in the CNS, NMDA receptors are characterized by voltage-dependent block by Mg^{2+}, a high permeability to Ca^{2+}, and slow gating kinetics (Ozawa, Kamiya, & Tsuzuki, 1998). An abundance of literature demonstrates that opioid-induced hyperalgesia, tolerance, and neuropathic pain are all prevented by application of NMDA receptor antagonists (Coderre & Melzack, 1991; Juni, et al., 2006; Laulin, et al., 2002; Mao, et al., 1994; T. Yamamoto & Yaksh, 1992). However, the mechanism by which morphine activates NMDA receptors in the absence of opioid synaptic activity remains to be determined. Evidence reveals that opioids are able to potentiate the actions of the excitatory amino acid (EAA) glutamate at NMDA receptors. At the molecular level, the μ-opioid receptor agonist DAMGO increases *in vitro* NMDA receptor-mediated glutamate response via a PKC removal of the magnesium blockade of the NMDA receptor channel in nociceptive neurons (L. Chen & Huang, 1991, 1992; Lan, et al., 2001). This increase in intracellular Ca^{2+} concentration then further stimulates PKC activity, leading to a long-lasting enhancement of glutamate synaptic efficiency (Lan, et al., 2001; Nicoll & Malenka, 1999; Ossipov, et al., 2004). In addition, chronic opioid treatment leads to a down-regulation of spinal glutamate transporters, presumably enhancing glutamate availability at spinal NMDA receptors (Mao, et al., 2002). This increase in the

availability of glutamate to bind to NMDA receptors signifies one possible mechanism of OIH, independent of opioid receptor activity.

Intracellular processes initiated by the activation of NMDA receptors may also contribute to the underlying non-opioid receptor mediated mechanisms of OIH. The binding of glutamate to NMDA initiates the opening of gated calcium channels, which leads to the hydrolysis of phospholipids mediated by guanosine triphosphate-binding proteins. Products of phospholipid hydrolysis include diacylglycerol and inositol 1,4,5-triphosphate (Schoepp & Conn, 1993). Inositol 1,4,5-triphosphate (IP_3) mobilizes calcium from intracellular compartments, increasing the availability of calcium (Schoepp & Conn, 1993). Diacylglycerol mediates activation of PKC (J. E. Haley, Dickenson, & Schachter, 1992), which produces nitric oxide (NO) that diffuses presynaptically to release more EAA (Meller, Pechman, Gebhart, & Maves, 1992). Again, the increased availability of glutamate activates NMDA receptors and thus, may signify a non-opioid receptor mediated mechanism of OIH.

Furthermore, the translocation and activation of Ca^{2+}-mediated protein kinase C (PKC), particularly the gamma isoform PKCγ, is associated with functional CNS changes that occur by means of phosphorylating ion channel proteins (Mao, Price, Phillips, Lu, & Mayer, 1995; Mayer, Mao, & Price, 1995). This phosphorylation leads to increased NMDA-mediated glutamate responses and to long-term potentiation of synaptic transmission (L. Chen & Huang, 1991, 1992; Suen, et al., 1998). In attempts to investigate these events upstream of the NMDA receptor, GM1 ganglioside, an intracellular inhibitor of PKC translocation and activation in both *in vivo* and *in vitro* studies (Magal, et al., 1990; F. Vaccarino, et al., 1987), attenuates thermal hyperalgesia induced by peripheral nerve injury and reduces formalin-induced hyperalgesic responses. Studies utilizing mice lacking the PKCγ gene also support the critical role of PKCγ in the

mechanism underlying OIH. PKCγ knockout mice do not develop opioid-induced hyperalgesia after systemic opioid administration (Zeitz, et al., 2001). Independent of opioid receptor activity, this NMDA/PKCγ pronociceptive system may represent the mechanism underlying OIH.

Furthermore, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKIIα), a multifunctional serine/threonine protein kinase, has been shown to be another required component for the development and maintenance of OIH (Y. Chen, Yang, & Wang, 2010). Ca^{2+} influx via the activation of NMDA receptors results in CaMKIIα activation and autophosphorylation of CaMKIIα at position Thr286 (Fukunaga, Soderling, & Miyamoto, 1992; Strack, McNeill, & Colbran, 2000). With this elevated intracellular Ca^{2+}, CaMKIIα can phosphorylate and activate the NMDA receptor, leading to the influx of more Ca^{2+} through the channels. Studies demonstrate that CaMKIIα activity is increased after chronic treatment of opioids (Tang, Shukla, Wang, & Wang, 2006), as well as morphine failed to induce OIH in $CaMKII\alpha^{T286A}$ point mutant mice (Y. Chen, et al., 2010). Therefore, an essential role of CaMKIIα and its relationship to NMDA receptors as a cellular mechanism leading to and maintaining OIH should be explored further.

NMDA antagonism attenuated morphine-induced hyperalgesia in acute and chronic models (Plesan et al., 1999; Li et al., 2001a; Mao et al., 2002). However, NMDA antagonists 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, MK-801 is less effective in reversing hyperalgesia in NTX-pelleted mice relative to placebo-pelleted controls. Therefore, the present demonstration of MK-801 reversal of hyperalgesia in NTX-pelleted mice provides additional

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

Contribution of NMDA receptors at spinal and supraspinal loci. The data from Specific Aim 2 demonstrating that morphine injected directly into spinal and supraspinal loci can cause hyperalgesia that is not mediated by opioid receptors prompted the investigation of the role of NMDA receptors at these loci. MK-801 administered directly at the supraspinal level completely blocked OIH after systemic morphine injection, but only partially reduced OIH when administrated at the spinal level. Therefore, these findings suggest that NMDA receptors localized in the brain may contribute to a greater extent to the acute morphine-induced hyperalgesia than NMDA receptors in the spinal cord.

Ascending and descending projections from the spinal cord and brain areas, including the PAG and RVM, form a regulatory loop to control nociceptive transmission in the spinal cord (Suzuki, Morcuende, Webber, Hunt, & Dickenson, 2002). In terms of ascending projections, NMDA receptors are located postsynaptically and extrasynaptically in the membrane of the dorsal horn neurons and are activated by the presynaptic release of glutamate from nerve terminals of dorsal root ganglia cells, depolarized by pain-provoking mechanical or chemical stimuli. Activation of NMDA receptors facilitate the influx of cations, such as Na^{2+} and Ca^{2+}, causing the dorsal horn neurons to depolarize and fire action potentials, resulting in the further release of glutamate and propagation of the pain signal from neuron to neuron through the spinal cord to the brain. Furthermore, NMDA receptor-containing neurons are widely distributed in the RVM and PAG, and NMDA receptor-dependent descending pain facilitation contributes to the development of hyperalgesia after injury (Guo, et al., 2006).

The contribution of NMDA receptors, independent from opioid receptor activity, in the central nervous system to acute morphine-induced hyperalgesia has been confirmed by these studies. However, at this time, the mechanism by which morphine activates NMDA receptors in the spinal cord or brain in the absence of opioid synaptic activity remains to be determined. In addition to the intracellular cascades described above, specific activities, involving NMDA receptors, at the spinal and supraspinal levels may also underlie the mechanism of acute morphine-induced hyperalgesia. At the spinal level, the augmented release of glutamate, the production of nitric oxide (NO), the activation of NK-1 receptors, and dynorphin may contribute to the role of NMDA receptors in OIH. In contrast, the increased activation of on-cells in the RVM, the activation of NK-1 receptors, and the augmented release of excitatory neuropeptides, including CCK, may impact of the role of NMDA receptors at the supraspinal level.

Behavioral data in mice and rats suggest that morphine-induced hyperalgesia may be mediated through an increase of glutamate from primary afferent terminals in the dorsal horn of the spinal horn, and thus, subsequent activation of NMDA receptors. Furthermore, evidence demonstrates that an NMDA-NO cascade in the spinal cord may mediate OIH (Watanabe, Okuda, et al., 2003; Watanabe, Sakurada, et al., 2003). High-dose i.t. morphine produces a significant increase of NO release in the spinal cord, and thus, this increased generation of NO leads to increased activation of spinal NMDA receptors (Malmberg & Yaksh, 1993; Sorkin, 1993). The presence and activation of NK-1 receptors on spinal lamina I projection neurons are also essential for the induction of activity-dependent LTP that requires a SP-induced rise in Ca^{2+}, likely by Ca^{2+} release from intracellular stores, and a SP-facilitated Ca^{2+} influx through NMDA receptors. This synaptic plasticity in spinal lamina I neurons has been shown to mediate hyperalgesia and may be enhanced by the presence of opioids (Ikeda, et al., 2003). Lastly,

dynorphin, an endogenous peptide with significant non-opioid activity, evokes long-lasting allodynia, and scratching, licking, and biting behaviors through NMDA receptors (Vanderah, et al., 2000; Vanderah, et al., 1996). Administration of opioids may upregulate spinal dynorphin, which evokes an increased release of excitatory neurotransmitters from primary afferent fibers that promotes exaggerated pain through an NMDA-dependent mechanism (Gardell, et al., 2002; Vanderah, Suenaga, et al., 2001). Thus, all of these different activities at the level of the spinal cord may contribute to the non-opioid receptor-mediated role of NMDA receptors in OIH.

At the supraspinal level, the importance of NMDA receptors in the brain areas, such as the RVM and PAG, in OIH has been confirmed through these studies. Opioids elicit hyperalgesia through tonic activation of descending facilitation from the RVM and opioid-induced neuroplastic changes in these supraspinal areas may underlie the mechanism of OIH (Vanderah, Suenaga, et al., 2001). Evidence demonstrates that the NMDA receptors may have a direct and indirect involvement in this descending facilitation and in these neuroplastic changes. Direct blockade of medullary NMDA receptors or of NO generation attenuates somatic and visceral hyperalgesia (Coutinho, et al., 2001; Urban & Gebhart, 1999). Dynamic plasticity in excitability of RVM has been observed during inflammatory hyperalgesia and is related to changes in NMDA receptor activation (Guan, Terayama, Dubner, & Ren, 2002; Terayama, Dubner, & Ren, 2002). Furthermore, the activation of NK-1 receptors in the RVM enhances excitability of on-cells evoked by NMDA (Budai, et al., 2007). These findings suggest that SP acting on these NK-1 receptors can facilitate EAA transmission either by enhancing their response from presynaptic terminals or by enhancing responses of on-cells to EAAs. NK-1 receptors are also found to be coexpressed with NMDA receptors on a subset of neurons in the RVM (Budai, et al., 2007). Thus, activation of NK-1 and NMDA receptors in the RVM and the

ensuing sensitization of on-cells may contribute to the development of central sensitization and OIH.

Furthermore, descending influences dependent on the release of excitatory peptide neurotransmitters, such as cholecystokinin (CCK), may also underlie the supraspinal involvement in OIH and may indirectly activate NMDA receptors. Acute and chronic morphine treatment caused a dose-dependent increase of CCK reactivity in the RVM and the PAG (Ding & Bayer, 1993; Rosen & Brodin, 1989). CCK selectively activates CCK_2 receptors on on-cells in the RVM, which produces behavioral allodynia and hyperalgesia (Ambriz-Tututi, Cruz, Urquiza-Marin, & Granados-Soto; W. Zhang, et al., 2009), and leads to a time-dependent dynorphin and CCK release in the spinal cord (Ambriz-Tututi, et al.; Heinricher & Neubert, 2004; Kovelowski, et al., 2000; Xie, et al., 2005). Since CCK_2 receptor activation increases the basal release of endogenous excitatory amino acids in rat hippocampal slices (Migaud, Roques, & Durieux, 1994; Tatsuo Yamamoto & Nozaki-Taguchi, 1995), the increased release of these pronociceptive agents may lead to further activation of NMDA receptors. Therefore, due to the findings that NMDA receptor blockade at the supraspinal level completely abolishes acute morphine-induced hyperalgesia, these activities in the RVM and PAG that directly and indirectly involve NMDA receptors may contribute to a greater extent to the acute morphine-induced hyperalgesia than the involvement of NMDA receptors in the spinal cord.

IV. Study 4: To determine the contribution of genotype on acute morphine-induced hyperalgesia.

1. Introduction

In order to explain interindividual differences in analgesia and nociception, the influence of genetics in clinical and animal populations has gained attention in the literature. Inbred strains comparisons are utilized to illustrate and understand the contribution of genetics to nociception. An inbred strain is created by repeated sibling matings for at least 20 generations (Mogil, Sternberg, et al., 1996). The offspring from a given strain are virtually genetically identical to each other. The specific allele that becomes fixed at each genetic locus is randomly determined. Therefore, individuals from different inbred strains will likely differ at many genetic loci and can be used to address nature-versus-nurture issues.

Differences between inbred strains indicate likely genetic factors when strains are raised and tested under the same conditions. To investigate the contribution of genetics on acute morphine-induced hyperalgesia, nociception before and after acute morphine injection was tested in 10 inbred mouse strains pretreated with NTX. We also aimed to determine if these inter-strain differences exist across a wide range of cumulative morphine doses.

2. Materials and Methods

Subjects. Male mice of the following inbred strains were obtained from the Jackson Laboratory (Bar Harbor, ME): 129P3/J, A/J, AKR/J, BALB/c/J, C3H/He/J, C57BL/6/J, CBA/J, DBA/2/J, SJL/J, and SWR/J. All mice were housed four to a cage with same sex/strain mates in the College of Staten Island Animal Facility. Mice were allowed free access to food and water in a temperature-controlled (22°C) environment maintained on a 12:12 hour light/dark cycle. All

testing was performed following an acclimation period of at least 1 week after arrival and at 7–9 weeks of age. Each subject was used once and for all groups, $n \geq 8$.

Nociceptive assay. Nociception was assessed using a modified version of the tail-withdrawal test as described previously.

Drugs and drug delivery. Morphine and naltrexone were dissolved in a saline vehicle and delivered using a 10ml/kg injection volume and were obtained from the NIDA Drug Supply Program. Morphine and naltrexone were injected subcutaneously.

Morphine hyperalgesia time-response study. Groups of mice from each strain were assayed for nociception on the tail-withdrawal test and then immediately injected subcutaneously with a single NTX (15 mg/kg) dose. Nociception was assayed again 30 minutes later, representing their nociceptive baseline latency (time 0). Thirty minutes later, they were injected with a single morphine dose (15 mg/kg) or saline and nociception was reassessed every 30 min for the next 120 minutes.

Morphine hyperalgesia dose-response study. Groups of mice from each strain were assayed for nociception on the tail-withdrawal test and then immediately implanted with a single NTX pellet. Nociception was assayed 24 hour later and this assay represented their nociceptive baseline latency (time 0). Mice were then injected with a single morphine dose (1 mg/kg) or saline and nociception was reassessed 30 minutes later. Mice were then immediately injected with a larger morphine dose (2 mg/kg) and again assayed for nociception 30 min later. This procedure of assaying nociception, morphine injection, and retesting was repeated, each time using increasing morphine doses (All doses: 1, 2, 3.6, 6.5, 11.7, and 21 mg/kg) or saline until all doses were tested.

Data analysis. Withdrawal latencies obtained in the time-response studies were analyzed using a three-way (strain X treatment X time) analysis of variance followed by a Fisher's LSD (protected t-test) for post-hoc comparisons. P-values < 0.05 were considered significant. For the dose-response study, latencies obtained after each test dose were used to calculate the latency values at which each strain achieved 50% of their maximum hyperalgesic response (i.e., HD_{50}). Data were fitted with a one-phase exponential decay function, which showed better goodness-of-fit than any other non-linear regression function available (Prism v5.04). The maximum hyperalgesia response was defined as the plateau of this function for each strain, and the HD_{50} as the half-life.

3. Results

In the time-course study, some strains displayed 30-50% reduction in withdrawal latency following a single morphine dose (15 mg/kg) whereas other strains failed to become hyperalgesia at any time during the 120 minutes post-morphine testing interval (Figure 10). Specifically, inbred strains, including AKR, C3H/He, and SJL, displayed significant hyperalgesia from 30-120 minutes post-morphine injection. In contrast, strains 129P3 and A demonstrated no reduction in their withdrawal latencies from baseline, after an acute injection of morphine and pre-treatment with NTX. The five remaining strains, BALB/c, C57BL/6, CBA, DBA/2, and SWR, demonstrated varying degrees of hyperalgesia along the 120 minutes time period.

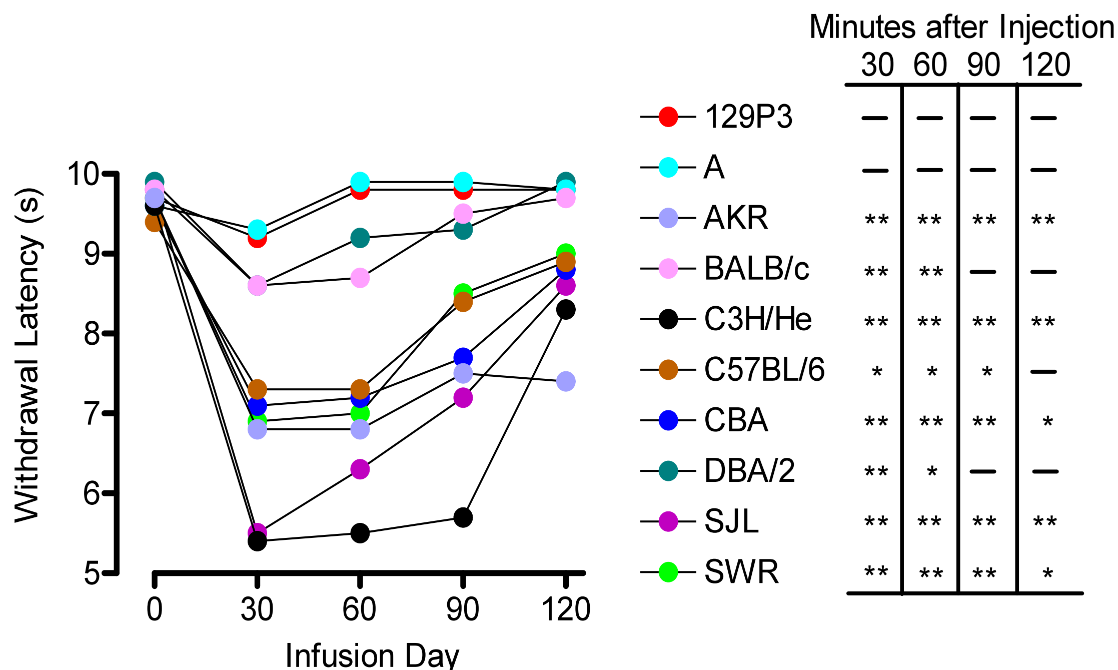

Figure 10. Time course of acute morphine hyperalgesia in 10 inbred strains pretreated with NTX. Mice were subcutaneously injected with NTX (15 mg/kg) prior to morphine injection. Data are mean latencies obtained prior to morphine injection (baseline; time 0) and at the various post-morphine injection intervals indicated. S.E.M. legends were omitted for the sake of clarity. The post-morphine injection intervals where latencies were significant reduced relative to pre-injection baseline values are indicated in the table with their corresponding *p* values (*< 0.05; **<0.001).

In the dose-response study, variation in the degree of hyperalgesia is also demonstrated among the different strains (Figure 11). Certain strains, including the CBA, C3H/He, and C57BL/6, showed a hyperalgesic response after a small acute dose of morphine, whereas other strains required a higher dose of morphine to reach their maximal hyperalgesic response. Findings also indicate that once most strains reach their maximum hyperalgesic response, additional morphine does not further reduce their withdrawal latencies. Table 2 also shows the HD_{50} values, which are the doses at which each strain achieved 50% of their maximum hyperalgesic response. Significant variation exists among these values for the different inbred strains.

Tail-Withdrawal Latency (s)

12
10
8
6
4

0 10 20 30 40 50

Dose (mg/kg)

129P3
A/J
AKR
BALB/c
C3H/He
C57BL/6
CBA
DBA/2
SJL
SWR

% Hyperalgesia

60
40
20
0

0 10 20 30 40 50

Dose (mg/kg)

129P3
A/J
AKR
BALB/c
C3H/He
C57BL/6
CBA
DBA/2
SJL
SWR

Figure 11. Hyperalgesia dose-response data from 10 inbred strains pretreated with NTX. Mice were subcutaneously injected with NTX (15 mg/kg) prior to morphine injection. Data are mean (± S.E.M.) latencies obtained prior to morphine injection (baseline; dose 0) and at the various doses indicated. Dose-response data for each strain are fitted with a one-phase exponential decay function ($r^2 = 0.10 – 0.87$).

Mouse Strain	**HD_{50} (95% CI)**
129P3	14.4 (3.4-Infinity)
A/J	≈ 80,000 (*interrupted*)
AKR	2.3 (1.3-8.0)
BALB/c	54.8 (8.7-Infinity)
C3H/He	1.8 (1.2-2.9)
C57BL/6	1.9 (1.5-2.7)
CBA	1.8 (1.4-2.9)
DBA	12.4 (5.0-Infinity)
SJL	0.9 (0.4-Infinity)
SWR	5.7 (4.2-8.9)

Table 2. Morphine hyperalgesic potency (HD_{50} values) based on dose-response data for each inbred strain. Latencies obtained after each test dose were used to calculate the latency values at which each strain achieved 50% of their maximum hyperalgesic response (i.e., HD_{50}). Data were fitted with a one-phase exponential decay function, which showed better goodness-of-fit than any other non-linear regression function available. The maximum hyperalgesia response was defined as the plateau of this function for each strain, and the HD_{50} as the half-life.

4. Discussion

The major findings of Specific Aim 4 were as follows: 1) Time-response studies reveal marked genetic variation in acute morphine-induced hyperalgesia across a 120-minute time course; 2) Dose-response studies reveal that some strains do not show hyperalgesia across a wide range of cumulative morphine doses whereas other strains show, at least at initial morphine doses, a dose-dependent hyperalgesic response; 3) These inter-strain differences are not likely attributable to initial differences in nociceptive sensitivity since baseline withdrawal latencies did not significantly differ between strains; and 4) Hyperalgesia manifest in these studies is not attributable to NTX-preventable mechanisms of morphine withdrawal since mice were pretreated with NTX.

This is the first systematic, multiple-strain analysis of genetic variation in acute morphine-induced hyperalgesia, and thus the reduction of withdrawal latencies, indicative of acute OIH, for most of the strains in the present study is characterized for the first time. Inter-strain differences in hyperalgesia were apparent in the time-course study, where some strains displayed 30-50% reduction in withdrawal latency following a single morphine dose (15 mg/kg) and other strains failed to become hyperalgesic at any time during the 120 minutes post-morphine testing interval. Several strains were highly insensitive or all together refractory to acute OIH, including 129P3 and A, and by contrast, some strains, including AKR, C3H/He, and SJL, displayed significant reductions in withdrawal latencies after an acute systemic morphine dose. Furthermore, HD_{50} values, the acute morphine dose at which each strain achieved 50% of its maximum hyperalgesic response, reflect tremendous variation among the different strains.

This study is the first to date to investigate acute morphine hyperalgesia utilizing inbred strains; therefore, no comparisons to other studies can be conducted. However, strain surveys

have been used to investigate morphine analgesia, morphine tolerance, and acute and chronic morphine and heroin dependence (Kest, Hopkins, et al., 2002; Kest, Palmese, et al., 2002; Klein, et al., 2008; Wilson, et al., 2003). These studies provide evidence that the presence and magnitude of morphine analgesia, morphine analgesic tolerance, and acute and chronic morphine and heroin dependence are all genotype-dependent and identify strains with widely divergent liabilities that should facilitate identification of trait-relevant genes. The findings from these strain surveys also demonstrated a strong genetic correlation between physical dependence to morphine and heroin following acute and chronic treatment and thus, imply that genes associated with variable sensitivity in the two traits are the same (Kest, Palmese, et al., 2002; Klein, et al., 2008).

Specifically, Wilson and colleagues tested 12 inbred mouse strains for antinociceptive sensitivity to systemic administration of 5 different μ-opioid analgesics, including morphine (Wilson, et al., 2003). Eight of the 10 same strains were used in their study as utilized in this dissertation. Therefore, in determining if a relationship between strain sensitivity on acute morphine-induced hyperalgesia and morphine analgesia exists, the half-maximal antinociceptive doses (AD_{50}), calculated from dose-response curves, from their study and the half-maximal hyperalgesia doses (HD_{50}) from this dissertation were utilized. These values were ranked from smallest to highest according to effect size and Spearman rank correlation (r_s) was calculated. No relationship exists between strain sensitivity on acute morphine-induced hyperalgesia and morphine analgesia. Therefore, this evidence suggests that the genes underlying the interindividual differences in OIH are not identical to the genes underlying the differences in acute morphine analgesia. These data align with the findings that OIH operates through a non-opioid receptor mechanism whereas morphine analgesia is mediated by opioid receptors.

However, a significant correlation between morphine analgesia and hyperalgesia may explain the strain differences in analgesia, since the different levels of hyperalgesia among the strains may reduce their analgesic sensitivity accordingly. Since only 8 strains were compared and experimental variables and procedures differed, identical methods and morphine doses should be used to calculate HD_{50} and AD_{50} for all 10 strains in order to further investigate the genetic correlation between morphine analgesia and morphine-induced hyperalgesia.

Despite the paucity of literature regarding strains of the present study, 129P3 mice deserve additional discussion. Acute and chronic naloxone-precipitated withdrawal resistance has previously been demonstrated in this strain (Kest, Palmese, et al., 2002), as well as the 129S6 mice (previously referred to as 129/SvEv), another 129 substrain, are resistant to morphine analgesic tolerance and naloxone-precipitated withdrawal jumping (S. M. Crain & K. Shen, 2000; Kolesnikov, Jain, Wilson, & Pasternak, 1998). Both substrains show similar phenotypes and are highly related genetically (Simpson, et al., 1997) and therefore, findings from studies utilizing 129S6 mice may be of value to understanding 129P3 mice. 129S6 mice have been shown to possess deficiencies in the NMDA receptor system and/or the biochemical cascade activating nitric oxide synthase consequent to its activation (Kolesnikov, et al., 1998). Additional evidence that tolerance/dependence-resistance following chronic morphine treatment results from deficiencies in GM1 ganglioside-regulated excitatory opioid receptor-mediated functions (S. M. Crain & K. Shen, 2000). Therefore, since the importance of NMDA receptor system in OIH has been demonstrated in this dissertation, these deficiencies may contribute to the ability of 129P3 mice to resist acute morphine-induced hyperalgesia.

Overall, this strain survey provides preliminary data to support that genetic factors play a role in susceptibility to acute morphine-induced hyperalgesia. Although caution is obviously

warranted when generalizing from a survey of 10 inbred mouse strains to the variation in human responses, the syntenic conservation between the mouse and human genomes suggests that the study of mouse genetics can be of significant heuristic value. Furthermore, inbred strains with consistent and relatively large differences in hyperalgesic response after acute morphine treatment can be utilized in a linkage mapping study to facilitate the identification of the trait-relevant gene(s) mediating variable OIH liability.

Chapter 4.

I. General Discussion

The overall aim of this dissertation was to assess at a behavioral level whether acute morphine injection, like continuous morphine infusion, causes hyperalgesia in mice, and to characterize this phenomenon. My findings here demonstrate that: 1) Acute morphine injection indeed causes hyperalgesia, and that it does so independently of concurrent or prior μ, κ, and δ opioid receptor activation, as indicated by the persistence of hyperalgesia in CD-1 mice pretreated with the opioid-receptor antagonist NTX and in knockout mice lacking μ, κ, and δ-opioid receptors; 2) Acute morphine hyperalgesia is not mediated by the morphine metabolite, M3G, as demonstrated in the $Mrp3^{(-/-)}$ mice; 3) Supraspinal and spinal loci are directly involved in acute morphine hyperalgesia; 4) Systemic administration of NMDA receptor blockade with MK-801 completely abolished acute morphine-induced hyperalgesia, independent of μ, κ, and δ opioid receptor activity; 5) NMDA receptor blockade with MK-801 at supraspinal loci completely blocks hyperalgesia induced by systemic injection of morphine in NTX-treated mice; 6) NMDA receptor blockade with MK-801 at spinal loci reduces, but does not completely block, hyperalgesia induced by systemic injection of morphine in NTX-treated mice; 7) Marked genetic variation exists in acute morphine-induced hyperalgesia, which may account for interindividual differences in this phenomenon.

There are several limitations to the studies detailed within this dissertation. All assessments were conducted using morphine, a substance that preferentially binds to the μ opioid receptor. Morphine and the μ opioid receptor agonists have been reported to cause hyperalgesia in humans and rodents (Ossipov, et al., 2004). Since here we only have evidence that an acute administration of morphine causes hyperalgesia independently of μ, κ, and δ opioid receptor

activity or M3G, we can not predict whether the present findings can be extrapolated to include delta or kappa receptor opioids. Further studies that assess the hyperalgesic tendencies of different opioids are required before such comparisons can be made. Furthermore, our findings do not rule out the involvement of opioid receptor-like (ORL1) receptors in OIH. Even though morphine does not express any affinity for this receptor, activation of ORL1 caused both nociception and antinociception at the supraspinal and spinal levels (Khroyan, Polgar, Jiang, Zaveri, & Toll, 2009; Mogil & Pasternak, 2001). Therefore, the role of ORL1 and its endogenous ligand, orphanin FQ/nociception (OFQ/N) on nociceptive transmission still remains unclear (Mogil & Pasternak, 2001). However, since NTX is not known to block this receptor (Mathis, et al., 1997), future investigations should specifically address the involvement of the ORL1 receptor in acute OIH. Lastly, since the dependent nociceptive measure in most of 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 spontaneous pain (Mogil, et al., 1999a). Applicability beyond the narrow conditions described above should thus not be assumed.

In aiming to characterize this phenomenon, this dissertation demonstrated that acute OIH is independent of μ, κ, and δ opioid receptor activity and the actions of the morphine metabolite, M3G. Previous studies support that the stimulation of μ-opioid receptors triggers the activation of NMDA receptors by increasing intracellular PKC activity (L. Chen & Huang, 1991) as well translocation of the cytosolic PKC to the plasma membrane, leading to the phosphorylation of the NMDA receptors implicated in pain promotion (Suen, et al., 1998). However, this dissertation demonstrates that this is not the case, since μ-opioid receptors are blocked by NTX and acute OIH continues to exist. Therefore, the mechanism by which morphine activates

NMDA receptors in the absence of a μ, κ, and δ opioid receptor synapse remains to be determined. Furthermore, with no detectable affinity to opioid receptors, M3G 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), i.t. (Woolf, 1981; Yaksh et al., 1986), and systemic (Ekblom, Gardmark, & Hammarlund-Udenaes, 1993; Lipkowski et al., 1994) doses, that are naloxone insensitive (LaBella et al., 1979; Yaksh et al., 1986). However, through the use of mice lacking circulating M3G, our findings demonstrate that M3G does not logically contribute to OIH. Therefore, with this evidence, significant attention shifted to the investigation of the role of NMDA receptors in OIH and their involvement was confirmed by this dissertation.

Therefore, if the stimulation of opioid receptors or the actions of M3G does not lead to the activation of NMDA receptors, then the intermediary between morphine and NMDA receptors remains unknown. The excitatory neurotransmitter glutamate has been implicated in central sensitization and studies demonstrate that morphine leads to the increased availability of extracellular glutamate (Belanger, et al., 2002; Ibuki, et al., 2003). With this increased extracellular glutamate in the synapse, activation of NMDA receptors initiates the opening of gated calcium channels, which leads to the hydrolysis of phospholipids mediated by guanosine triphosphate-binding proteins. Products of phospholipid hydrolysis include two second messengers, diacylglycerol and inositol 1,4,5-triphosphate (Schoepp & Conn, 1993). Inositol 1,4,5-triphosphate (IP_3) mobilizes calcium from intracellular compartments, increasing the availability of calcium (Schoepp & Conn, 1993). Diacylglycerol (DAG) mediates activation of PKC (J. E. Haley, et al., 1992), which produces nitric oxide (NO) that diffuses pre-synaptically to release more EAA (Meller, et al., 1992; Watanabe, Sakurada, et al., 2003). Again, the increased availability of glutamate, elicited from these extracellular and intracellular processes,

activates NMDA receptors and thus, may signify a non-opioid receptor mediated mechanism of OIH. Since this dissertation does not directly identify glutamate as this intermediary, future studies should primarily focus on understanding the mechanism by which morphine activates NMDA receptors in the absence of an opioid synapse at the molecular level.

Furthermore, enhanced activation of specific kinases, including PKC and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKIIα), has been shown after chronic treatment of opioids and may contribute to the glutamatergic/NMDA receptor mediated-mechanism of OIH (Celerier, et al., 2004; Y. Chen, et al., 2010; Tang, et al., 2006). Specifically, PKCγ catalyzes NMDA receptor phosphorylation, leading to enhancement of the NMDA receptor glutamate response and to long-term potentiation of synaptic transmission (Celerier, et al., 2004; L. Chen & Huang, 1992; Coderre & Katz, 1997; Mao, et al., 1995; Suen, et al., 1998). CaMKIIα can also phosphorylate and activate the NMDA receptor, leading to more influx of Ca^{2+} through the channels. Furthermore, crosstalk may exist between PKC and CaMKIIα in OIH. The NR2A subunit of the NMDA receptor serves as the CaMKIIα docking site that binds to autophosphorylated CaMKIIα, a process known to be regulated by PKC (Gardoni, Bellone, Cattabeni, & Di Luca, 2001; Leonard, et al., 2002). Therefore, the actions of these kinases and their relationship to NMDA receptors as a cellular mechanism leading to the development and maintenance of OIH should be further investigated.

The role of NMDA receptors at spinal and supraspinal loci in the mechanism underlying OIH has been thoroughly demonstrated by this dissertation, as well as the more significant role of supraspinal NMDA receptors. Different mechanisms may exist at the supraspinal and spinal levels and further studies should be conducted to differentiate these mechanisms. In the central nervous system, high affinity, sodium-dependent glutamate transporters provide the major

mechanism for extracellular glutamate uptake and homeostasis (Danbolt, 2001). To date, five such transporters have been cloned and characterized, including GLT-1, which is primarily expressed in astrocytes and responsible for glutamate clearance in the spinal cord (Kanai & Hediger, 1992). Activated by repeated opioid administration, spinal astrocytes can exhibit reduced GLT-1 expression and glutamate uptake (Song & Zhao, 2001). This impaired glial glutamate uptake in spinal cord could contribute to neuroexcitability in subjects with chronic pain or exhibiting OIH (Liao & Chen, 2001; Zhang, Xin, & Dougherty, 2009). Increasing spinal GLT-1 expression attenuates opioid-induced paradoxical pain, alleviates neuropathic pain, and suppresses associated glial activation in rodents (Z. Chen, He, & Wang, 2012; Ramos, et al., 2010). Specifically, ceftriaxone, a beta-lactam antibiotic, reversed the downregulation of spinal GLT-1 expression and inhibited the development of OIH (Z. Chen, et al., 2012). Therefore, in addition to its involvement in the neuroplastic changes at the NMDA receptor, morphine's influence on the functioning of GLT-1 may play a role in the development and maintenance of OIH at the level of the spinal cord.

NMDA receptors in relevant brain areas, such as the RVM and PAG, and their role in descending facilitation contribute significantly to acute OIH. Increased availability of glutamate and enhanced NMDA receptor activation may contribute to the neuroplastic changes in these supraspinal sites and thus, to the development and maintenance of OIH (Guan, et al., 2002; Terayama, et al., 2002). Additionally, limited evidence exists that the cascade of intracellular events associated with NMDA receptor activation, including the activation of nitric oxide synthase (NOS) and subsequent production of NO, in the RVM modulates transmission of opioid pain-inhibitory signals from the PAG (Javanmardi, et al., 2005). Furthermore, concurrent activation of NK-1 and NMDA receptors in the RVM and the ensuing sensitization of on-cells

may also contribute to the development of central sensitization and OIH (Budai, et al., 2007; T. King, et al., 2005). Lastly, other excitatory neurotransmitters, such as CCK, which are released by neurons in the RVM, may indirectly activate NMDA receptors by activating spinal pathways that upregulate spinal dynorphin (Ding & Bayer, 1993; Laughlin, et al., 1997; Xie, et al., 2005; Tatsuo Yamamoto & Nozaki-Taguchi, 1995). Thus, a comprehensive understanding of the indirect and direct role of NMDA receptors in these supraspinal areas in OIH will help inform the development of therapeutic interventions for OIH.

The current findings also have interesting implications within the greater context of pharmacological interventions for acute OIH. With the evidence that NMDA receptors contribute to OIH, clinical work in attenuating and preventing OIH expression has mainly focused on manipulation of the glutaminergic system, either through direct or indirect modulation of the NMDA receptor. The use of low-dose ketamine, a non-competitive NMDA receptor antagonist that binds to the phencyclidine site, in the management of OIH has conflicting support (Lee, Silverman, Hansen, Patel, & Manchikanti, 2011; Ramasubbu & Gupta, 2011). Some randomized controlled trials (RCTs) show no change in the hyperalgesic state with coadministration of low-dose ketamine (Engelhardt, et al., 2008; Joly, et al., 2005; Luginbuhl, et al., 2003), whereas others RCTs demonstrate that perioperative administration of ketamine reduce expression of OIH (Angst, et al., 2003; Guignard, et al., 2002; Menigaux, et al., 2000). Specifically, in relation to the work of this dissertation, Angst and colleagues conducted a double-blind, randomized, crossover and placebo-controlled design to test whether co-administration of S-ketamine would prevent the development of acute OIH in opioid naïve, healthy human volunteers (Angst, et al., 2003). They demonstrated that S-ketamine abolished observed enlargement of the hyperalgesic skin area and thus, that NMDA-receptor mediated

mechanism may underlie acute OIH. Additionally, minimal clinical support exists for the use of dextromethorphan, the D-isomer of the methyl ether derivative of levorphanol and thus, a NMDA receptor antagonist (P. A. Compton, Ling, & Torrington, 2008; Galer, Lee, Ma, Nagle, & Schlagheck, 2005). Other possible treatment regimens include nonsteroidal anti-inflammatory drugs (NSAIDS), amantadine, buprenorphine, methadone, and α_2 agonists and inconclusive findings have been detected for these treatments (Lee, et al., 2011; Ramasubbu & Gupta, 2011). Lastly, emerging evidence shows that beta-Lactam antibiotics, one of the most widely used antibiotic classes in the world, may selectively treat the GLT-1 target and thus, prevent or treat OIH (Z. Chen, et al., 2012). Despite the extensive basic science evidence for OIH, current literature lacks high quality prospective, randomized, controlled, clinical trials examining the different therapeutic treatments for OIH and the neuroplastic changes leading to OIH expression.

The present strain survey data also identified two inbred strains, 129P3 and C3H/He, with highly divergent hyperalgesic responses to acute systemic morphine administration. Specifically, 129P3 was the most insensitive strain and C3H/He was the most significant hyperalgesic response. A full genome scan of F_2 mice derived from these strains can be used to identify quantitative trait locus, or chromosomal regions (and ultimately candidate genes) associated with acute morphine hyperalgesia. This approach has been successfully used to identify qualitative trait loci (QTL) on Chromosomes 1, 5, and 10 that account for 43% of the total variance in naloxone-precipitated withdrawal (NPW) jumping frequencies in morphine dependent F_2 hybrid mice derived from C57BL/6 and 129P3 progenitors (Kest, Palmese, Juni, Chesler, & Mogil, 2004). More recently, to identify genes contributing to variation in morphine physical dependence, NPW jumping frequencies of 30 strains of the AcB/BcA recombinant congenic mouse strain set, including their A/J and C57BL/6J (B6) progenitors, were utilized to

identify a QTL localized to Chromosome 8 (Kest, et al., 2009). The most salient candidate gene within this QTL was *Gnao1*, guanine nucleotide binding protein, and thus, the likely contributor to interindividual variability in physical dependence of opioids in mice. As tail-withdrawal latency has been shown here to be a valid and sensitive measure of morphine-induced hyperalgesia, the present identification of highly divergent strains such as 129P3 and C3H/He should facilitate success in identifying such QTLs. Once candidate genes are identified a variety of pharmacologic and behavioral studies can be utilized to identify particularly salient candidate genes that underlie the divergent behavioral responses to acute OIH. The goal is that this will ultimately lead to better a better understanding of acute morphine-induced hyperalgesia and the development of more efficacious interventions.

Based on the current findings, acute morphine-induced hyperalgesia appears to be an active process not mediated by μ, κ, and δ opioid receptors or the morphine metabolite, M3G, independent of prior analgesia, and dependent on NMDA receptors. 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 acute 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 the NMDA receptor system, hold great promise as possible adjuvants to opioid therapy by reducing the likelihood of pain escalation and sensitization.

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